Biochimica et Biophysica Acta, 1098 (1992) 275–335

BBABIO 43547

Review

Protein phosphorylation in regulation of photosynthesis

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(Received 8 April 1991)

Key words: Protein phosphorylation; Photosynthesis; Molecular recognition; Light harvesting; Redox sensor/effector; Protein kinase/phosphatase

Contents

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I. Introduction

I-A. Protein phosphorylation

Regulation of function of proteins by phosphorylation of constituent amino acids has been studied most extensively in enzymes from mammalian cell and tissue extracts. In 1956, Krebs and Fischer showed that conversion of glycogen phosphorylase of rabbit skeletal muscle from the inactive *b* form to the active *a* form is a result of its phosphorylation [1]. This is probably the most clearly understood example of regulation of enzyme activity by covalent modification, the structural changes caused by phosphorylation having been identified by X-ray crystallography of the two forms of the protein [2]. In regulation of metabolism serine and threonine are the amino acids usually phosphorylated [3], though tyrosine and histidine phosphorylations are also known, especially in cell signalling pathways [4]. Numerous examples from mammalian physiological biochemistry of regulation of enzyme activity by protein phosphorylation can be cited [3–6].

In the 1980s it became clear that protein phosphorylation is also widespread in plants [7,8] and prokaryotes [9,10]. In addition to regulation of metabolism by control of such enzymes as pyruvate dehydrogenase and isocitrate dehydrogenase [3,7,10], phosphorylation of receptors and of components of cell signalling pathways has been studied in plant and prokaryotic systems [4,7,11]. It therefore seems likely that protein phosphorylation is a ubiquitous mechanism of control of protein structure and function, and that in any single cell it may involve many individual proteins. It may therefore provide independent or co-ordinated control of a large number of different processes at a number of different levels of gene expression.

The phosphotransferase catalyzing a protein phosphorylation is usually a protein kinase, that is, the phosphate group donor is either ATP or GTP. The enzyme catalyzing dephosphorylation is described as a protein phosphatase. The name of the specific protein substrate or phosphate group acceptor is used (e.g., 'phosphorylase kinase') where more than one kinase or phosphatase may be encountered. In a protein kinase reaction the γ-phosphate of ATP or GTP reacts with the hydroxyl group of the amino acid side chain to form a phosphate ester linkage, giving phosphothreonine or phosphoserine, for example, and ADP or GDP is released. Both monoester and diester linkages may occur, the latter including attachment of nucleoside phosphates by adenylyl or uridylyl transferases. In the protein phosphatase reaction, the phosphate ester is hydrolyzed to release inorganic phosphate, and the threonine or serine side chain is restored.

Threonine phosphorylation is summarized in Fig. 1. A conspicuous effect of phosphorylation is increased negative charge, analogous to replacement of the threonine or serine by glutamate or aspartate. Effects of

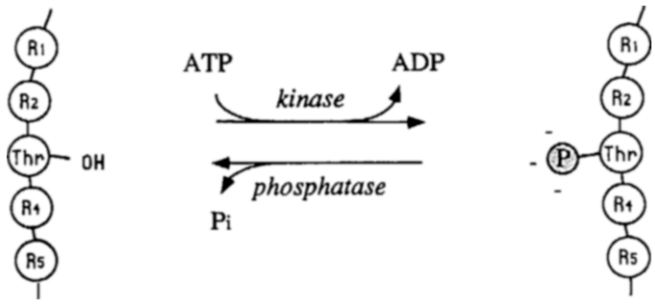

Fig. 1. A naïve view of protein kinase and phosphatase reactions catalyzing phosphorylation and dephosphorylation of a threonyl residue.

phosphorylation on molecular shape and bond geometry are shown in Fig. 2. The specific addition of negative charge is likely to be the basis of structural and functional effects of protein phosphorylation.

I-B. Chloroplast phosphoproteins

Phosphoproteins were first shown to be present in chloroplasts by Bennett who in 1977 described a number of chloroplast phosphoproteins spanning the range of molecular weight 7–70 kDa [11]. Work on protein phosphorylation in plants had previously concerned proteins whose counterparts in animals had already been described [12] and Bennett's results [11] were therefore the first concerning phosphoproteins involved in photosynthesis.

Chloroplast thylakoid membrane proteins were shown by Bennett to be phosphorylated in vivo in ^{32}P-labelled pea leaves and in vitro in isolated pea chloroplasts illuminated in the presence of [^{32}P]P_i. The two polypeptides most conspicuously phosphorylated were a major thylakoid membrane component at about 26 kDa and a minor component (as judged by

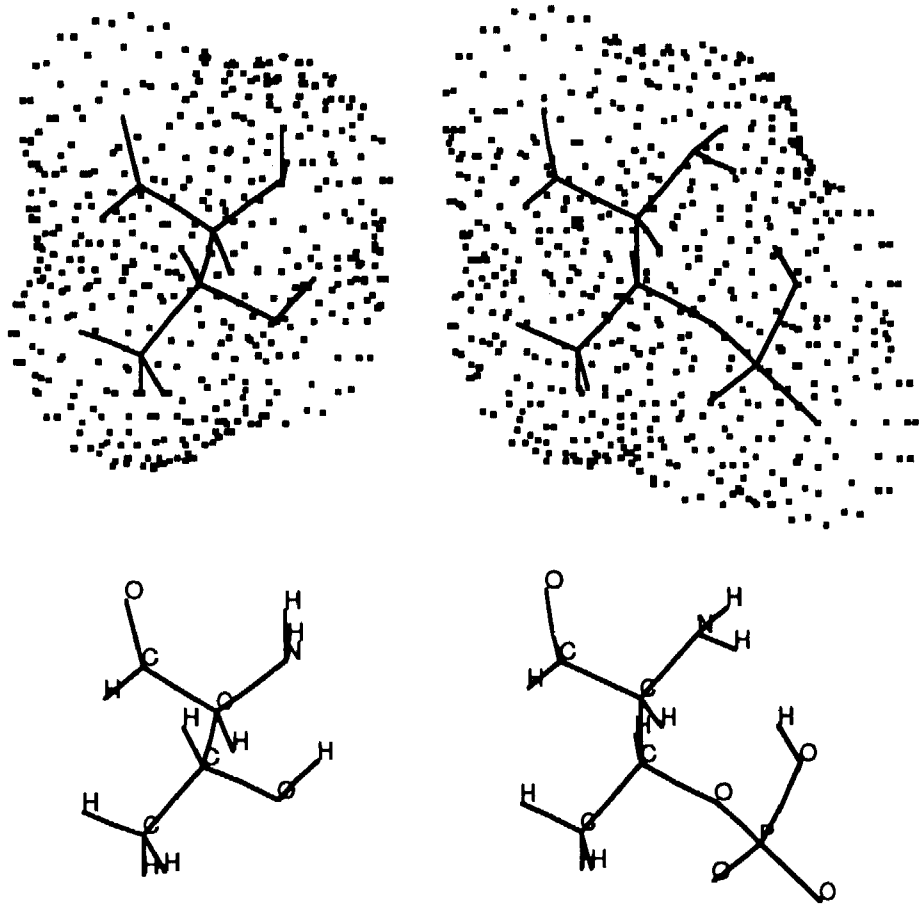

Fig. 2. Threonine (left) and phosphothreonine (right) depicted as labelled stick models (below) and as dot surfaces (above). The phosphate group (lower right) decreases the electrostatic potential of the side chain. This can be visualized when the dots are coloured according to electrostatic potential (not shown). The phosphate group also affects the geometry produced by energy optimization. A conspicuous example of this is rotation of the amino group (above right), which could contribute to changes in protein secondary structure. Structures optimized and drawn using the program *Nemesis* (Oxford Molecular Ltd., Oxford, U.K.)

Coomassie blue staining) at about 9 kDa. Both were phosphorylated on threonine residues [11]. Bennett identified the 26 kDa thylakoid phosphoprotein as a polypeptide (LHCP) of the light-harvesting, chlorophyll *a*/*b* binding, pigment-protein complex of Photosystem II (LHC II), and suggested that thylakoid protein phosphorylation "may be involved in establishing the high energy state of the thylakoids in state 1–state 2 transitions" [11]. A role for LHC II phosphorylation in state 1–state 2 transitions is now established (subsection II-A.4.), and has essentially replaced the 'high energy state' in current thinking on this topic, at least for chloroplasts. LHC II phosphorylation is reviewed here in subsections II-A, II-D and Section IV. The identity and role of the 9 kDa phosphoprotein is less clear, and is considered in subsection II-B.

Bennett subsequently confirmed his identification of the 26 kDa phosphoprotein as a component of LHC II [13]. He also showed that phosphorylation of both thylakoid proteins is light-dependent in vitro where [γ-^{32}P]ATP is used as the phosphate group donor and even in the presence of an uncoupler of ATP synthesis [14]. The requirement of the chloroplast protein phosphorylation reaction for light does not therefore reside only in photosynthetic phosphorylation of ADP to make ATP as the phosphate group donor: the thylakoid protein kinase reaction is itself light-dependent. In further experiments Bennett showed that both the protein kinase [14] and protein phosphatase [15] activities are present, like their protein substrates, in isolated thylakoid membranes. Following Bennett's results with pea (*Pisum sativum*) thylakoid phosphoproteins, similar findings were reported for bean (*Vicia faba*) [16] and spinach (*Spinacia oleracea*) [17].

In the mid-1980s the subject of chloroplast protein phosphorylation and its regulatory role in photosynthesis in green plants was extensively reviewed [18–25]. Chloroplast protein phosphorylation has also been evaluated in reviews on regulation of photosynthetic electron transport [e.g., 26–30] and on state 1–state 2 transitions [31,32]. The present review covers more recent work on this topic, for example characterisation of the LHC II kinase (subsection II-D), and is intended to provide a new perspective on some of the central problems from the viewpoint of recent developments in structural studies of light-harvesting complexes and reaction centres. In addition, enough is now know about protein phosphorylation in photosynthetic prokaryotes (Section III) to permit explicit comparisons with what happens in chloroplasts. It is also proposed here (Section IV) that membrane protein structural changes underlie the functional effects of their phosphorylation, and that protein phosphorylation has a role in control of expression of genes encoding components of the photosynthetic apparatus (Section V).

I-C. The photosynthetic unit and membrane organisation

Of the several hundred pigment molecules in each photosynthetic unit [33], almost all function as light-harvesting pigments that can absorb light energy and transfer it to and from neighbouring pigments [34]. The pathway of excitation energy transfer may terminate with re-emission of light energy as fluorescence [35]; with non-radiative decay as thermal deactivation; or with a photochemical reaction in which a proportion of the energy of each absorbed quantum is conserved as electrochemical potential [36]. Photoelectrochemistry takes place in the reaction centre component of the photosynthetic unit where excitation causes a specialized chlorophyll molecule to be oxidized and a primary electron acceptor to be reduced, and where the charge separation is stabilized by secondary electron transfer. Excitation energy transfer can be reconstituted between light-harvesting and reaction centre complexes [37,38].

High-resolution structures of purple bacterial reaction centres have been solved from X-ray crystallographic data [38–42]. These structures show four chlorophylls, two phaeophytins, one iron atom and two quinones, giving a two-fold symmetry. A single carotenoid is also present. The polypeptides of the reaction centre are the L, M and H chains. L and M each have five non-polar, α-helical domains between the amino-terminus on the cytoplasmic, acceptor side and the carboxy-terminus on the periplasmic, donor side. L and M together form a heterodimer that ligates all the chromophores. The chromophores and the L/M heterodimer have a common axis of symmetry. The primary electron transfer occurs between the L-chain chlorophyll and the L-chain phaeophytin. The M-chain quinone, Q_A, accepts an electron from the L-chain phaeophytin and donates one to the L-chain quinone, Q_B: the L and M chains are twisted around each other such that the M-quinone is nearest to the L-phaeophytin and vice versa. The H-polypeptide chain is approximately the same length as the L and M chains, but has only one membrane-spanning α-helix. Most of the H-chain forms a polar, carboxy-terminal domain on the acceptor (cytoplasmic) side of the reaction centre.

The role of the H-chain is unclear: it has been suggested that it stabilizes binding of the second quinone [40,43] and that it interacts with light-harvesting polypeptides [44]. It is suggested in Section IV that protein phosphorylation regulates recognition and binding of complementary docking surfaces of neighbouring pigment-protein complexes, and this raises the possibility that the H-chain is involved in this process. A further feature of the reaction centre which may be important in this respect is the existence of the pair of accessory or 'voyeur' chlorophylls, which could be involved in excitation energy transfer from the light-

harvesting system [45] or in electron transfer [46]. A single carotenoid adjacent to the M-chain accessory chlorophyll is also seen.

The reaction centre of purple non-sulphur bacteria has many features in common with that of Photosystem II (PS II) [47], and it seems likely that the D1 and D2 polypeptides of PS II form a heterodimer analogous to L/M and hold a similar chromophore disposition and orientation [40,48–50]. D1 and D2 show sequence homology with each other and with L and M respectively of purple bacterial reaction centres [48–50]. A D1/D2 complex [51,52] shows flash-induced P680 photooxidation and phaeophytin reduction [46].

Photosystem I (PS I) has a different reaction centre, with monomeric chlorophyll *a*, phylloquinone, and iron-sulphur centres functioning as electron acceptors [53]. PS I resembles the reaction centre of green photosynthetic bacteria [54]. It is likely that structural models at atomic resolution will also become available in the near future for PS I [55]. High-resolution structural studies should eventually provide a definitive answer to questions about the putative phospho-LHC II-PS I complex discussed here in subsections II-A and IV-E.

Four main types of light-harvesting antenna system have been described [56–59]. These are (i) highly non-polar, membrane-bound and chlorophyll-based systems such as those found in purple photosynthetic bacteria; (ii) membrane-bound, chlorophyll-based systems with large polar regions indicating an extensive surface-exposed region, as seen in chloroplasts and in some oxygenic prokaryotes (*Prochloron* and possibly also *Prochlorothrix hollandica*); (iii) water-soluble, chlorophyll-based systems found in green photosynthetic bacteria such as *Chlorobium* and *Prosthecochloris*; (iv) phycobilin light-harvesting systems which are water-soluble and may be organized into globular phycobilisomes as in cyanobacteria and red algae or contained within the thylakoid in some other (as yet uncharacterized) structure as in cryptomonads. Regulation of light-harvesting function by protein phosphorylation has been most extensively characterized for group (ii) (Section II) and there is evidence for analogous reactions in groups (i) and (iv) (Section III). To date there is no report known to the reviewer on protein phosphorylation in green bacteria (iii), nor in reaction centre or light-harvesting proteins of PS I (ii).

Each type of antenna system is associated with a particular membrane architecture. Purple bacteria have chromatophores, which are specialised invaginations of the cell membrane [60]. Chloroplasts have topologically discrete thylakoids which are often appressed to neighbouring thylakoids for at least part of their surface area [61]. Green bacteria have light-harvesting chlorosomes or 'chlorobium vesicles' attached to the cytoplasmic surface of the cell membrane [62]. Cyanobacteria have unstacked thylakoids with attached phycobilisomes, hemispherical or hemidiscoidal objects typically 50 nm in diameter and containing 500 or more phycobilin chromophores [58].

The concentration of attention on phosphorylation of chloroplast light-harvesting proteins has led to a view [63] that protein phosphorylation is a plausible mechanism for regulation of light-harvesting function only in chloroplast-type membrane systems, specifically in thylakoids stacked to form discrete appressed (grana) and non-appressed (stroma) regions. I propose that the evidence on protein phosphorylation in purple bacteria and cyanobacteria now makes protein phosphorylation a plausible mechanism of control even in membrane systems devoid of lateral heterogeneity in distribution of protein complexes within the thylakoid membrane. If this is correct, then effects of protein phosphorylation may also have no necessary connection with lateral heterogeneity in chloroplast thylakoids. This possibility is considered further in Section IV.

II. Phosphoproteins of chloroplast thylakoid membranes

II-A. Light-harvesting chlorophyll a / b protein complex (LHC II)

II-A.1. Structure and function of LHC II

'LHC II' describes a pigment-protein complex that contains about half the chlorophyll and a third of the protein of green plant thylakoids. LHC II is probably the most abundant species of membrane protein complex in nature, and is the major green band on non-denaturing gels of chloroplasts. Its significance as a light-harvesting complex of PS II was recognised by Thornber [64]. It typically contains 8 chlorophyll *a*, 7 chlorophyll *b* and 1–2 xanthophyll molecules per polypeptide of 24–27 kDa [65,66]. LHC II contains a number of closely-related LHCP polypeptides encoded by a family of nuclear genes, called *cab* genes, whose cDNA and genomic DNA has been sequenced from many higher plant species [67–69]. Each polypeptide has an extensive amino-terminal sequence on the stromal side of the thylakoid membrane, and a shorter carboxy-terminal sequence on the inside of the thylakoid membrane. Hydropathy plots [69,70] indicate three membrane-spanning α-helices giving a membrane disposition such as that shown in Fig. 3A. A recent three-dimensional structure at 6 Å resolution (Fig. 3B) confirms this prediction. In Thornber's nomenclature this complex is described as LHC IIβ or LHC IIb [65, 71] with the 'α' or 'a' form containing a larger polypeptide and known as 'CP29'. The consensus of LHC II protein sequences shows conserved regions around helices I and III. These two regions show sequence similarity with each other, consistent with internal symmetry and perhaps

with an evolutionary origin from a single-helix light-harvesting protein by gene duplication.

The LHC II complex can be identified with a freeze-fracture membrane particle of 8 nm diameter that is found predominantly in stacked regions of membrane [23,72]. It is thought that the amino-terminal surface-exposed regions of the complex are sites of membrane adhesion that cause thylakoid stacking [23,72–74]. This is consistent with the three-dimensional structure proposed by Kühlbrandt on the basis of Fourier analysis of electron micrographs of two-dimensional crystals [75]. This structure has three-fold symmetry and a platform at one surface that could provide for interaction with a neighbouring platform through van der Waals' forces. Three-dimensional crystals obtained so far are small and give X-ray diffraction only at low resolution [76]. A more recent structure, at 3.7 Å resolution in projection, has been obtained by Kühlbrandt and Downing from cryo-electron microscopy [77]. This structure (Fig. 4) shows a trimeric arrangement, consistent with ultracentrifugation studies, and internal symmetry within each monomer, reflecting sequence similarities within the polypeptide. A trimeric arrangement of monomers each corresponding to a single polypeptide is concluded from similar studies by Lyon and Unwin [78].

Kühlbrandt and Wang have obtained a three-dimensional reconstruction of LHC II at 6 Å resolution [79]. This shows three membrane-spanning helices and 15 chlorophylls, and the arrangement of the helices is

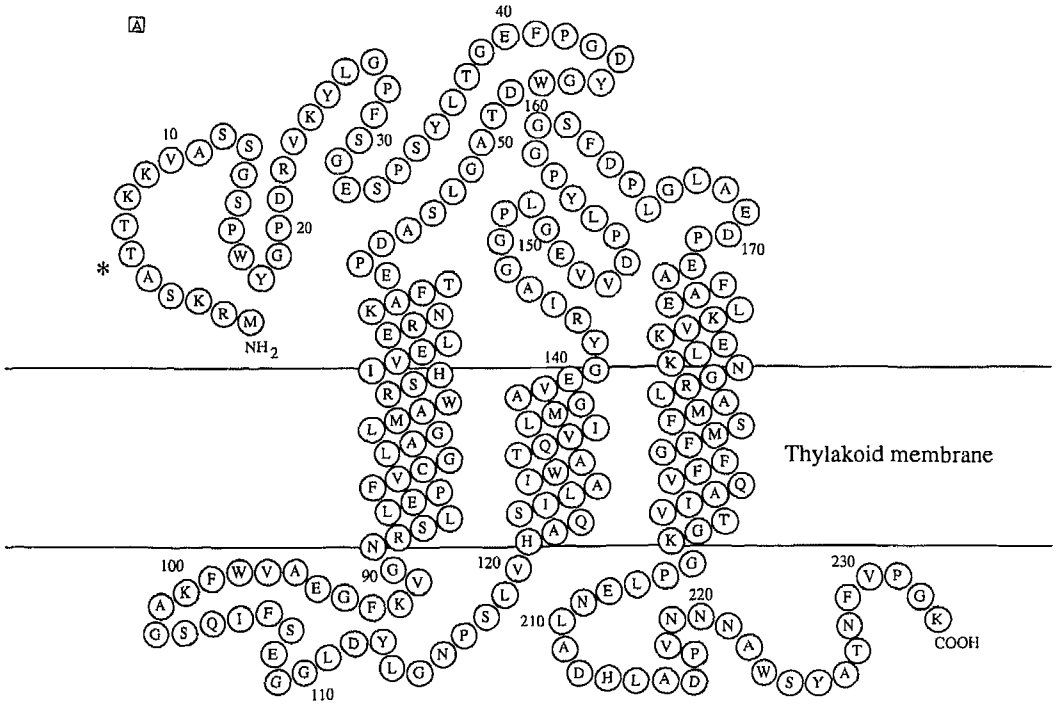

Fig. 3. (A) A model for the organization of LHC II with respect to the thylakoid membrane, based on that of Bürgi et al. [70], but with helices I and III extended to prolines 56 and 171. This produces helices of 33 amino acids, with length 49.5 Å, close to the estimated lengths of helices B (49 Å) and A (46 Å) in the structure in Fig. 3B. Abrupt turns in the sequence correspond to regions of high turn probability. * indicates the phosphorylation site, thr 6. The sequence is mostly from Edman degradation of a pea LHCP. Adapted from Ref. 70. (B) Topography of the LHC II polypeptide proposed by Kühlbrandt and Wang [79], based on their three-dimensional electron density map at 6 Å resolution. Helices I, II and III (numbered from the N-terminus) probably correspond to helices B, C and A respectively. A (49 Å) and B (46 Å) are longer than C (30 Å), are tilted with respect to the membrane plane (at angles of 25° and 31° respectively), are twisted around each other slightly (not shown), and protrude from the membrane. A and B also have hook-like extensions at the stromal side of the membrane, and together with the hooks show a 2-fold symmetry about an axis (dash-dotted line) perpendicular to the membrane plane, and corresponding to the polypeptide segments showing internal sequence homology. Redrawn from Ref. 79.

shown in Fig. 3B. Helices I and III (termed B and A, respectively, by Kühlbrandt and Wang) are 49 Å and 46 Å long, longer than any helix described previously for a membrane protein, and protrude beyond the membrane surface. They extend for 9 Å and 7 Å beyond the top of helix II (helix C). The 15 chlorophylls (*a* and *b* cannot be distinguished at 6 Å) all have chlorin rings roughly perpendicular to the membrane plane. The chlorophylls are arranged on two levels, the upper level of 8 chlorophylls being related by the local 2-fold symmetry found in helices I and III, indicating conserved chlorophyll binding sites in the internally homologous polypeptide segments.

Kohorn and Tobin [80] conclude from studies on *cab* genes expressed in vitro to permit incorporation of pre-LHCPs into *Lemna* chloroplasts that opposite charge on helices I (positive) and III (negative) are required for correct accumulation and assembly of this polypeptide into the thylakoid LHC II complex.

Proteolysis of thylakoid membranes shows that phosphorylation of LHC II polypeptides occurs on the amino-terminal surface-exposed segment that is also associated with membrane stacking [13,73]. It was concluded that phosphorylation occurs at one or both of adjacent threonines in positions 6 and 7 in pea [73]. The location of the phosphorylation site is considered further in subsection II-D.

The consensus at present [22,23,30] is that phosphorylation alters the net electrical charge of the complex at the membrane surface, causing a change leading to electrostatic repulsion that can overcome the resultant of van der Waals' and other forces otherwise holding neighbouring LHC IIs at 180° to each other on a common axis perpendicular to each membrane plane. The mechanism by which phosphorylation of LHC II exerts its effects is considered in Section IV.

II-A.2. Functional effects of LHC II phosphorylation on excitation energy distribution

Fluorescence spectra. The functional effect of phosphorylation of LHC II first identified was a change in the chlorophyll fluorescence emission properties of isolated thylakoids. Phosphorylation is accompanied by a decrease in total chlorophyll fluorescence yield at room temperature [81–83] which is consistent with decreased emission from PS II. Fluorescence spectroscopy at 77 K shows that LHC II phosphorylation produces a decrease in yield in the PS II emission bands at 685 and 695 nm relative to that of the PS I band at 735 nm [81,82]. It was concluded by Bennett et al. [81] and by Horton and Black [82] that excitation energy from light absorbed by LHC II becomes diverted away from PS II as a result of phosphorylation, and that all or part of that energy reaches PS I instead.

Normalisation procedures intially showed only a relative increase in the ratio F_{735}/F_{685} [81,82]. Using fluorescein as an external standard in isolated spinach thylakoids, Krause and Behrend showed both a de-

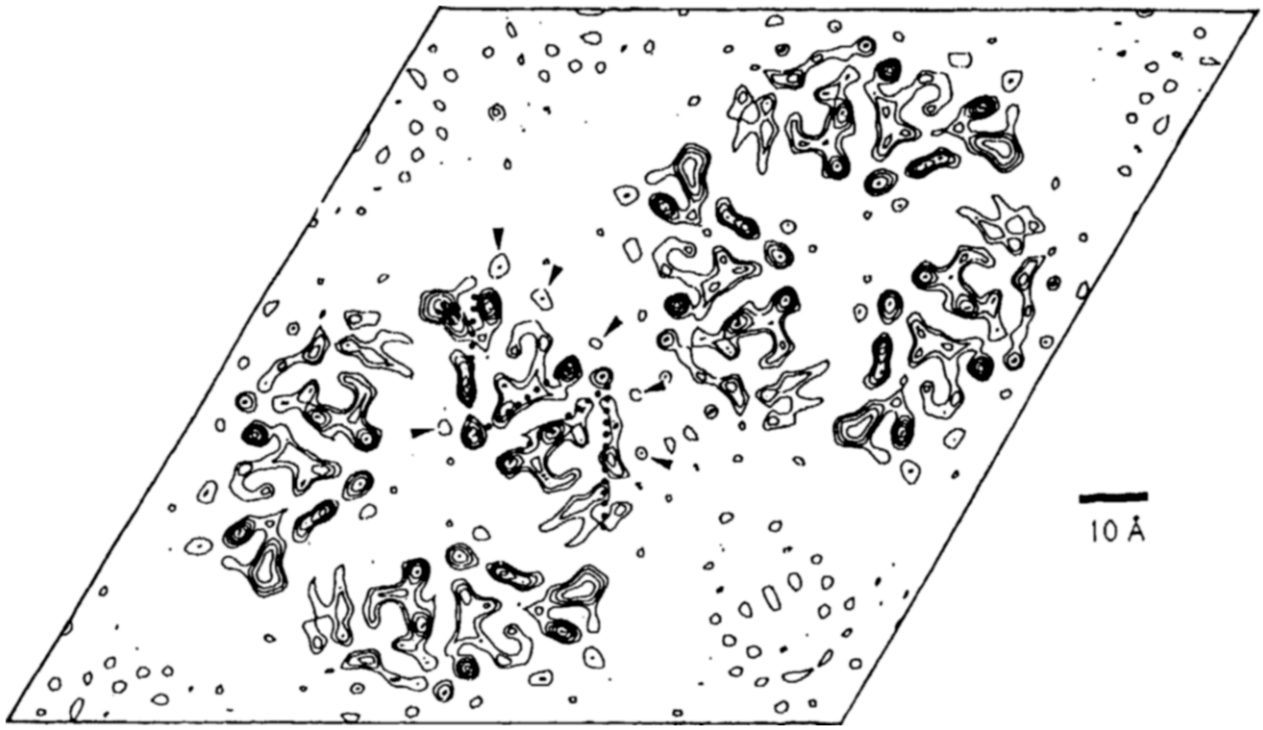

Fig. 4. Projection map obtained by Kühlbrandt and Downing, of one unit cell of two-dimensional LHC II crystals at 3.7 Å resolution, showing a dimer of trimers. * indicates a feature corresponding to an α-helix roughly normal to the membrane plane. Dotted lines show triangular features with two-fold symmetry corresponding to structural domains with homologous polypeptide sequences. From Ref. 77. See also Fig. 3.

crease in F_{685} and an increase in F_{735} [84]. The same conclusion was reached by Saito et al. using phycoerythrin as an external standard with *Chlorella* cells and thylakoids [85]. These results were taken as further evidence that excitation is redirected to PS I at the expense of PS II as a consequence of phosphorylation of LHC II. Evidence from photoacoustic spectroscopy [86] and modulated fluorescence [87] has also been interpreted as favouring redirection of excitation energy in this way. This conclusion is in accordance with expectations deriving from the assumed physiological role of redistribution of absorbed excitation, as discussed in subsection II-A.4. Whether redirected excitation energy can be trapped by PS I photochemistry is therefore an important question for understanding the function of LHC II phosphorylation.

PS I antenna size. Attempts have been made with various systems to demonstrate the increased antenna size of PS I that is implied by increased PS I fluorescence emission. Horton and Black [88] measured kinetics of cytochrome *f* photooxidation in phosphorylated and unphosphorylated pea thylakoids in the presence of DCMU and found no significant difference. Melis and co-workers [89,90] found no difference between phosphorylated and control spinach thylakoids with respect to kinetics of P-700 photooxidation. In contrast, Telfer and co-workers [91,92] measured P-700 photooxidation at 820 nm by single-turnover flashes in pea thylakoids, and concluded that phosphorylation increases the absorption cross-section of PS I by 12% [92]. Measurement of steady-state PS I electron transport rates at limiting light intensity have also produced different estimates of the increase in PS I yield ranging from negligible [93] to 8% even with 650 nm excitation [94], which should produce the maximum effect by exciting through chlorophyll *b*. If all LHC II detached from PS II upon phosphorylation becomes attached instead to PS I [95,96], then these results fall short of the required increases in absorption cross-section of PS I of at least 16% for broad-band excitation and of 40–70% for Chl *b* excitation. However, Farchaus et al. [97] reported a decrease in PS II electron transport rate of 18% and an increase in PS I electron transport rate of 14% upon phosphorylation of spinach thylakoids, illuminated for electron transport measurement by white light. Forti and Vianelli [98] have reported up to a 40% increase in the steady-state rate of NADP reduction by spinach thylakoids illuminated with light selective for chlorophyll *b*. In an attempt to measure increased excitation energy transfer to P-700 in vivo, Allen and Melis [99] found no difference in P-700 kinetics between *Scenedesmus* cells containing phosphorylated and dephosphorylated LHC II as judged by room-temperature fluorescence transients.

Delepelaire and Wollman obtained the largest estimate of increased yield of PS-I-associated reactions using flash kinetic spectroscopy of a *Chlamydomonas* mutant lacking PS II reaction centres [100]. They measured flash-induced absorption changes at 515 nm which are proportional to the $\Delta\psi$ (membrane potential) component of the proton motive force. The observed increase in ΔA_{515} upon LHC II phosphorylation was 50%. The absence of PS II was taken to suggest that all of this increase arose from increased energy transfer to PS I from phospho-LHC II. Several factors apart from PS I light-harvesting capacity could affect $\Delta\psi$ in this system, however. In particular, anaerobic conditions and light were used to obtain phosphorylation of LHC II by redox-activation, with aerobic conditions and darkness giving the unphosphorylated experimental control. In the absence of PS II activity, cyclic electron transport and photophosphorylation are known to be inhibited under aerobic conditions by over-oxidation of electron transport components [27]. An increase in $\Delta\psi$ between anaerobic and aerobic PS-II-deficient cells may therefore reflect changes in LHC II phosphorylation only indirectly.

Enhancement. When photosynthesis is driven experimentally by two lights of different wavelength, the rate of photosynthesis with both lights may exceed the sum of the rates obtained with each beam alone. This enhancement effect [101] occurs when the two wavelengths used are selective for each of the two photosystems, and it therefore indicates that excitation energy distribution between PS I and PS II is unequal for each beam acting alone. If phosphorylation of LHC II tends to equalize distribution of excitation energy between PS I and PS II then it should decrease enhancement.

Sinclair and Cousineau [102] showed an ATP-induced decrease in enhancement by PS II illumination (640 nm) of oxygen evolution by spinach thylakoids under PS I illumination (700 nm) and with NADP and ferredoxin as electron acceptors. They concluded that LHC II phosphorylation redirects excitation energy, but they found no increase in the total yield of oxygen evolution after enhancement had decreased. This result suggests that phosphorylation of LHC II decreases the antenna size of PS II so that the 640 nm illumination is no longer selective for PS II, but that PS I receives little of the redirected excitation energy.

In contrast, Forti and Fusi [103] used a different enhancement protocol, measuring the enhancement effect of PS I illumination (722 nm) on NADP reduction by spinach thylakoids under a variety of different PS II illuminations. They found a maximum enhancement when the wavelength of the PS II illumination corresponded to the chlorophyll *b* absorption maximum of LHC II at 475–500 nm. They confirmed that enhancement was eliminated by conditions giving rise to LHC II phosphorylation, and concluded that phospho-LHC II can act as an antenna for PS I. This conclusion was

supported by a measured 20% decrease in the antenna size of PS II and a proportional increase in the antenna size of PS I. The problem of different experiments giving rise to different conclusions about the fate of redirected excitation energy therefore extends also to enhancement studies.

Spillover and absorption cross-section (Part I: chloroplasts). Fluorescence induction measurements (this technique is reviewed in [104]) seemed initially to support a movement of a phospho-LHC II-PS II complex into functional interaction with PS I [81,105]. This model allowed 'spillover' of excitation energy from PS II to PS I, and envisaged the functional effect of LHC-II phosphorylation as an increase in spillover. Spillover fits within the bipartite and tripartite models of Butler and co-workers [106] by which the photosystems and their antenna complexes may comprise a supercomplex within which excitation energy transfer can proceed by a number of different routes.

Spillover is a diversion of excitation energy away from PS II, reflecting a change in the rate constant k_t of a reaction (transfer to PS I) competing at all times with photochemistry and therefore fastest when photochemistry is saturated at F_m ($Q = 0$, where Q is the proportion of open traps) and slowest at F_o (when $Q = 1$) in accordance with

$$\Phi_f = \frac{k_f}{k_f + k_d + k_t + k_p[Q]} \qquad (1)$$

where Φ_f is fluorescence yield, and k_f, k_d, k_t and k_p are the rate constants for fluorescence, thermal deactivation, energy transfer, and photochemistry respectively. The prediction of the spillover change model is that the change in k_t will have a proportionately greater effect on fluorescence yield at F_m (when $k_pQ = 0$) that at F_o (when $k_pQ = k_p$). In contrast, a change in absorption cross-section will not affect fluorescence yield but will simply give a change in total fluorescence emission that is independent of Q and proportionately the same at F_m and F_o. Effects of changes in spillover and absorption cross-section on fluorescence induction are outlined in Fig. 5.

Several laboratories obtained fluorescence induction transients with F_v/F_m ratios ($F_v = F_m - F_o$) apparently independent of the phosphorylation state of LHC II, consistent with a change in absorption cross-section of PS II rather than in spillover [95,107–108]. In contrast, changes in free cation concentration alter the F_v/F_m ratio, suggesting control of spillover. Decreased cation concentration unstacks thylakoid membranes and may randomize the distribution of PS I and PS II in such a way as to permit spillover between them (subsection IV-A). In the writer's view it is doubtful now whether such cation effects on spillover serve as a useful model

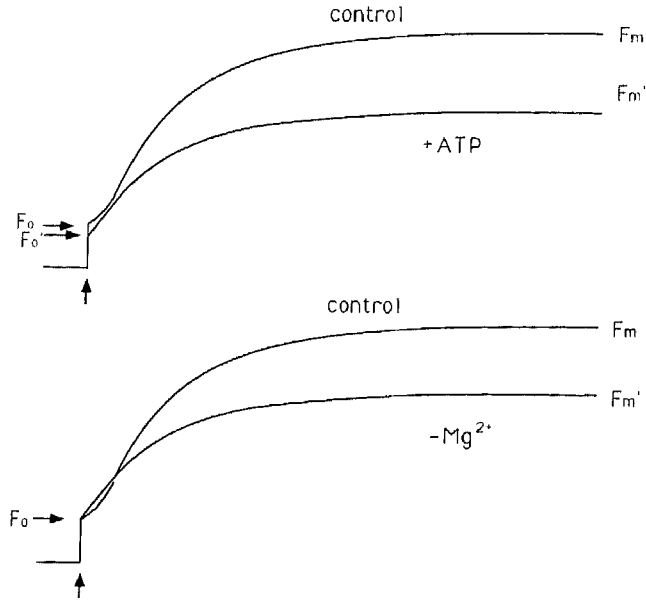

Fig. 5. Model fluorescence induction transients, illustrating the decrease in both F_m and F_o upon phosphorylation of LHC II (+ATP) and in F_m alone on cation depletion ($-Mg^{2+}$). The upper transients suggest a decrease in the absorption cross-section of PS II, the lower transients an increase in spillover from PS II to PS I. Such transients are obtained in the presence of DCMU. The *y*-axis is room-temperature fluorescence emission in arbitrary units, the *x*-axis is time on a millisecond scale. The rapid rise from the baseline to F_o occurs upon switching on the light (vertical arrow). Changes in sigmoidicity also occur. The $-Mg^{2+}$ and plus ATP (state 2) induction curves are markedly less sigmoidal than the controls, i.e., than those for $+Mg^{2+}$ and without ATP (state 1).

for effects of LHC II phosphorylation (Section IV), though at low, subphysiological cation concentrations (such as 1 mM Mg^{2+}) phosphorylation may promote thylakoid destacking, randomization of PS I and PS II, and hence increase spillover [109–110]. The Mg^{2+}-dependency of the LHC II kinase and phosphatase [13,15] is a further factor in analysis of such effects. At more physiological cation concentrations (20–30 mM Mg^{2+}) thylakoid stacking may be less dependent on the phosphorylation state of LHC II [109], and absorption cross-section changes therefore predominate.

Absorption cross-section changes upon phosphorylation of LHC II are easier to accommodate by lateral heterogenity and mobility of phospho-LHC II (subsection II-A.6). It may be that changes in both spillover and absorption cross-section can operate under different circumstances, and this possibility depends also on the role of LHC II phosphorylation in PS II heterogeneity. Furthermore, factors other than LHC II phosphorylation may contribute to changes in excitation energy distribution in vivo, where spillover-type effects can certainly be observed [111].

Fluorescence lifetime studies also support the conclusion that PS II absorption cross-section is lowered upon phosphorylation of LHC-II in thylakoids [112–

113], but they generally shown no effect of phosphorylation on the fast decay component attributed to PS I [113,114]. Contrasting fluorescence lifetime data have been obtained by Hodges et al., however [115].

PS II heterogeneity. Studies on thylakoids [113] and on *Chlorella* [114] have suggested that a major effect of LHC II phosphorylation is on PS II heterogeneity, either to convert α-centres into β-centres [113], as proposed Kyle et al. [116], or to cause complementary changes in the absorption cross-sections of PS II-α and PS II-β, as proposed by Holzwarth [114]. Fluorescence induction in studies of PS II heterogeneity is reviewed by Krause and Weis [104].

An additional effect of LHC II phosphorylation may therefore be control of excitation energy distribution between different types of PS II centre [113–116]. Kyle et al. [116] analysed room-temperature chlorophyll fluorescence of isolated pea thylakoids in the presence of DCMU, the thylakoids having previously been incubated in the presence of ATP under conditions for LHC II phosphorylation. Kyle et al. found a shift from a sigmoidal to an exponential fluorescence rise and a decreased proportion of the fast α component compared with the slow β component. Similar conclusions have been reached from similar data obtained in different systems by other groups [82,109–110]. The basis of such changes is a decrease in the average connectivity of PS II photosynthetic units for excitation energy transfer. This may reflect movement of phospho-LHC II from PS II-α centres that are highly connected for excitation energy transfer and their re-association with PS II-β centres that function independently of each other with respect to energy transfer [104,117]. Similar results could also arise from conversion of PS II-α to PS II-β as a result of LHC II phosphorylation. If the cores of PS II-α centres are connected for energy transfer through shared peripheral LHC II complexes then dissociation of phospho-LHC II from PS II could produce this effect. Altered excitation energy distribution within PS II may be an earlier functional effect of LHC II phosphorylation than increased energy transfer to PS I, and the two effects should be capable of being separated experimentally. As discussed in Section III, changes in sigmoidicity of fluorescence induction are also seen under similar circumstances in cyanobacteria and possibly also in purple bacteria. In chloroplast thylakoids, PS II-α and -β are located in different membrane domains, and these functional changes may depend on lateral migration of PS II and phospho-LHC II (subsections II-A.6–7).

Timmerhaus and Weis have put forward a model for the organisation of PS II which might explain the conflicting data from various laboratories on the question of phosphorylation-induced changes in PS I yield as well as PS II heterogeneity [118]. They propose four states, arising from combinations of phosphorylation-dephosphorylation of PS II and temperature effects (Fig. 6). They propose that phospho-LHC II moves away from PS II cores at moderate temperature, with a decrease in connectivity of PS II but with no reassociation of phospho-LHC II with PS I. At high temperatures the model includes movement of individual, PS II-β centres to the vicinity of PS I, where phospho-LHC II may connect PS II-β with PS I and allow spillover of excitation energy between them. If this model is correct then the discrepancies between the results of the various laboratories concerning PS I yield [88–94] and the F_v/F_m ratio [81–82] could be explained as undefined differences in relative temperature or some other factor perhaps connected with cell growth or thylakoid

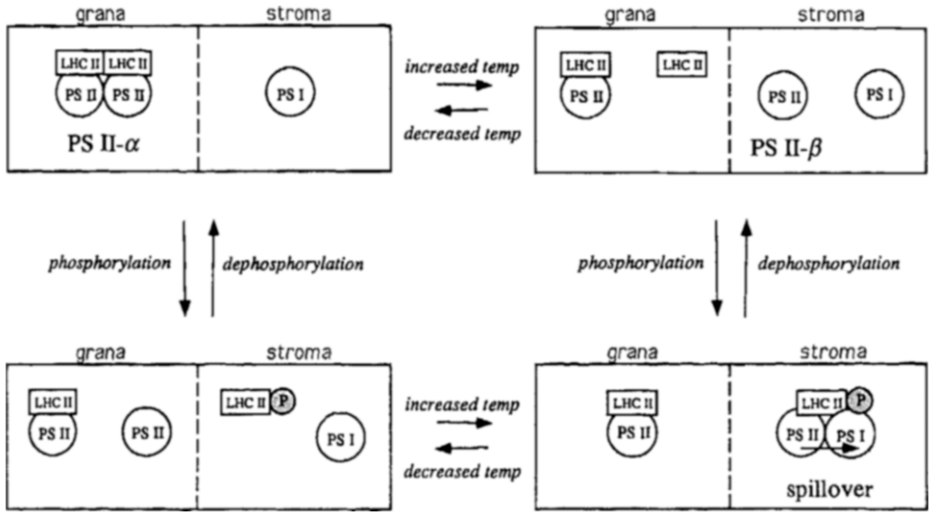

Fig. 6. Effects of LHC II phosphorylation and of temperature changes on distribution of pigment-protein complexes and their functional interactions, according to the model of Timmerhaus and Weis. The transition from the state in the top left-hand box to any of the other three may represent a state 2 transition. See also FIg. 10. Adapted from Ref. 118.

isolation techniques. This model must also be reconciled with the occurrence of state transitions in PS-II-less mutants [100].

An additional proposal for a factor governing the fate of excitation energy transfer is that of Horton [119], who suggested that energy transfer from phospho-LHC II to PS I occurs only in LHC II complexes in which violoxanthin is present. In this proposal the conversion of LHC II violoxanthin to zeaxanthin by de-epoxidation [120] generates a form of phospho-LHC II incapable of excitation energy transfer to PS I but acting as a preferred substrate for the phospho-LHC II phosphatase. If reversible carotenoid epoxidation were under temperature control, this model [119] might be compatible with that of Timmerhaus and Weis [118].

Further proposals for functional effects of LHC II phosphorylation include control of PS II cyclic electron transfer [121], decreased PS II electron transfer via alteration of quinone binding on the acceptor side of PS II [122–124], alteration of binding of herbicides to Q_B [125], protection from photoinhibition of PS II [126,127], identification ('flagging') of LHC polypeptides selected either for or against proteolysis, and signalling in transcriptional control (Section V). A role in assembly of PS II has also been proposed [128]. These reactions may interrelate, are not mutually exclusive (see subsections II-A.3–5) and may concern phosphorylation of other thylakoid proteins (subsections II-B and II-C).

Functional effects of LHC II phosphorylation also arise in connection with lateral migration of a proportion of phospho-LHC II complexes from PS-II-rich to PS-I-rich domains of the chloroplast thylakoid membrane (subsections II-A.6 and 7).

II-A.3. Redox control at the level of plastoquinone

The light-activation of the thylakoid LHC II kinase [11,14] is due to an activation by photosynthetic electron transport, as first indicated by Bennett, who demonstrated the sensitivity of the kinase to inhibition by DCMU [14], an inhibitor of electron transport between Q_A and Q_B on the acceptor side of PS II. Activation of the kinase in darkness by reduced ferredoxin supported this conclusion, and was also consistent with a preliminary suggestion that the site of control lay on the acceptor side of PS I [14], as with thioredoxin-regulated enzymes of the Calvin cycle and CF_1 [129].

The LHC II kinase activity of isolated pea thylakoids was shown by Allen et al. to be dependent instead on the redox state of plastoquinone [130], a component of the photosynthetic electron transport chain situated between PS I and PS II. This conclusion was based initially on the observation that the kinase is activated in darkness by reducing agents such as dithionite or duroquinol while its light-activation is reversed by electron acceptors such as potassium ferricyanide or methyl viologen. Electron donors such as reduced DCPIP that donate directly to PS I do not activate the kinase in darkness or in the presence of DCMU [130] Such donors would be expected to reverse inhibition by DCMU if the site of activation lay on the acceptor side of PS I. Further support for the idea of redox control at the level of plastoquinone was the low DBMIB-sensitivity of kinase activation, since DBMIB blocks plastoquinone oxidation at a high-affinity binding site [130]. Furthermore, it was found that activation of the thylakoid kinase by single-turnover flashes of light showed a dependency on flash number which exactly matched that of reduction of the plastoquinone pool as measured independently as the area above the room-temperature chlorophyll fluorescence induction curve in the absence of DCMU [130]. Allen et al. [130] also showed a similar redox control of redistribution of excitation energy in favour of PS I and at the expense of PS II, as judged from 77 K fluorescence emission spectra. The conclusion from these experiments is that LHC II phosphorylation couples excitation energy distribution between PS I and PS II to the redox state of electron carriers that connected them, and that this coupling gives rise to a feedback regulation of photosynthetic energy conversion known as state 1–state 2 transitions (section II-A.4.). The model proposed by Allen et al. [130] is shown in Fig. 7.

The donor and inhibitor studies were extended by Allen and Horton [131] who showed that pre-reduced duroquinol activates both LHC II phosphorylation and

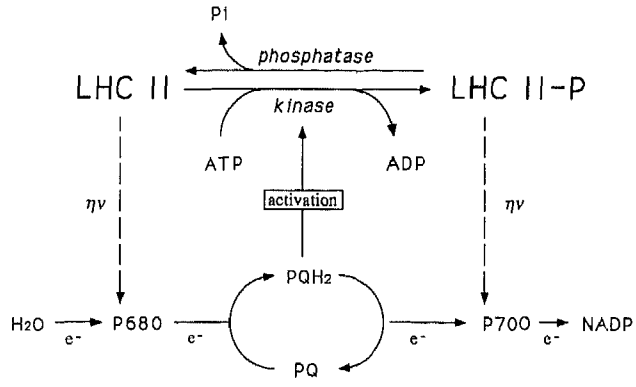

Fig. 7. A scheme for control of the LHC II protein kinase by the redox state of plastoquinone. LHC II transfers absorbed excitation energy primarily to PS II, driving electron transport at the reaction centre chlorophyll, P680. Reduction of plastoquinone (PQ) to plastoquinol (PQH_2) by imbalance in excitation energy distribution leads to activation of the LHC II protein kinase. Phospho-LHC II (LHC II-P) transfers absorbed excitation energy primarily to PS I, where it drives electron transport at the reaction centre chlorophyll, P700. Increased excitation energy transfer to PS I will tend to oxidise plastoquinone, inactivate the kinase, and allow the LHC II phosphatase reaction to predominate. Excitation energy distribution between PS I and PS II will therefore tend to be self-regulating.

the ATP-dependent RT chlorophyll fluorescence decrease in uncoupled thylakoids. In contrast, duroquinone with ascorbate functions like DCPIP with ascorbate, giving PS I electron transport but no activation of LHC II phosphorylation or ATP-dependent fluorescence lowering in darkness [131]. Redox control therefore lies between the primary sites of inhibition of electron transport by DCMU and DBMIB.

Redox control at the level of plastoquinone was also reported by Horton and Black [132], who carried out a potentiometric redox titration of the ATP-dependent fluorescence decrease previously described [82]. Horton et al. [133] extended the redox titration to measurement of LHC II phosphorylation. They obtained results consistent with control of the LHC II kinase by a two-electron carrier with a redox potential measured at pH 7.8 of 0 mV. Horton et al. also showed a strong correlation between LHC II kinase activation, the ATP-dependent fluorescence decrease, and plastoquinone redox state measured from fluorescence induction [133]. Similar results of a redox titration of LHC II kinase activity were reported by Millner et al. [134]. In the experiment of Horton et al. [133] phosphorylation of the 9 kDa protein (subsection II-B) showed a dependence on redox potential similar to that of LHC II.

Activation of the LHC II kinase by reduced ferredoxin in the experiment of Bennett [14] is consistent with redox control at the level of plastoquinone, since plastoquinone becomes reduced slowly by ferredoxin in darkness, as demonstrated by decreased photochemical quenching of PS II chlorophyll fluorescence [83].

Alternatives to redox control of activity of the LHC II kinase at the level of plastoquinone have been suggested. These include control by adenine nucleotide concentration [135], though it is unlikely that such control could be exerted independently of inhibition of CO_2 assimilation [136].

The occurrence of LHC II phosphorylation in vivo in *Chlamydomonas* cells irrespective of illumination or the presence of DCMU [128] and even in *Chlamydomonas* mutants lacking PS II altogether [100] appears to be inconsistent with redox control, but in fact the established maintenance of reduced plastoquinone in darkness by respiratory or 'chlororespiratory' electron transport in *Chlamydomonas* and other green algae provides a more satisfactory explanation, particularly since green algal thylakoids show plastoquinone redox control of LHC II phosphorylation and excitation energy distribution in vitro. [85,100,128]. Similar considerations apply to cyanobacteria (subsection III-A.5). The interface of redox control with assimilatory reactions, photophosphorylation and ΔpH is discussed further in subsection II-A.5.

In systems more complex than thylakoid membranes it is possible to see effects that are difficult to explain purely on the basis of redox control [137]. However, there seems little doubt from studies with isolated thylakoids that redox control is exerted on the LHC II kinase, with the redox titration experiments [132–134] being probably the most compelling direct evidence. Further studies with mutants and site-specific inhibitors now strongly implicate the cytochrome *b/f* complex in redox control (subsection II-D.1). Studies on the purified kinase itself support a direct regulation by plastoquinone redox state, and are discussed in subsection II-D.2.

Heil et al. [138] have studied phosphorylation of LHC II in cell extracts of the green alga *Scenedesmus obliquus*, using monochromatic light of different wavelengths but constant photon flux density to obtain action spectra. Their objective was to distinguish between the redox control hypothesis for LHC II kinase activation [130] and the possibility that some photoreceptor other than chlorophyll is involved. They obtained action spectra with maxima at 450 and 679 nm and a minimum at 580 nm. These resembled closely the absorption spectra of the cells themselves and of a 3:1 chlorophyll *a:b* solution in methanol. They conclude that the photoreceptor for thylakoid protein phosphorylation is chlorophyll, and that the simplest explanation for this is that photosynthetic electron transport is a necessary link between the stimulus (light) and the response (protein phosphorylation). Taking this result [138] together with redox titration experiments [132–134] and donor and inhibitor studies [130–131] it seems reasonable to pursue plastoquinone redox control as a useful working hypothesis for the mechanism of control.

II-A.4. Light-state transitions in green plants

The enhancement of photosynthesis under one wavelength of light by a second, supplementary light of different wavelength depends on the connection, in series, of PS I and PS II, each having a distinct pigment system with distinct absorption characteristics and a distinct action spectrum [26,139]. In fact, the action spectrum of enhancement corresponds to the absorption spectrum of the rate-limiting photosystem, and for PS II especially it is specific to the pigment system of the organism (subsection I-C) [101].

As described above (subsection II-A.2), enhancement therefore occurs only when excitation energy distribution between PS I and PS II is unequal under both wavelengths used and where the two wavelengths complement each other. From a time-dependent variability in enhancement it is clear that organisms with two photosystems are able to redistribute absorbed excitation energy in such a way as to minimize the inequality in distribution [101]. This redistribution serves to increase overall quantum yield by decreasing delivery of surplus excitation energy to one photosys-

tem while increasing delivery of excitation energy to the other.

The operation of a mechanism serving to redistribute excitation energy was demonstrated in the green alga *Chlorella pyrenoidosa* by Bonaventura and Myers [140] and in the red alga *Porphyridium cruentum* by Murata [141], and has subsequently been studied in a number of ways and found in all two-light-reaction organisms examined [18,31–32]. Fig. 8, adapted from the results of Bonaventura and Myers, illustrates the process. Dark-adapted cells are illuminated with modulated light at 645 nm, absorbed predominantly by chlorophyll *b* in the LHC II complex. Chlorophyll fluorescence falls slowly from an initial maximum, and oxygen yield increases with approximately the same kinetics. This indicates a redistribution of excitation energy in favour of PS I, which is initially rate limiting but which becomes more effective in capturing light energy and electrons as the redistribution proceeds. The chlorophyll fluorescence from PS II decreases as a result of the combined decrease in excitation energy transfer to PS II and increased photochemical quenching of PS II fluorescence. The state of maximum oxygen yield under PS II light, light 2, is termed state 2. The transition to state 2 is thus a process of redistribution of excitation energy in favour of PS I. Upon addition of continuous light 1 at 710 nm, further quenching of PS II fluorescence occurs and there follows a slow increase in oxygen yield accompanied by a fluorescence rise that indicates redistribution of excitation energy back to PS II. The new state of maximum oxygen yield under PS I light is termed state 1. The transition to state 1 is thus a process of redistribution of excitation energy in favour of PS II. In whole cells it is apparent that both the state 1 and state 2 transitions result in increased yield of oxygen [140] (Fig. 8).

As outlined in Fig. 9, the transition to state 2 can be explained by redox-controlled phosphorylation of LHC II, as follows. Where light 2 drives PS II momentarily faster than PS I, plastoquinone becomes reduced, the LHC II kinase is activated, LHC II becomes phosphorylated, and phospho-LHC II moves away from PS II and supplies excitation energy to PS I instead. Conversely, the transition to state 1 occurs because light 1 drives PS I momentarily faster than PS II, plastoquinone becomes oxidised, the LHC II kinase is inactivated, and the LHC II phosphatase dephosphorylates LHC II thereby returning excitation energy to PS II.

Direct evidence in favour of this scheme [130] was provided by Telfer et al. [142] who used modulated fluorescence to follow state 1–state 2 transitions in pea thylakoids with ATP present at 0.15 mM to provide a substrate for protein phosphorylation. The fluorescence rise indicating the state 1 transition was inhibited by the protein phosphatase inhibitor NaF [142]. Moreover, state 2 was shown to be a state of high LHC

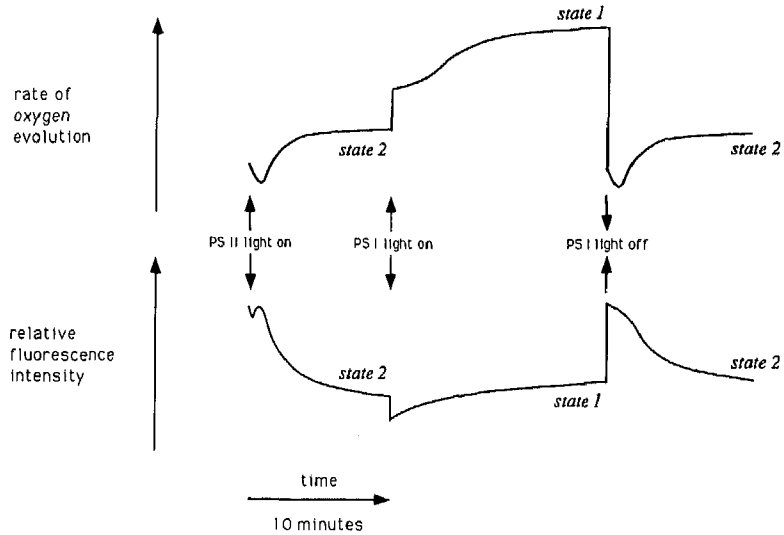

Fig. 8. Model state 1–state 2 transitions, drawn to illustrate the phenomenon, and resembling the data of Bonaventura and Myers with *Chlorella pyrenoidosa*. PS II light (e.g., $\lambda = 645$ nm) is modulated (e.g., $n = 13$ Hz) and the oxygen and fluorescence signals are obtained through a lock-in amplifier. Effects of continuous PS I light (e.g., $\lambda = 710$ nm) are therefore indirect, and indicate redistribution of excitation energy between PS I and PS II, as described in the text. For fluorescence, qualitatively similar results can be obtained with any oxygen-evolving, two-light-reaction species, from cyanobacteria and cryptomonads to leaves of higher plants. The phenomenon can be demonstrated in isolated chloroplasts, and in isolated thylakoids provided ATP is present (at e.g., 200 μM) as a substrate for the LHC II kinase. The wavelengths described above are suitable for LHC II-containing organisms. In phycobilin-containing organisms PS II light should be specific for phycobilin absorption (e.g., within the range 500–610 nm) and any chlorophyll-absorbed light (e.g., blue, centred on 440 or 480 nm, or red, above 640 nm) will function as PS I light. A terminal electron acceptor must be available (e.g., $NADP^+$ regenerated by assimilatory reactions in intact systems) if light absorbed by the PS I antenna is to function as PS I light, and the light intensity should not be saturating. At saturating intensities or in the absence of PS I acceptors, any light functions as 'PS II light' by reducing plastoquinone.

II phosphorylation and state 1 a state of low LHC II phosphorylation, with the kinetics of changes in LHC II phosphorylation matching exactly the kinetics of the fluorescence changes, with a half-time of 4 min for the state 2 transition and 6 min for the state 1 transition [142]. Only the 9 kDa phosphoprotein (subsection II-B) and another polypeptide at 40–50 kDa (see subsection II-D) showed similar kinetics of phosphorylation-dephosphorylation [142].

Other schemes put forward to explain light-state transitions have been reviewed elsewhere [31,32]. Markwell et al. [143] described a stimulation of LHC II phosphorylation by Zn^{2+} ions at low ATP concentrations without a corresponding increase in chlorophyll fluorescence at room temperature or change in low-temperature fluorescence emission spectra indicating redistribution of excitation energy between PS I and PS II. They suggested [143] that this indicated the absence of a direct relationship between LHC II phosphorylation and state 1–state 2 transitions. However, Zn^{2+} binds tightly to ionizable groups on thylakoids and may therefore inhibit structural and functional effects of phosphorylation [144]. In contrast, Farchaus et al. obtained a clear correlation between inhibition of LHC II phosphorylation and of the ATP-induced fluorescence decrease by the ATP analogue 5′-*p*-fluorosulphonylbenzoyladenosine (FSBA) [144].

The most obvious and direct function of state transitions would be to allow photosynthetic cells to maintain high efficiency of photosynthesis, despite otherwise detrimental changes in spectral composition of incident light at limiting intensity. Such changes in spectral composition can occur in response to variations in shading in both terrestrial [145] and aquatic [146] environments, where shading causes selective attenuation of PS II light. In terrestrial environments reflection can have similar effects [147]. McTavish [148] used 77 K fluorescence spectroscopy to demonstrate induction of a state 1 transition upon shading of plants and algae, and induction of state 2 by direct sunlight. Water by itself had no effect on excitation energy distribution in green algal cells [148].

In natural environments it may often be more important for plants or algae to be able to dissipate excitation energy under transient conditions of high irradiance [145]. Dissipation of excitation energy would result from decreased antenna size of PS II without a complementary increase in antenna size of PS I, though increased antenna size of PS I would tend to minimize photoinhibition of PS II provided assimilatory reactions were able to accommodate increased PS I electron transport. For this reason additional reactions that determine whether phospho-LHC II can provide excitation energy to PS I (section II-A.2.) make good physiological sense. A mechanism for switching between excitation energy transfer to PS I and non-photochemical quenching would be expected to determine whether the state 2 transition functions to in-

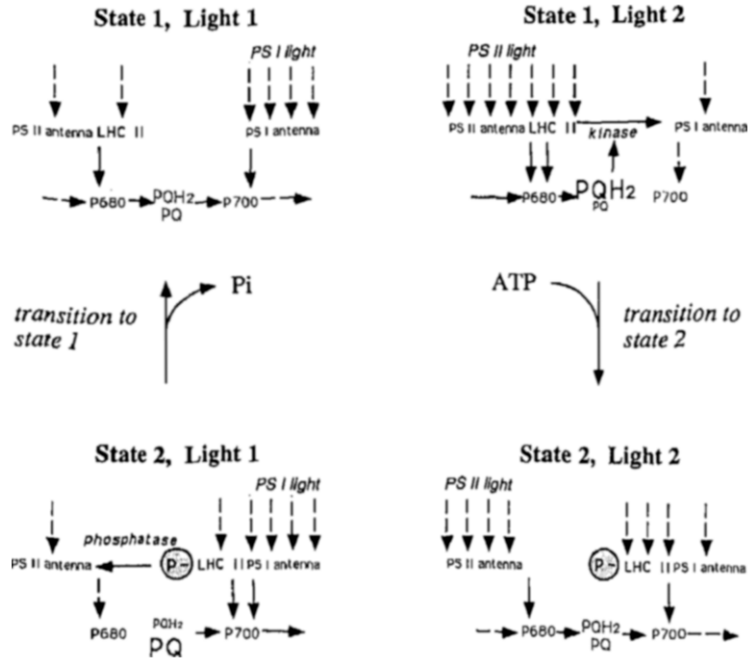

Fig. 9. A model for state 1–state 2 transitions (e.g., Fig. 8), based on redox control of excitation energy distribution via phosphorylation (state 2 transition) and dephosphorylation (state 1 transition) of LHC II, as depicted in Fig. 7. The redox state of the plastoquinone pool is indicated qualitatively by the relative size of the symbols PQ and PQH_2. The changes in fluorescence and oxygen evolution in the experiment of Bonaventura and Myers (Fig. 8) are predicted by moving clockwise around the model, starting with the scheme in the upper left-hand section (for state 1, light 1).

crease or decrease quantum yield of photosynthesis, that is, to conserve or dissipate excitation energy. Such a reaction would be expected to respond to the balance between light energy capture and the capacity of the system for energy storage by assimilatory metabolism.

II-A.5. Regulation of photophosphorylation and assimilatory metabolism

Even at constant irradiance and spectral composition it is possible to imagine a functional role for LHC II phosphorylation as both an energy conserving and an energy dissipating process. This is because the metabolic capacity for utilization of ATP and NADPH may vary according to other internal and external environmental constraints, and LHC II phosphorylation may then serve to control the efficiency of ATP and NADPH production where altered stoichiometry would not otherwise be possible without decreased quantum yield. The specific role of protein phosphorylation in relation to other regulatory processes in carbon assimilation has been discussed by Horton, Foyer and co-workers [149–150].

One area where redox control of the LHC II kinase might be sufficient to explain adaptations to variable assimilatory requirements concerns regulation of the balance between non-cyclic electron transport generating both NADPH and ATP and PS I cyclic electron transport generating ATP alone [27]. An increase in PS I cyclic photophosphorylation will require redistribution of excitation energy to PS I if maximum efficiency is to be obtained [151]. This could result from reduction of plastoquinone by donation of electrons from the acceptor side of PS I. Thus factors increasing the demand for ATP would be expected to increase LHC II phosphorylation and induce a state 2 transition. Increased LHC II phosphorylation has been observed on addition of uncouplers at low concentration, mimicking increased metabolic demand for ATP [152–153]. Hypothetical figures for quantum yields of ATP synthesis by non-cyclic, pseudocyclic and PS-I-cyclic photophosphorylation with and without a mechanism for redistribution of excitation energy illustrate that PS-I cyclic is energetically advantageous only when excitation energy distribution can be shifted towards PS I, since otherwise energy would be wasted exciting PS II [151]. In the absence of this shift an amplification of energetic losses by over-excitation of PS II should be expected to arise from over-reduction of the PS I cyclic chain and from photoinhibition of PS II electron transport.

Addition of pyruvate to intact maize mesophyll chloroplasts increases demand for ATP, and has been shown by Horton et al. [154] to stimulate LHC II phosphorylation as predicted by redox control at the level of plastoquinone. Increased enhancement on addition of pyruvate in these experiments [154] indicates increased imbalance in excitation energy distribution which may occur because of the increased rate of return of electrons to plastoquinone by PS I cyclic electron transport. Since altered metabolic demand for ATP will determine relative rates of cyclic and non-cyclic electron transport purely by kinetic means, it is possible that redox control of the LHC II kinase is sufficient to explain the induction of LHC II phosphorylation under conditions of increased ATP demand. Nevertheless, Horton and co-workers interpret such effects as evidence in favour of direct control of the LHC II kinase by thylakoid ΔpH [154]. Direct control of LHC II phosphorylation by ΔpH has also been supported by the in vivo studies of Demmig et al. [155]. Clearly ΔpH decreases as the ATP/ADP ratio declines on addition of an extra ATP sink. However, because of the coupling of NADPH oxidation and ATP hydrolysis in the Calvin cycle, additional ATP demand would also be expected to lead to an increased NADPH/NADP ratio and hence to reduction of plastoquinone via the PS I cyclic chain. Redox activation of the LHC II kinase may therefore account for the inverse correlation between LHC II phosphorylation and thylakoid ΔpH. Thylakoids reconstituted with soluble stromal extract have also been used to probe such effects [156]. Similar considerations apply to the observed effect of oxaloacetate addition in causing dephosphorylation of LHC II in intact chloroplasts [157].

In unicellular algae, which are metabolically flexible, redox control of LHC II phosphorylation may be particularly important in adaptation to altered metabolic demand for ATP. Using fluorescence spectroscopy Turpin and Bruce [158] have shown that addition of NH_4^+ produces a state 2 transition in the green alga *Selenastrium minutum*. They interpret this effect entirely in terms of redox control of the LHC II kinase coupled with increased PS I cyclic as NH_4^+ assimilation increases cellular demand for ATP. Similar effects occur in cyanobacteria though there is evidence there for addition levels of coupling between photosynthetic electron transport and nitrogen metabolism (subsection III-A.3).

Bulté et al. have shown control of state transitions by ATP demand in the green alga *Chlamydomonas reinhardtii* [159]. They found that a variety of treatments inhibiting mitochondrial oxidative phosphorylation induced a state 2 transition, as judged by fluorescence emission properties as well as ^{32}P-labelling of LHC II in vivo. Bulté et al. [159] propose that state 1–state 2 transitions are concerned primarily with maintaining balanced production of ATP and NADPH. As proposed above, it is perhaps more likely that ATP : NADPH ratios change for purely kinetic reasons in response to altered metabolic demand, and that

phosphorylation-dephosphorylation of LHC II maintains quantum yield [27,151].

The existence of chloroplast NAD(P)H dehydrogenases [160] is also a factor that must be expected to influence dark plastoquinone redox state and therefore to control thylakoid protein phosphorylation. Bulté et al. [159] suggest that dark NAD(P)H-plastoquinone oxidoreductases in chlororespiration [160] may serve to maintain state 2 in vivo in readiness for PS I cyclic phosphorylation which supplies ATP for the onset of assimilatory reactions upon illumination.

Control of excitation energy distribution via LHC II phosphorylation by spectral composition on the one hand and by relative ATP demand on the other are in no way mutually exclusive. This single series of protein phosphorylation-controlled regulatory reactions can legitimately be viewed as a short-term mechanism serving to readjust relative excitation of PS I and PS II whenever this ceases to be optimally adjusted, and for whatever reason.

II-A.6. Lateral heterogeneity and mobility of phospho-LHC II

Chloroplast thylakoids exhibit heterogeneity in lateral distribution of their intrinsic protein complexes [161]. In particular, aqueous two-phase partition of fractionated thylakoids developed by Albertsson [162] indicates a marked heterogeneity in the distribution of PS I and PS II. Earlier studies based on differential centrifugation had led to the conclusion that PS II was exclusively located in grana which it shared with PS I, while PS I was also found in stroma lamellae, allowing a compartmentalization of non-cyclic photophosphorylation in grana and PS-I-cyclic in the stroma [163–164].

The phase partition technique of Albertsson and co-workers separates vesicles on the basis of surface charge properties in turn dependent on orientation, and has also been useful in probing the transverse organisation of the thylakoid membrane. Lateral heterogeneity is apparent as a greater density of PS II in inside-out vesicles originating from appressed domains of the thylakoid membrane and corresponding to the grana or thylakoid stacks than in right-side-out vesicles originating from unstacked stroma-exposed thylakoids, and as the opposite distribution for PS I, present predominantly in unstacked thylakoids [165–166]. There are further heterogeneities within both PS I and PS II [161], notably a localisation of PS II-α (as described in subsection II-A.2.) in appressed membranes and PS II-β in unappressed membranes [167–169] in addition to heterogeneities within PS II-α [161]. The proposed lateral distribution of these subpopulations is shown in Fig. 10.

If phosphorylation of LHC II polypeptides decreases the interaction of LHC II with PS II and

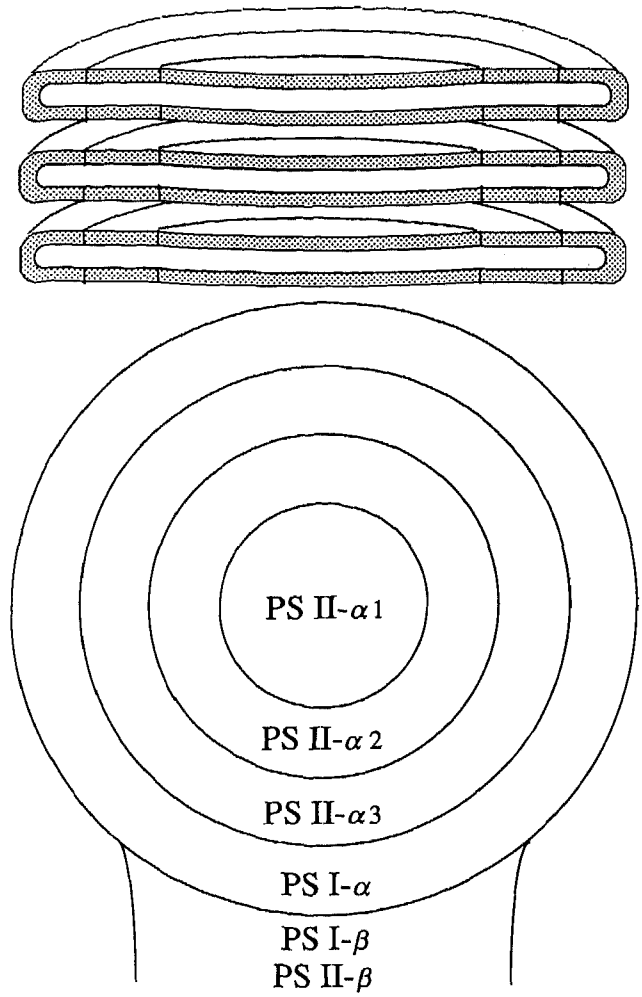

Fig. 10. The model of Albertsson et al. for the organization of the thylakoid membrane of higher plant chloroplasts. Above is an end projection of three stacked thylakoids, below is a plan which shows the proposed domains. PS II-α_1, PS II-α_2 and PS II-α_3 stand for domains with PS II-α units having different antenna sizes, such that $\alpha_1 > \alpha_2 > \alpha_3$. This could also correspond to an order of increasing degree of phosphorylation of LHC II, $\alpha_1 < \alpha_2 < \alpha_3$. PS I-$\beta$ and PS II-β are located in the unappressed, stroma domain. PS I-α is involved in non-cyclic electron transport. The state 2 transition could therefore depend primarily on lateral movement of phospho-LHC II within the grana stack, from inner domains to the margin. Other movements and interconversions may occur - c.f. Fig. 6. Redrawn from Ref. 161.

increases its interaction with PS I (subsections II-A.2.–4) then lateral reassociation of the three complexes must occur. In principle, this could take the form of movement of PS I into LHC II-rich domains or of movement of LHC II into PS-I-rich domains. The latter mechanism commands most experimental support, and is in accord with the idea of complementary absorption cross-section changes (II-A.2). The true domain organisation of the thylakoid may be more complex than this simple bipartite model suggests, however, with six discrete domains being possible [161]. The grana margin [170–171] may be an important site

of protein traffic and of alteration in protein-protein interactions.

Andersson et al. [172] labelled intact spinach chloroplasts using [^{32}P]P_i according to the procedure of Bennett [15], isolated thylakoids by osmotic lysis and then subjected the thylakoids to Yeda press treatment and the fragments to aqueous phase partition. They found a six-fold higher specific activity of LHC II polypeptides (at 23 and 25 kDa) in the 'Y-100' fraction of vesicles derived from unstacked thylakoids than in the 'B3' fraction from grana stacks, although most of the LHC II material as judged by Coomassie brilliant blue staining was located in the grana stacks, along with PS II polypeptides. They concluded that the small pool of LHC II in non-appressed thylakoids is considerably more phosphorylated after illumination than the larger LHC II pool in appressed thylakoids, but were unable to decide on these results alone whether the pattern of labelling arose from lateral migration of phospho-LHC II from appressed to unappressed thylakoid domains or from a greater kinase activity in the latter.

Kyle et al. [173] used both digitonin-solubilized and French press-treated pea thylakoids labelled in vitro with [γ-^{32}P]ATP and subsequently fractionated by differential centrifugation according to the earlier protocols [163]. When conditions were right for LHC II phosphorylation, they found that stromal vesicles showed a decrease in chlorophyll *a*/*b* ratio, an increase in LHC II polypeptides judged by Coomassie blue staining, and an increase in energy transfer from chlorophyll *b* to PS I as judged from 77 K fluorescence emission and excitation spectra. The grana fraction showed the complementary changes indicating loss of a portion of the LHC II pool upon phosphorylation. They proposed that a proportion of LHC II acts as a 'mobile antenna' which migrates upon phosphorylation from grana to stroma domains of the thylakoid membrane [23,173]. Kyle et al. also found an apparent decrease in the PS I content of the stroma fraction upon phosphorylation of LHC II [173], keeping open the additional possibility of some movement of PS I.

Freeze-fracture electron microscopy carried out on barley [174] and pea [175] thylakoids gives additional support to the concept of the mobile antenna. Simpson [174] observed a change in particle density and size distribution between unstacked and stacked membrane regions that suggested movement of LHC II-containing particles from grana to stroma membranes upon phosphorylation of LHC II polypeptides. He also saw an increased number of larger particles on the protoplasmic (intrathylakoid) fracture face of stacked domains suggesting either a conformational change in LHC II or inward migration of PS I [174]. The change in particle density was consistent with a 5% decrease in stacking upon phosphorylation. Similar results were obtained by Kyle et al. [175], who identified as LHC II an 8 nm particle which became depleted from stacked domains under conditions favouring phosphorylation. Both groups carried out phosphorylation at 5 mM Mg^{2+}, which may be sufficiently low for LHC II phosphorylation to have an effect on stacking. A further conclusion from freeze-fracture electron microscopy [174–175] is the absence of movement of PS II itself, which is additional evidence in favour of an altered absorption cross-section of PS II and against a phosphorylation-regulated change in spillover of excitation energy from PS II to PS I (subsection II-A.2).

Thylakoid fractionation studies also give support to the functional interaction of phospho-LHC II with PS I. Fluorescence spectroscopy [173], electron transport measurements [88,94] and incorporation of LHC II polypeptides into PS I preparations from stroma membranes [175] are consistent therefore with the energy-conserving function of LHC II phosphorylation by redirection of excitation energy to PS I (subsections II-A.2. and II-A.4.) at least under the experimental conditions employed.

Bassi et al. [176] carried out a comprehensive study of effects of LHC II phosphorylation on the properties and organisation of purified stroma membranes from maize mesophyll chloroplasts isolated from plants in darkness and illuminated by high intensity white light. The combined data from fluorescence and absorbance spectroscopy, electron transport, electrophoresis, and freeze-fracture electron microscopy leaves little room for doubt that the illumination conditions gave rise to light states 1 and 2 respectively. NaF was present to inhibit dephosphorylation and the thylakoid reaction medium contained 10 mM Mg^{2+} to maintain stacking. They observed an increase in the chlorophyll/P-700 ratio corresponding to 21–24 chlorophyll molecules as LHC II becoming functionally attached to the PS I reaction centre, a value in agreement with an increased PS I antenna size of 15% apparent from kinetics of P-700 photooxidation [176]. Freeze-fracture electron microscopy showed an increased particle density consistent with a 4% decrease in stacking, but small particles associated with LHC II did not appear, which the authors attribute to incorporation of LHC II into larger PS I particles. Incorporation of LHC II into PS I is also suggested by fluorescence spectroscopy [176], as in the work of Kyle et al. [173]. Four distinct LHCPs became phosphorylated and appeared in stroma thylakoids, while the same LHCPs were retained in grana thylakoids provided an additional, unphosphorylated 26 kDa polypeptide was also present [176], a result pointing to an organisation of LHC II into discrete mobile and immobile pools (subsection II-A.7).

Kohorn and Yakir [177] used in vitro synthesis and incorporation of pre-LHCP [80] specifically to address the question of LHC II mobility. They used a detergent fractionation technique to separate stacked and un-

stacked thylakoids from chloroplasts which had taken up, processed and incorporated ^{35}S-labelled pre-LHCPs, and they obtained a preferential distribution of newly-incorporated LHCPs consistent with lateral movement from unstacked to stacked membranes. Replacement of threonines 6 and 7 with alanines was without effect on either incorporation or lateral movement, though it increased the polypeptides' breakdown by an endogenous protease. Kohorn and Yakir [177] concluded that phosphorylation at threonines 6 or 7 can have no effect on lateral migration of LHCPs, and furthermore that phosphorylation is unrelated to state transitions, proposing that LHCP turnover is involved instead. There are several alternative explanations of their results, however. The first is that lateral migration of functional LHC II upon phosphorylation of LHCPs is unrelated to movement, in the opposite direction, of newly-processed polypeptides, and governed by different intermolecular forces. Another possibility is that newly-incorporated LHCPs resistant to phosphorylation could be moved by phosphorylation of pre-existing polypeptides of the LHC IIs into which they are taken up. The obvious way to test functional effects of replacement of the phosphorylation site is to carry out state transition measurements on the modified and unmodified chloroplasts. Kohorn and Yakir [177] report light-dependent changes in modulated chlorophyll fluorescence that indicate state 1–state 2 transitions, but only for the control (unmodified) chloroplasts. What happens to state transitions in the modified chloroplasts is crucial for the interpretation that Kohorn and Yakir provide, and it would be interesting to know the outcome of such an experiment.

LHC II mobility can be suppressed at low temperatures (e.g., 0–5°C) without effect on phosphorylation of LHC II [178–179], which demonstrates that decoupling of LHC II from PS II and lateral migration may be separate events (see Section IV). This inhibition of migration of LHC II may be a contributory factor in increased sensitivity to photoinhibition at low temperatures [178–179].

II-A.7. Function and relative mobility of LHC II isomers

LHC IIs contains a number of different LHCP subunits, all in the 24–30 kDa region of molecular mass [180] and encoded by a family of nuclear *cab* genes [68]. It is apparent from gel electrophoresis of grana and stroma membrane proteins that there is a heterogeneity in the polypeptide composition of LHC IIs [181,182] analogous to the phenomenon of isoenzymes.

Sub-populations of LHC II with different phosphorylation states and different mobilities were reported for pea by Kyle et al. [173], though differences in specific activity between the sub-populations may simply reflect the proportion of unphosphorylated LHCPs they contain [183]. Andersson and co-workers resolve two main classes of LHCPs in spinach, at 25 and 27 kDa [184–186]. Data on relative lateral mobilities suggest a higher proportion of lower-MW polypeptides (25 kDa) in the mobile, peripheral LHC II pool than in the pool tightly bound to PS II [184–185]. In addition, the kinetics of phosphorylation may differ between the two pools, with phosphorylation of the mobile pool more closely matching the kinetics of the state 2 transition [185]. Larsson et al. [185] propose that phosphorylation of both inner and peripheral LHC II pools may be required for detachment of the peripheral LHC II and its lateral migration, with the unphosphorylated CP29 polypeptide acting as a spacer to prevent detachment of the inner pool from the phosphorylated PS II core. Increased temperature may also serve to detach the mobile pool from PS II and permit it to migrate into unappressed thylakoid domains [185], and it is also seen that long-term adaptation to low light increases total LHC II size largely by increasing the proportion of 25 kDa LHCPs [186].

Other groups obtain different patterns for the two pools, some of which may be attributable to different isolation and fractionation techniques, different gel systems, different plant species, or to a combination of such factors. Islam [183] describes more rapid phosphorylation of a 24 kDa polypeptide compared with a 25 kDa polypeptide in spinach, and concludes the immobile PS-II-bound pool may be further subdivided into phosphorylated and non-phosphorylated pools. Dunahay et al. [187] observed specific labelling of only one LHCP in spinach thylakoids, the LHCP comigrating on non-denaturing gels with an unphosphorylated 27 kDa polypeptide. Bassi et al. [188] working with maize resolve four phosphorylated polypeptides and a fifth, unphosphorylated polypeptide of 26 kDa which is seen only in the immobile pool. Further characterisation by flat-bed electrofocussing [188] showed seven polypeptides, with phospho-LHC II comprising 30% of the total chlorophyll-*a*/*b*-binding polypeptides and the mobile fraction 20% of total LHC II chlorophyll. The 26 kDa polypeptide of the immobile, inner pool of maize is characterised by absence of an N-terminal segment containing the phosphorylation site [188].

Di Paulo et al. [189] carried out immunological studies on maize thylakoids fractionated by French press treatment and differential centrifugation, and their results confirm a lateral heterogeneity for PS I and PS II chlorophyll-protein complexes in addition to LHC II complexes. Upon LHC II phosphorylation during the state 2 transition, lateral heterogeneity was maintained for all complexes with the exception of a specific LHC II pool, lacking the 26 kDa polypeptide. This pool was seen to migrate from grana to stroma membranes upon phosphorylation. Bassi and Dainese [190] carried out an extensive analysis of LHC II poly-

peptides isolated by different techniques from maize. They propose three LHC II sub-populations: LHC II *a* bound to the core antenna CP43; LHC II *b* also connected to CP43 but via the non-phosphorylatable CP26; and LHC II *c* which connects to CP43 via the unphosphorylated CP24 and CP29, and which comprises the mobile pool that dissociates from PS II completely upon phosphorylation and part of which then connects with PS I via LHC I.

The question arises whether the LHC II isomers contain different proportions of different gene products or are the result of altered processing or turnover at some stage of synthesis or assembly. Lamppa et al. [191] describe import into pea chloroplasts of a single wheat pre-LHCP synthesised in vitro. They find evidence for two processing sites. Cleavage at the primary site results in removal of the 34 amino acid transit peptide leaving a mature polypeptide of 26 kDa. Cleavage at the secondary site results in removal of both the transit peptide and an N-terminal segment giving a mature polypeptide of 25 kDa. Lamppa et al. [191] suggest that this smaller polypeptide gives rise to the mobile pool of LHC II [181–182,184–185], since the secondary cleavage removes a basic segment and renders the mature protein more susceptible to charge repulsion by neighbouring phosphoproteins. However, since the phosphorylation site is located within the N-terminal segment that would be removed by secondary cleavage [13,73], then one would expect it not to be phosphorylated, which the polypeptides of the mobile LHC II pool clearly are [181–185] The possibility exists that the secondary cleavage site gives rise instead to the unphosphorylated polypeptide characteristic of the immobile LHC II pool and described as CP 26 by Bassi et al. [190].

In contrast to the suggestion of specific processing, Jansson et al. [192] propose that the 27 kDa and 25 kDa LHCPs which predominate in the inner, immobile, and outer, mobile pools [182,185,188] respectively are products of different genes. Amino acid sequencing of proteolytic fragments from each type of LHCP correspond to nucleotide sequences of different types of *cab* gene. Type 1 and type 2 *cab* genes are distinguished by sequence differences and by the presence of an intron in type 2. Using sequences from Scots pine [193], Jansson et al. identify the 27 kDa LHCPs as products of type 1 genes and 25 kDa LHCPs with type 2 genes. This proposal strengthens the analogy with isozymes, and suggests that long-term adaptation of the LHC II system [e.g., 186] according to irradiance and spectral composition could arise from selective transcriptional control of gene expression.

II-A.8. Phosphate group donors

The thylakoid LHC II kinase shows high specificity for ATP among nucleoside phosphates, with a K_m for ATP of 90 μM or less [13,81,194] compared to 600 μM for GTP [194]. The kinase will also use ATP analogues such as ATP-γ-S [195] (see also subsection II-D.4).

Pramanik et al. have demonstrated phosphorylation of LHC II and other spinach thylakoid polypeptides in vitro using labelled pyrophosphate ([^{32}P]PP_i) in place of [γ-^{32}P]ATP [196]. The apparent K_m for PP_i-dependent phosphorylation is 50–80 μM, which is within the physiological range of stromal PP_i concentration [197]. However, the maximum extent of protein phosphorylation with PP_i is only 3–5% of that with ATP [197]. PP_i-dependent protein phosphorylation is inhibited by DCMU and by the electron acceptor phenyl-*p*-benzoquinone, suggesting redox control at the level of plastoquinone. It is also stimulated in darkness by reducing agents such as reduced ferredoxin or dithionite, but only to 10–15% of the activity obtained in the light, in contrast to ATP-dependent phosphorylation which is fully activated by reducing agents in darkness [130]. The reaction is not inhibited by inhibitors of photophosphorylation or stimulated by cofactors of cyclic photophosphorylation, which eliminates the possibility that ATP is being made by photophosphorylation using [^{32}P]P_i generated by hydrolysis of [^{32}P]PP_i. Unlabelled ATP stimulates the labelling with [^{32}P]PP_i within a narrow ATP concentration range at about 100 μM, while at about 400 μM ATP is inhibitory, with complete inhibition above 2 mM. The stimulation at low ATP concentrations prevents analysis of the mode of inhibition [197].

Pramanik et al. propose that the phosphorylation sites on the thylakoid proteins are the same for pyrophosphate phosphorylation as for ATP phosphorylation [197]. Although care was taken to exclude stromal contamination, there may remain the possibility that the apparent pyrophosphate-LHC II phosphotransferase is the result of the LHC II kinase operating in conjunction with a phosphate exchange mechanism which produces [γ-^{32}P]ATP at low concentrations. This could account for the low levels of labelling, and explain the stimulation by low concentrations of ATP. Labelling of LHC II by this route need not involve photophosphorylation. Phosphate exchange is an established method of enzymatic synthesis of [γ-^{32}P]ATP [198], and is know to occur as a light-stimulated reaction in isolated chloroplasts [199]. Certainly pyrophosphate-dependent protein phosphorylation would be an important additional regulatory process, especially in photosynthetic prokaryotes where pyrophosphate functions as an energy storage compound analogous to ATP [200]. Preliminary attempts to demonstrate pyrophosphate labelling of cyanobacterial thylakoid and purple bacterial chromatophore proteins in vitro using protocols reported earlier for spinach thylakoids [201] have been unsuccessful (Holmes, N.G., Sanders, C.E. and Allen, J.F., unpublished data).

II-B. The psbH gene product or 9 kDa phosphoprotein

II-B.1. Occurrence, location and regulation of phosphorylation

A thylakoid membrane polypeptide with an apparent molecular mass of 9 kDa was reported as one of the substrates of chloroplast protein phosphorylation in the original studies of Bennett [11]. This polypeptide is phosphorylated in the same way as the 25 kDa LHC-II polypeptide [13], that is, in a light-dependent reaction [14] catalysed by a membrane-bound kinase [13] and with threonine as the phosphate group acceptor [11]. Dephosphorylation of the 9 kDa polypeptide is generally slower than that of LHC-II [202,203] and requires higher Mg^{2+} concentrations [15] but it is nevertheless thought to be catalysed by the same membrane-bound phosphatase [15].

Phosphorylation of the 9 kDa polypeptide appears to be under the same kind of redox control as phosphorylation of LHC II. In the experiment of Telfer et al. [142] on correlation of protein phosphorylation with state transitions in pea thylakoids supplied with ATP, the 9 kDa phosphoprotein became phosphorylated with apparently the same kinetics as that of LHC II [142]. Furthermore, the redox titration of the LHC II kinase [133] gave an identical curve whether labelling of the 9 kDa polypeptide or LHC II was plotted as a function of redox potential (Allen and Horton, unpublished). This effect can be seen in single autoradiographs of PAGE tracks corresponding to samples incubated in a range of redox potentials close to 0 mV [133].

The 9 kDa phosphoprotein has been proposed as subunit III of CF_0 (the 'DCCD-binding protein') [204] and also the apoprotein of cytochrome b_{559} [205,206]. The former suggestion is inconsistent with the location of the phosphoprotein as a component of Photosystem II [105,128,207–208], and both are inconsistent with subsequent determinations of amino acid composition and sequence.

Allen and Findlay reported an amino acid composition of the 9 kDa phosphoprotein of pea thylakoids [209] which was quite different from compositions predicted from published sequences of the genes for CF_0 subunit III and cytochrome b_{559}. They proposed that the 9 kDa phosphoprotein is a chlorophyll-binding protein structurally related to a segment of LHC-II. By analogy with the known amino acid sequence of LHC-II and structural predictions based thereon [69], it was specifically suggested [209] that the 9 kDa phosphoprotein has a structure similar to that of the part of LHC-II polypeptides represented by its N-terminal, surface-exposed segment together with the first hydrophobic domain.

Farchaus and Dilley [210] obtained an amino acid composition for the spinach 9 kDa phosphoprotein isolated from Photosystem II particles. They also ruled out CF_0 subunit III and cytochrome b_{559} on the basis of their analysis. Farchaus and Dilley also obtained an N-terminal sequence of nine amino acid residues, but draw no comparison with LHC-II, concluding that the 9 kDa phosphoprotein was a unique, as yet unidentified, polypeptide associated with Photosystem II [210].

II-B.2. Sequence determination, phosphorylation site and distribution of psbH genes

The amino acid sequence obtained by Farchaus and Dilley [210] shows homology with one predicted from the nucleotide sequence of an open reading frame seen in the complete sequence of chloroplast DNA from tobacco [211] and the liverwort *Marchantia polymorpha* [212]. Amino acids 41 to 60 of the tobacco ORF 73 sequence [211], also designated *psbH* [213–216], have non-polar side chains, and this segment corresponds to a helical region according to structural predictions, consistent with the gene product being a membrane protein.

In higher plants, *psbH* genes have also been sequenced from rice [213], barley [214], spinach [215] wheat [216] and rye [217]. For rice the gene can be mapped precisely since a complete rice chloroplast DNA sequence is now also known [218]. In these species the predicted relative molecular mass of the *psbH* gene product is 7.8 kDa. Until a name signifying function can be agreed, the term '9 kDa phosphoprotein' is therefore not too misleading and is retained here on grounds of common usage, though '8.3 kDa' and '10 kDa' are also encountered. The gene itself was first designated, inappropriately, *psbF* [211,213]. *psbH* genes have also now been identified and sequenced in two species of cyanobacteria [219–221]. These sequences confirm that the segment containing the phosphorylation site in chloroplasts (subsection II-B.2) is missing in cyanobacteria [219], as discussed in subsection III-A.4.

The phosphorylation site of the 9 kDa phosphoprotein is the threonine in position 2. This conclusion was first established for the spinach protein by Michel and Bennett [222], who obtained from PS II particles a tryptic fragment of ten amino acids corresponding exactly to the sequence obtained by Farchaus and Dilley [210]. Trypsin treatment removes all the label [15] indicating that trypsin cleaves a fragment containing the phosphorylation site. The fragments were separated by Fe^{3+}-chelating Sepharose affinity chromatography and reversed-phase HPLC, with ^{32}P-labelling being almost wholly concentrated in threonine 2 [222]. The same conclusion was reached for *Chlamydomonas reinhardtii* by Dedner et al. [223] who obtained a 48 amino acid sequence from the N-terminus. The *Chlamydomonas* sequence contains an insert of 7 amino acids and is missing 4 amino acids near the N-terminus

in comparison with the liverwort and higher plant sequences [223], as shown in Fig. 11. Despite these major differences at the N-terminus, the phosphorylation site and a cluster of basic amino acids beginning 7 amino acids afterwards are conserved. The possible significance of the conserved basic amino acids also found in reaction centre proteins is discussed in subsection IV-C. An analogous segment of the *R. viridis* H chain [224] is also shown in Fig. 11.

II-B.3. What is its function in Photosystem II?

There is currently no agreement concerning the function of the 9 kDa phosphoprotein or the regulatory significance of its phosphorylation. Effects of thylakoid protein phosphorylation in light-saturated PS II electron transport rates have been attributed specifically to phosphorylation of the 9 kDa protein by Packham [225]. Packham also suggests an analogy with the H subunit of the purple bacterial reaction centre (subsection I-C) which would help to account for the proposed effects of its phosphorylation on binding of Q_A on the acceptor side of PS II [226]. By this model [226] inhibition of electron transfer out of PS II might occur by repulsion of Q_A^- by the phosphothreonine. A prediction of this hypothesis is therefore that the N-terminal phosphothreonine should be in close proximity to the quinone-binding pocket of D2.

This effect might also contribute to an explanation of a role of the phosphoprotein in photoinhibition of electron transport in PS II. Kuhn et al. [227] observed a selective loss of the 9 kDa phosphoprotein upon high light treatment of spinach PS II particles, and suggest that the protein is involved in the fluorescence decrease that is an early event in photoinhibition. Switching of electron flow on the acceptor side of PS II could be imagined, for example from Q_A to cytochrome b_{559} instead of to Q_B, a mechanism of protection from photoinhibition proposed by Brudvig [45]. A role for phosphorylation in photoinhibition might also be supported by the observation of Sundby [228] who observed that 20 mM bicarbonate provides protection from photoinhibition at the same time as selectively decreasing phosphorylation of the 9 kDa protein in isolated spinach thylakoids. Sundby et al. [229] suggest that the N-terminal cluster of basic amino acids (see Fig. 11) might provide a binding site for the bicarbonate anion, which could account for competition between bicarbonate and phosphorylation. For the same reasons a protective effect of phosphorylation from photoinhibition of PS II electron transfer might be produced in vitro by bicarbonate binding as an alternative method of charge compensation.

The proposal of Allen and Findlay [209] that the 9 kDa phosphoprotein is involved in regulation of excitation energy transfer might also be compatible with a functional role in protection from photoinhibition. Some support for a role for the 9 kDa phosphoprotein in regulation of excitation energy distribution is provided from the observation of Hird et al. of limited sequence homology between the N-terminus of LHCPs and amino acid residues 27 to 63 of the 9 kDa phosphoprotein [216]. However, Black et al. concluded from a study of intermittent-light-grown pea thylakoids (which are depleted in LHC II) that phosphorylation of the 9 kDa protein is at all times independent of ATP-induced chlorophyll fluorescence quenching, the latter reflecting only the phosphorylation state of LHC II [230]. In thylakoids from intermittent-light-grown peas the apparent K_m of the 9 kDa kinase activity is lower (100 μM) than in mature, LHC II-containing thylakoids (500 μM). In contrast, during greening of intermittent-light-grown peas the apparent K_m of the LHC

psbH gene product:

	1 5 10
Tobacco	M A **T*Q** T V E N S S **R** S G P **R R**
Spinach	A **T*Q** T V E S S S **R** S **R** P **K** P
Chlamydomonas (spaces added)	A **T*G** T - - - - S **K** A **K** P S **K** V N S D
Chlamydomonas	A **T*G** T S **K** A **K** P S **K** V N S D

R. viridis reaction centre H polypeptide:

M Y H G A L A Q H L D I A Q L V W Y A Q W L V I W **T** V V L L Y L **R R** E D **R R** E

1 5 10 15 20 25 30 35

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periplasm *helix* *cytoplasm*

Fig. 11. Aligned N-terminal sequences of three chloroplast *psbH* gene products. * indicates a known phosphorylation site. Phosphorylated threonines and basic amino acid residues are shown in bold. An analogous segment of the H-subunit of the reaction centre of *R. viridis* is also shown.

II kinase decreases (from 100 μM to 40 μM). This means that the 9 kDa phosphoprotein becomes the most conspicuous phosphoprotein of intermittent-light-grown pea thylakoids [230]. The idea of involvement of the 9 kDa phosphoprotein in regulation of excitation energy transfer is also called into question by Farchaus et al. [144] on the basis of experiments with the ATP analogue and protein kinase inhibitor 5′-*p*-fluorosulphonylbenzoyladenosine (FSBA). FSBA treatment giving 95% inhibition of LHC II phosphorylation but only 35% inhibition of 9 kDa phosphorylation gave complete inhibition of ATP-dependent chlorophyll fluorescence quenching [144]. However, the results of both Black et al. [230] and Farchaus et al. [144] indicate only that LHC II phosphorylation is necessary for functional effects such as ATP-dependent fluorescence decrease and the state 2 transition, and the possibility therefore remains that phosphorylation of the 9 kDa protein is involved. If both the 9 kDa protein and LHC II must be phosphorylated in order for the latter to dissociate from PS II, then inhibition of either phosphorylation might be sufficient to inhibit functional decoupling. One process that might make phosphorylation of both proteins necessary for function effects is suggested by the proposal of mutual electrostatic repulsion between phospho-LHC II and phosphate groups attached to PS II polypeptides such as the 9 kDa protein, as discussed in subsection IV-B. However, more recent results described by Harrison and Allen [231–232] suggest that phosphorylation of LHCPs alone is sufficient to induce a state 2 transition.

Harrison and Allen describe an experimental protocol for the separation of phosphorylation of LHC II from phosphorylation of PS II polypeptides [231–232]. This involves the preparation of phosphorylated thylakoids (with both LHC II and PS II proteins phosphorylated) followed by dark incubation for 20 min (selectively dephosphorylating LHC II), alkaline phosphatase treatment (selectively dephosphorylating PS II proteins), or both (dephosphorylating all thylakoid membrane proteins). This procedure therefore yields four membrane types: membranes with both LHC II and PS II polypeptides phosphorylated; membranes with only PS II polypeptides phosphorylated; membranes with only LHC II phosphorylated; and membranes with neither class of proteins phosphorylated. A number of informative comparisons then become possible. The idea that phosphorylation of the 9 kDa phosphoprotein is necessary for the functional decoupling of LHC II from PS II is eliminated by the full effect of phosphorylation of LHC II on light-limited PS II electron transport rates in membranes with a dephosphorylated 9 kDa protein [232]. This conclusion can be extended to the other PS II phosphoproteins (section 2.3.). Another finding is that phosphorylation of PS II polypeptides maintains PS II electron transport at high light intensities [232], a further observation consistent with a role in protection from photoinhibition. Phosphorylation of LHC II in the absence of PS II phosphorylation has two inhibitory effects on PS II electron transport, one at low light intensity characteristic of the state 2 transition, and an unexpected, second effect at high light intensity, serving to counteract the protective effect of PS II protein phosphorylation. Q_B-binding was unaffected by either type of phosphorylation [232]. Is is not possible from these results to distinguish between the PS II phosphoproteins that could be involved (subsection II-C).

Sundby et al. [233] found opposing effects of bicarbonate addition on phosphorylation of the 9 kDa phosphoprotein, which was inhibited, and on LHC II phosphorylation, which was stimulated. The stimulatory effect was specific to the 25 kDa, mobile LHCP of the spinach thylakoids used, bicarbonate having no effect on phosphorylation of the 27 kDa component. Sundby et al. [233] suggest bicarbonate concentration as a physiological factor regulating excitation energy distribution by means of LHC II phosphorylation, and serving to couple these reactions to ATP and NADPH usage in assimilation. No information was recorded on effects of bicarbonate on the other PS II phosphoproteins. The opposing effects on phosphorylation of LHC II and the 9 kDa protein [233] are reminiscent of effects of insulin on proteins regulating glycogen metabolism, where the explanation is that a protein kinase has as its substrate a protein phosphatase which it activates by phosphorylation, thereby promoting dephosphorylation of a third protein [234]. It is tempting to consider that the results of Sundby et al. [233] could be explained if the 9 kDa protein were an LHC II phosphatase that became activated by phosphorylation (subsection II-D.3). The additional possibilities of a signalling role and of a role in PS II assembly are considered in Section V.

II-C. Other phosphoproteins of chloroplast thylakoids

II-C.1. CP 43 of the PS II core antenna system

Phosphoproteins of PS II other than LHC II (subsection II-A) and the 9 kDa phosphoprotein (subsection II-B) were reported in *Chlamydomonas* by Owens and Ohad [128] and Delepelaire [208] and for pea by Steinback et al. [105]. One is CP 43 or 'CPa-2' [235], a chlorophyll-binding protein identified with the PS II reaction centre prior to the discovery of the D1-D2 complex [51] but now generally agreed to function as a PS II core antenna protein. Various models exist concerning its precise relationship with a similar protein, CP 47, with LHC II polypeptides and with the reaction centre, though it is generally agreed to function in some way (in series or in parallel with CP 47) in the pathway of excitation energy transfer from LHC II to

the reaction centre [235,236]. CP 47 is the chloroplast *psbB* gene product and CP 43 the chloroplast *psbC* gene product, the genes being located between those of D1 (*psbA*) and D2 (*psbD*) [211–212].

Ikeuchi et al. [237] used immunoelectrophoresis to identify phosphorylated PS II polypeptides isolated from pea and spinach by detergent solubilization and sucrose gradient centrifugation. They concluded that CP43, D1, D2 and the 9 kDa protein were phosphorylated [237].

Michel et al. [238] used tandem mass spectrometry of fragments from tryptic digestion of phosphorylated PS II core particles from spinach to obtain N-terminal sequences of the three PS II phosphoproteins other than the *psbH* product, and to identify their phosphorylation sites. They found the phosphorylation site of CP 43 to be threonine in position 3 (counting the N-terminal methionine). They also reported that the CP 43 kinase was redox activated at the level of plastoquinone. The phosphorylation sites were not bounded by basic amino acids as in LHC II, which they interpret as evidence in favour of a separate kinase or kinases (subsection II-D.4), but which also may indicate a separate mechanism by which phosphorylation exerts any effect on protein-protein interactions (subsection IV-C). Cyanobacterial *psbC* genes encode CP 43 proteins with a conserved threonine [239], indicating the possibility that these too could be phosphorylated (subsection III-A.4.). Unlike D1 and D2, the phosphorylation sites do not stand in any obvious relation to basic amino acid residues.

Both Ikeuchi et al. [237] and Michel et al. [238] suggest a function for phospho-CP 43 in providing negative charge for electrostatic repulsion of phospho-LHC II during the state 2 transition, in accordance with the model discussed here in subsection IV-B. As in the case of the 9 kDa phosphoprotein, however (section II-B.3), the experiment of Harrison [231–232] shows that phosphorylation of LHC II retains its effect on PS II absorption cross-section even in thylakoids from which phosphate groups have been removed from CP 43 by the action of alkaline phosphatase. CP 43 is likely to have a closer interaction with LHC II than CP 47 according to most models currently considered [236], so some effect of phosphorylation of CP 43 on binding of the inner, immobile pool of LHC II to the core of PS II is likely. Both groups [236–237] also agree that CP 47 is not phosphorylated.

In the absence of any functional change with which to correlate CP 43 phosphorylation specifically, most of the possibilities raised in connection with the 9 kDa phosphoprotein could also apply (subsection II-B.3). What is required to dissect the relative contributions of the PS II core phosphoproteins to regulation of PS II function, synthesis, or assembly, is specific inhibitors or mutagenesis directed to phosphorylation sites.

II-C.2. The reaction centre proteins D1 and D2

D1 and D2 are the products of the chloroplast *psbA* and *psbD* genes, respectively, and function together as a heterodimer binding the chromophores of the reaction centre of PS II (subsection I-C) Their phosphorylation was suggested for *Chlamydomonas* [128,208] and pea [105] along with that of CP 43, at which time they were generally described as Q_B- and Q_A-binding proteins, respectively. D1 was also described as a herbicide-binding protein. All these terms are accurate but less informative now the primary role of D1-D2 heterodimer is known. As described in subsection I-C, D1 and D2 show sequence and predicted secondary structural similarities with L and M of the purple bacterial reaction centre [48–49].

Phosphorylation of both D1 and D2 was demonstrated for spinach by Ikeuchi et al. [237] and D2 from

D1 polypeptide of PS II:

	1 5 10 15 20
Barley	M **T*A** I L E **RR** E S T S L W G **R** - - - - F C N W
Chlamydomonas	M **T*A** I L E **RR** E S T S L W A **R** - - - - F C E W
Synechococcus 7942	M **T** S I L **R** E Q **RR** D N V W D **R** - - - - F C E W

L polypeptide of purple bacterial reaction centre:

Rhodospirillum rubrum	A L L **S** F E **RK** Y **R** V **R** G G T L I G G D L F D F W
Rhodopseudomonas viridis	A L L **S** F E **RK** Y **R** V **R** G G T L I G G D L F D F W
	1 5 10 15 20 25

Fig. 12. Aligned N-terminal sequences of three D1 polypeptides of PS II. * indicates a known phosphorylation site, or, in the case of *Chlamydomonas*, the most likely site of a known phosphorylation. Phosphorylated or putatively phosphorylated threonines and basic amino acid residues are shown in bold. An analogous segment of the L-subunit of the reaction centre of purple bacteria is also shown.

pea by Millner et al. [240]. Pea D1 was shown to be phosphorylated near the N-terminus by Marder et al. [241]. Michel et al. identified the phosphorylation site as threonine in position 2 (counting methionine as 1) in both cases, a result comparable to that for CP 43 (threonine 2) [238]. Also like CP 43, D1 and D2 are phosphorylated in a plastoquinone redox-controlled reaction relatively insensitive to LHC II kinase inhibitors, and are relatively slowly dephosphorylated in comparison with LHC II [238].

Cyanobacterial *psbA* genes encode D1 proteins [242–244] with similarly placed threonines separated by four mainly non-polar residues from a pair or cluster of basic amino acid residues, as shown in Fig. 12. In contrast, purple bacterial reaction centre L subunits [245–246] contain no similarly-placed threonine, though serines in position 4 show a similar juxtaposition to basic residues, as discussed in subsection III-C.2 (Fig. 12). Cyanobacterial D1 and purple bacterial L polypeptides have a second arginine 3 to 4 residues further on. Chloroplast D2 sequences have the phosphorylated threonine separated from a single arginine by four non-polar residues (except in *Chlamydomonas* which has threonine in place of arginine) (Fig. 13). Again, this pattern is conserved in cyanobacteria [247–248] (Fig. 13), and a repeated arginine is found 3 or 4 residues further on from the putative phosphorylation site. The bacterial M subunit [245–246] has a threonine in position 8 that stands in the same relation (separated by four non-polar residues) from an arginine (Fig. 13). The possible significance of these patterns (Figs. 12–13) is discussed in subsection IV-C.

There is little information on the functional significance of the phosphorylation of D1 and D2. Any of the functions proposed for phosphorylation of the 9 kDa protein (subsection II-B.3) or of CP 43 (subsection II-C.1) might apply equally to D1 or D2 or both, since no experimental separation of these four phosphorylations has been reported. The proposal that PS II phosphorylation alters electron transfer on the acceptor side of PS II may turn out to concern D1 and D2 specifically [120–123] since the N-terminal phosphorylation sites are located on the acceptor side of the reaction centre which also contains the quinone pockets of the two proteins.

Mattoo et al. suggest a role for phosphorylation of D1 and D2 in spatial separation of the two populations of PS II reaction centres found in grana and stroma membranes [249]. This would be consistent with altered electron transport, since the PS II-β centres of stromal thylakoids (subsection II-A.2) have slower electron transport between Q_A and the plastoquinone pool than PS II-α centres [250]. There is a lateral cycling of PS II in thylakoids associated with a PS II repair cycle and rapid proteolysis of D1 during photoinhibition [251] which is likely to have effects on thylakoid membrane architecture [252]. It has been suggested that phosphorylation might serve to identify or 'flag' D1 polypeptides to the protease involved in turnover, although in vitro the breakdown and lateral movement of D1 does not require ATP [253].

Michel et al. suggest that the acceptor side of PS II may be modified by an interaction of the phosphate groups on D1 and D2 with the iron atom located between the quinone binding sites [238]. They also suggest that the phosphate groups may provide pH buffering and thereby affect protonation of Q_A and Q_B [238]. Either of these mechanisms could be imagined to play a role in maintenance of electron transport at high light intensity [232]. Here the prediction would be that phosphorylation of PS II proteins protects from the onset of photoinhibition, as proposed also for the other thylakoid phosphoproteins. This would seem to conflict with the idea that phosphorylation earmarks the proteins for breakdown and contributes to photoinhibition [253].

D2 polypeptide of PS II:

	1	5	10	15	20
Barley	M	**T***	I A L G	**R** V P	**K** E E N D L F D T M D D
Chlamydomonas	M	**T***	I A I G	**T** Y Q E	**K R** T W F D D A D D W
Synechococcus 7942	M	**T**	I A V G	**R** A P A E	**R** G W F D V L D D W

M polypeptide of purple bacterial reaction centre:

Rhodospirillum rubrum	S E Y Q N I L	**T**	G V Q V	**R**	T A P H S A P
Rhodopseudomonas viridis	A D Y Q T I Y	**T**	Q I Q A	**R**	G P H I T V S
	1	5	10	15	20

Fig. 13. Aligned N-terminal sequences of three D2 polypeptides of PS II. * indicates a known phosphorylation site. Phosphorylated or putatively phosphorylated threonines and basic amino acid residues are shown in bold. An analogous segment of the M-subunit of the reaction centre of purple bacteria is also shown.

De Vitry et al. observed absence of D1 and D2 phosphorylation in an LHC II-less *Chlamydomonas* mutant, and conclude that PS II can be phosphorylated only in an intact LHC II-PS II complex [255] They envisage the PS II kinase being associated with LHC II, the latter being required for the correct structural arrangement of kinase and substrate. An alternative suggestion [255] is that PS II polypeptides are phosphorylated by an LHC II-D1/D2 phosphotransferase. The implication of such a mechanism would be that phosphorylation of D1 and D2 might be involved in the mechanism of the state 2 transition, for example by providing the fixed negative charges to repel phospho-LHC II. However, as described in subsection II-B.3, the ability of chloroplasts to decrease PS II absorption cross-section upon phosphorylation of LHC II is independent of the phosphorylation state of PS II proteins. This is seen in vitro in alkaline phosphatase-treated pea thylakoids [232], as well as in PS-II-deficient *Chlamydomonas* mutants [254]. Perhaps most likely is a role for D1 and D2 phosphorylation in biogenesis and assembly of PS II [128,255].

II-C.3. Others

Phosphorylation of soluble proteins of the chloroplast stroma is most likely to concern regulation of assimilatory metabolism or of protein import or processing. More than ten soluble proteins of the chloroplast stroma can be seen to be phosphorylated by [γ-^{32}P]ATP in vitro in reconstituted chloroplasts [256]. Stromal protein substrates include both the large [257] and small [258] subunits of ribulose-1,5-bisphosphate carboxylase-oxygenase, as well as glyceraldehyde-3-phosphate dehydrogenase [257], and phosphoglucomutase [259]. These reactions almost certainly depend on specific soluble protein kinases, with no direct relationship to the thylakoid kinase-phosphatase system [256], though regulation of GA-3-*P* dehydrogenase in particular [257] might be expected to be an important additional factor in the role of thylakoid protein phosphorylation in regulation of photophosphorylation and assimilatory metabolism (subsection II-A.5). In this context it is also relevant to note that maize cytosolic phospho*enol*pyruvate carboxylase is phosphorylated by a light-activated serine kinase [260]. The Rubisco small subunit kinase is reported to be a Ca^{2+} and phospholipid-activated kinase located in the chloroplast envelope [258].

Bhalla and Bennett [261] demonstrated that a barley thylakoid-bound protein kinase under redox control catalyses phosphorylation of a soluble protein of the chloroplast stroma. This protein has an apparent relative molecular mass of 12 kDa, contains threonine as the phosphorylated amino acid residue, and seems to be released from association with the thylakoid membrane upon phosphorylation [261]. Bhalla and Bennett suggest that the 12 kDa-protein kinase is also the LHC II kinase on the basis of light-activation, sensitivity to inhibition by DCMU and activation by reducing agents or anaerobic conditions [261]. This phosphoprotein has not yet been identified, nor any suggestion made as to its identity, other than to note weak binding of a number of thylakoid membrane proteins [261].

Hodges et al. [262] report phosphorylation of ferredoxin-NADP oxidoreductase (FNR) in pea thylakoids, the kinase responsible also catalysing phosphorylation of added spinach FNR. The flavoprotein FNR is weakly bound to the stromal side of the thylakoid where it forms a cation-sensitive complex with ferredoxin. Its modification might be expected to alter binding to both ferredoxin and the membrane, and affect electron transfer to $NADP^+$. Hodges et al. report an increased recovery of FNR in the soluble fraction after incubation with ATP in the light or dark [262]. The kinase itself again shows the well-established properties of the LHC II kinase, indicating redox control at the level of plastoquinone [262]. Hodges et al. report an ATP-dependent inhibition of reduction of $NADP^+$ by PS I electron transport that is independent of effects of phosphorylation of LHC II. The inhibition could be reversed by antimycin A, which also inhibits cyclic electron transport. They suggest a role for FNR phosphorylation in regulation of the relative rates of cyclic and non-cyclic photophosphorylation [262]. FNR is a soluble or weakly membrane-bound protein, relatively rich in threonine and serine (e.g., in spinach 22 and 26 respectively out of 369 residues for the precursor [263]) and therefore with a number of possible phosphorylation sites. A conserved region in both spinach [263] and pea [264] precursors contains a serine (ser 66 in pea, ser 75 in spinach) bounded by lysines (KVEKHSK-KME). Hodges et al. [262] report both serine and threonine phosphorylation, indicating the possibility of multiple phosphorylation sites. Upon phosphorylation, FNR might be expected to behave like the 12 kDa phosphoprotein described by Bhalla and Bennett [261] though it is a much larger protein (around 35 kDa for the mature form) and the two cannot therefore be the same. Both phosphorylations are potentially of considerable regulatory significance.

II-D. The thylakoid kinase-phosphatase system

II-D.1. The site of redox control

As described in subsection II-A.3, redox control of LHC II phosphorylation in thylakoids, chloroplasts and cells is specifically the result of imbalance in electron transport through the two photosystems, and the site of redox control lies therefore in the region of plastoquinone, as indicated also by redox titration experiments. The site of redox activation of the kinase can also be determined from inhibitor titration studies

where it is seen to lie between the sites of inhibition by DCMU (Q_A to Q_B electron transfer) and DBMIB (the high affinity site inhibiting net plastoquinol oxidation) [130–131]. Whether activation involves plastoquinone itself or some other component at a similar potential and similar inhibitor sensitivity has been an open question. Dominy and Williams suggested from a study of state transitions in *Chlorella* that control was located between plastoquinone and PS II [265]. Studies with mutants deficient in components of the cytochrome *b/f* complex support the opposite conclusion, and implicate the kinase itself with the cytochrome *b/f* complex [266–274].

Direct evidence that redox activation of the LHC II kinase requires participation of the cytochrome *b/f* complex was obtained from studies of *b/f*-deficient mutants of *Chlamydomonas* by Lemaire et al. [266] and of *Lemna* by Gal et al. [267]. Lemaire et al. [266] found phosphorylation of both PS II and LHC II polypeptides in the *Chlamydomonas* mutant, but observed that phosphorylation and attendent fluorescence changes were no longer redox-sensitive. They suggested therefore that the LHC II kinase requires an intact *b/f* complex for redox activation. They also found that kinase activity could be separated from the *b/f* complex by sucrose gradient centrifugation [266]. Clark et al. found that LHC II kinase activity was associated with the *b/f* complex in spinach [275]. Gal et al. found a complete absence of LHC II phosphorylation and state transitions in the *b/f*-deficient *Lemna* mutant, while phosphorylation of the PS II polypeptides was unchanged [270]. It also seems from their data that the 9 kDa phosphorylation was inhibited in the mutant [270]. Histone phosphorylation was unaffected by the mutation. Gal et al. also carried out inhibitor studies on the *Lemna* wild-type, and concluded that the site of redox control lay with a quinone-binding site on the *b/f* complex. They also proposed that the selective effect of the mutation on LHC II phosphorylation indicated that the PS II phosphoproteins have a different kinase [270].

Inhibitor studies were carried out by Bennett et al. [268–269] on spinach thylakoids and a similar conclusion reached. Bennett et al. also observed the absence of LHC II phosphorylation from a maize mutant deficient in the *b/f* complex, whereas phosphorylation of all four PS II proteins, including the 9 kDa protein, was unaffected [268–269]. Lemaire et al. were able to extend their conclusion [266] to *Chlamydomonas b/f*-deficient mutants carrying either nuclear or chloroplast mutations, and demonstrated that D1/D2-deficient mutants were unaffected as regards LHC II phosphorylation or state transitions [254]. Gal et al. further localised the site of redox control of the LHC II kinase to the *b/f* complex using site-specific inhibitors with *Acetabularia* thylakoids [270]. They found that inhibitors of the quinol oxidase site of the *b/f* complex (DBMIB at around 1 mM, DIBB, DBBB, Bromanil) inhibited light- and reductant-dependent LHC II kinase activity, while inhibitors of the quinone reductase site (Antimycin A, NQNO) had little effect in the dark and stimulated kinase activity in the light [270]. Coughlan obtained similar results to Bennett et al. [271] using DBMIB and DNP-INT as inhibitors, finding stimulation of LHC II phosphorylation at around 1 μM and inhibition of phosphorylation of all thylakoid substrates for kinase activity, including the 64 kDa kinase itself, at 10 μM [271]. Inhibition of LHC II phosphorylation at this secondary, low-affinity site was among the earlier indications of redox control [130–131]. Coughlan also confirmed the effect of *b/f*-deficiency in selectively inhibiting LHC II kinase in the maize mutant used by Bennett et al. [268] and in the *Lemna* mutant used by Gal et al. [270], also showing by immuno-blotting that the mutants retained the 64 kDa kinase itself [271].

It is clear, therefore, that activation of the LHC II kinase in some way involves the redox state of a component of the cytochrome *b/f* complex. This does not preclude direct activation by binding of plastoquinol, and the two levels of redox control might operate according to the level of activation and range of substrates to be phosphorylated. The possibility of dual redox control at the *b/f* complex as well as the kinase itself is suggested by the results of Gal et al. [270] who have isolated a spinach thylakoid 64 kDa LHC II kinase and established conditions under which plastoquinol activation of LHC II phosphorylation can be achieved.

II-D.2. The LHC II kinase

The first chloroplast protein kinases to be isolated [272–273] are serine kinases that are soluble components of the stroma with stromal enzymes as likely substrates, though some proteins of non-appressed thylakoids may also be phosphorylated by these kinases in a light-independent reaction [274].

Partial purification of an LHC II kinase that would also catalyze phosphorylation of histones was reported by Clark et al. [275]. This kinase was isolated by detergent solubilization of spinach thylakoids and was therefore a membrane-bound protein, as originally shown by Bennett [13]. Similar procedures were subsequently used by Coughlan and Hind [276–278] who were able to distinguish three thylakoid kinases. Two were the 25 and 38 kDa kinases previously described [272–273]. The 38 kDa kinase was identified with FNR on the basis of immunological cross-reactivity and diaphorase activity [276]. A third protein kinase [277] accounted for 75–80% of total thylakoid protein kinase activity. It preferentially catalyzed phosphorylation of added histone but was also able to catalyze phosphorylation of added LHC II in detergent extracts, albeit at low rates

[277]. This LHC II kinase had an apparent relative molecular mass of 64 kDa, and was further distinguished from the serine kinases by lack of photo-affinity labelling with 8-azido-[^{32}P]ATP [276].

Coughlan and Hind showed that the 64 kDa LHC II kinase undergoes autophosphorylation [277] with threonine as the phosphorylated amino acid residue [278]. As for LHC II, the phosphorylated threonine was located on the stromal side of the membrane near one end of the protein as judged from the ability of trypsin to cleave it in situ. The 64 kDa LHC II kinase showed no redox control for LHC II phosphorylation, but was nevertheless proposed as the redox-controlled LHC II kinase involved in state transitions [277–279] (subsection II-A), a conclusion strengthened by the observation that antibodies to the purified kinase inhibited phosphorylation in situ of LHC II [278]. Phosphorylation of PS II polypeptides was also inhibited, which Coughlan and Hind interpreted as evidence for a single kinase acting on all PS-II-associated substrates of phosphorylation [278]. This question is considered further in subsection II-D.4. Subsequent work showed that the kinase was located primarily with its presumed substrate, LHC II, in appressed regions of thylakoid membrane, that autophosphorylation was an intramolecular event showing no concentration dependence, and that the histone phosphorylation it catalyzed was under redox control [279]. Autophosphorylation in situ showed kinetics comparable with those for LHC II phosphorylation, though the suggestion was made that autophosphorylation might serve to inactivate the kinase [279]. Forti et al. [280] report that the spinach LHC II kinase is autophosphorylated on serine in contrast to the threonine reported by Coughlan and Hind [278]. Forti et al. also report a slower dephosphorylation of the 64 kDa kinase (half-time 40 min) than of LHC II (20 min) [280].

Further studies on the LHC II kinase have used synthetic substrate analogues in order to determine the preferred sequence and locate a putative kinase recognition site. Bennett et al. [269,281] showed that a synthetic dodecapeptide (MRKSATTKKAVC) resembling the N-terminal segment of the phosphorylated pea LHCP [73] became phosphorylated in spinach thylakoids with the same characteristics as phosphorylation of the the protein itself. These included sensitivity to inhibition by DBMIB, and reversibility of this inhibition by ascorbate, the latter property not being shared by PS II protein phosphorylation. This peptide and a similar one corresponding to a tomato LHCP show maximum phosphorylation on threonine 6 with no labelling on serine 4 [282]. However, most LHCPs deduced from *cab* nucleotide sequences have no threonine or serine in positions 6 or 7, but retain the serine or threonine in position 4 [282]. Michel and Bennett showed that such peptides will also act as substrates for the spinach and pea thylakoid kinase, and both serine and threonine in position 4 can be phosphorylated [282]. With serine in both position 4 and 6, serine 4 is phosphorylated more, while with threonines in both these positions, threonine 6 is phosphorylated more [282]. This work was extended to show the need for basic amino acids on either side of the phosphorylation site for a low K_m [283] with this consensus of an effective substrate resembling that of mammalian protein kinase C [3]. Similar results were obtained by White et al. [284–285] who confirmed that synthetic substrates were also competitive inhibitors of LHC II phosphorylation. In subsection IV-C the question of sequence characteristics of phosphorylation sites is considered from the additional point of view of structural changes in the protein that may be induced by phosphorylation.

Gal et al. isolated and purified a spinach LHC II kinase which displays activation by plastoquinol and inactivation by plastoquinone provided the *b/f* complex is also present [286]. Gal et al. found that this property was dependent also on the detergent used for solubilization, with CHAPS yielding a kinase activity that was not regulated by plastoquinol whereas β-D-octyl glucoside gives a plastoquinol-activated kinase [286]. The kinase has an apparent relative molecular mass of 64 kDa, and will catalyze autophosphorylation and histone phosphorylation in a redox-sensitive reaction. As for LHC II phosphorylation, histone phosphorylation and autophosphorylation are sensitive to inhibition by DBMIB and DIBB, suggesting separate plastoquinone binding sites on the kinase itself and on part of the *b/f* complex required for activation of LHC II phosphorylation. This indicates that redox control of the LHC II kinase may operate at two distinct sites, with plastoquinol being required for formation of a functional kinase-*b/f* complex while binding of plastoquinone to the kinase itself may still inactivate the complex and prevent LHC II phosphorylation. Gal et al. also showed that the 9 kDa phosphoprotein appears to be phosphorylated under the same conditions by the LHC II kinase, with degree of phosphorylation being positively related to the amount of *b/f* complex in the preparation [286]. They suggest that the quinone binding site of the kinase may be involved in controlling substrate specificity. Gal et al. also used immunogold labelling with antibodies raised to the redox-sensitive LHC II kinase, and found the kinase located primarily at the edges of the grana stacks, consistent with the idea of its phosphorylation of LHC II at the outer domain of thylakoid stacks [286]. A schematic outline of the activation steps proposed by Gal and Ohad [287] is shown in Fig. 14.

At the time of writing it is likely that sequence information on the LHC II kinase will soon become available, and there is therefore little point here in

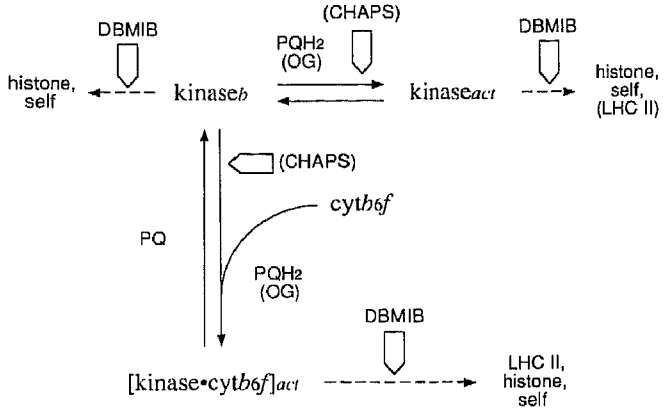

Fig. 14. The model of Gal and Ohad for the activation steps of the 64 kDa LHC II kinase, using histone, itself and LHC II as substrates of phosphorylation. DBMIB and the detergent CHAPS inhibit activation at the sites indicated by a broad arrow. Plastoquinol (PQH_2) activates when the complex is isolated with β-D-octyl glucoside (OG), and the cytochrome *b-f* complex ($cytb_6f$) is required as a cofactor for full LHC II kinase activity. 'Kinase b' is an intermediate, inactive form of the kinase.

speculation concerning the location of the phosphorylation site, membrane disposition, location of the kinase gene and the possible identity of the protein with known thylakoid components. In addition, the questions of substrate specificity and the requirement for other protein kinases in regulation of light-harvesting and energy transduction in photosynthesis will be more easily answered when information is available on the structure, function and control of the 64 kDa LHC II kinase. Plant genes encoding putative protein kinases have been described, including one designated PVPK-1 by Lawton et al. [288] which would encode a protein of about the correct size and with a putative site of autophosphorylation and at least one hydrophobic segment near the C-terminus. As with other developments in protein biochemistry, information on the kinase gene from DNA cloning is certainly going to provide important insight, as well as presenting the possibility of probing structure-function relationships by site-directed mutagenesis.

II-D.3. The LHC II phosphatase

Following the initial work of Bennett demonstrating thylakoid LHC II phosphatase activity [13] little work seems to have been done on the LHC II phosphatase, even though the lesson from other systems is that phosphatases are just as interesting as kinases and as likely to be involved in regulation [234].

Sun et al. [289] found a thylakoid-associated phospho-LHC II- and phosphohistone-phosphatase that was readily removed by salt washing. They discussed the relevance of this to the 12 kDa phosphoprotein of Bhalla and Bennett [261] released from the thylakoid upon phosphorylation, apparently by the LHC II kinase [289].

It has often been assumed that the LHC II phosphatase functions continually and independently of light or electron transport [130]. This is partly a simplifying assumption which facilitates interpretation of phosphorylation patterns and fluorescence changes in terms of redox-controlled kinase activity. After the onset of illumination it is possible to demonstrate in vitro that the presence of fluoride at 10 mM increases the net rate of LHC II phosphorylation and of the attendant decrease in RT chlorophyll fluorescence [142], consistent with the continued operation of the phosphatase, in the absence of fluoride, in parallel with the light-activated kinase. Fluoride is a non-specific inhibitor of phosphatase reactions and so the extension of this experiment to measurements made in vivo [87] may produce data consistent with a number of possible explanations in addition to inhibition of LHC II phosphatase activity.

There is a simple a priori reason for expecting the phosphatase itself to be subject to regulation, which is the avoidance of the unnecessary LHC II-mediated ATP hydrolysis that would result from the combination of the kinase and phosphatase activities under steady-state conditions. Bennett concluded from measurements of thylakoid protein dephosphorylation that the phosphatase is light-independent [13]. The assay was carried out over 15 min, starting with labelled thylakoids from [^{32}P]P_i-labelled intact chloroplasts which were osmotically shocked for 5 min, yielding thylakoids separated by centrifugation for 10 min. The results would therefore be consistent with light-activation of the LHC II phosphatase, provided the activity did not decline appreciably within 30 min or so after activation. CP 43, D1, D2 and the 9 kDa phosphoprotein would all be expected to retain label after such an incubation (subsections II-B–C) along with the LHC II kinase itself [280]. The possibility that any of these act as the LHC II phosphatase should be examined. In subsection II-B.3. it was suggested that the opposing effects of bicarbonate on 9 kDa and LHC II phosphorylation could be rationalized if the phosphorylated 9 kDa protein were required for activation of the LHC II phosphatase. The 12 kDa phosphoprotein of Bhalla and Bennett [261] might also be a good candidate for the LHC II phosphatase, and the different kinetics of dephosphorylation of LHC II and the PS II phosphoproteins might then be explicable in terms of a single phosphatase by different substrate accessibility, with the active, phosphorylated phosphatase selecting the phospho-LHC II of unappressed membranes.

The specific proposal advanced here is that the LHC II phosphatase is light-activated by redox-controlled phosphorylation, and that its activity persists in darkness for long enough for it to catalyze dephospho-

rylation of LHC II in a reaction that only appears to be light-independent. The suggestion is outlined more explicitly in Fig. 15. A suitable way of testing these possibilities is with the differentially phosphorylated thylakoids prepared by the technique of Harrison [231–232] and described here in subsection II-B.3. If this suggestion is correct, the alkaline phosphatase-treated thylakoids with dephosphorylated PS II polypeptides should have impaired LHC II phosphatase activity. Undoubtedly there is a urgent need for more information on the the LHC II phosphatase. An assay system based on synthetic peptides would help isolation. Inhibitor studies may also help, particularly the protein phosphatase specific inhibitors okadaic acid, a tumour promoter and toxin from dinoflagellates [290] and microcystin-LR, a toxin from certain cyanobacteria [291]. Both inhibitors prevent dephosphorylation of sucrose phosphate synthase in spinach leaves [292], indicating the involvement of a plant protein phosphatase 2A [293]. Possible effects of these inhibitors on state transitions and on the LHC II and PS II phosphatase(s) urgently require investigation.

II-D.4. How many kinases and phosphatases do we need?

Observations appearing to support a single thylakoid protein kinase.

(i) Similarities in redox control of phosphorylation of LHC II and other polypeptides [14,133].

(ii) Antibodies to the spinach 64 kDa kinase inhibit phosphorylation of both LHC II and PS II polypeptides [278].

(iii) DBMIB and related compounds inhibit phosphorylation of both LHC II and PS II polypeptides [268–271].

Observations appearing to support multiple thylakoid protein kinases.

(i) Differential sensitivity to inhibition with FSBA [144] and sulphydryl-directed reagents [134].

(ii) Mutations affecting the cytochrome b/f complex affect redox control only of LHC II [269–270].

(iii) Inhibition of phosphorylation by DBMIB affects all protein substrates, but inhibition is reversed by ascorbate only in the case of LHC II and its synthetic analogues [281]

(iv) GTP will support phosphorylation of LHC II

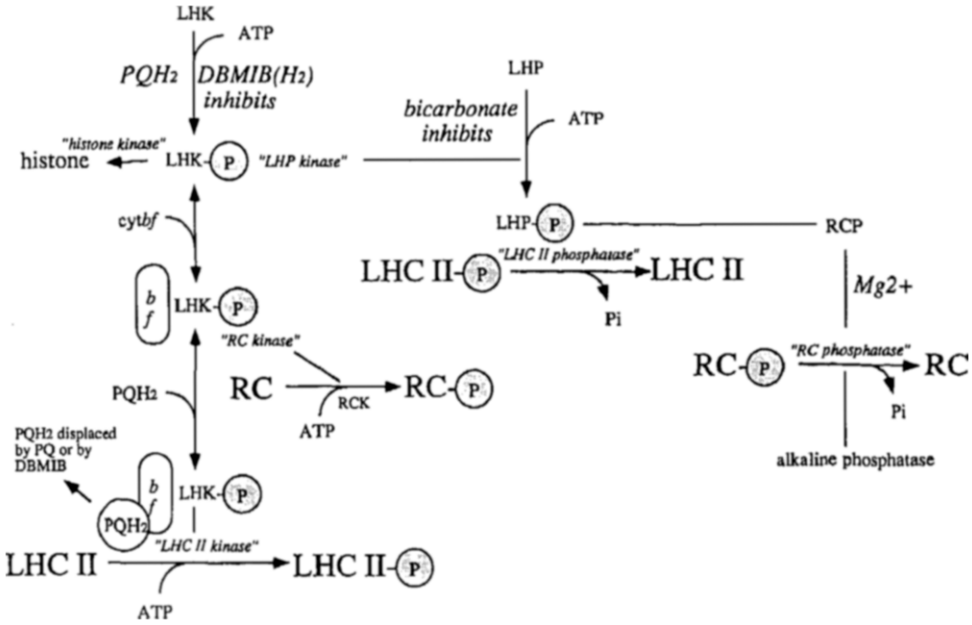

Fig. 15. A possible cascade for protein phosphorylation reactions involving LHC II and PS II reaction centre (RC) proteins. The left-hand vertical sequence of activation steps of the LHC II kinase is derived from that of Gal and Ohad (Fig. 14). However, it is proposed that the intermediates in activation of the LHC II kinase themselves have protein kinase activity, catalyzing phosphorylation of different substrates, as follows. LHC II kinase is one activity of the 64 kDa protein termed here 'LHK'. Other activities of LHK include RCK and LHPK. Abbreviations: LHK, light-harvesting kinase; LHP, light-harvesting phosphatase; LHPK, light-harvesting phosphatase kinase; RCK, reaction centre kinase; RCP, reaction centre phosphatase. It is proposed that LHK is autophosphorylated in a redox-controlled reaction, requiring PQH_2. It is also proposed that phospho-LHK functions as the LHPK (i.e., light harvesting phosphatase-kinase), activating by phosphorylation the enzyme required for rapid dephosphorylation of LHC II. The LHC II phosphatase is therefore redox-controlled indirectly, by virtue of activation of LHP kinase by phospho-LHK. Phospho-LHP either is, or activates, the slow RCP. Exogenous alkaline phosphatase also has RCP activity. Phospho-LHK when bound to the b/f complex also functions as, or activates, the RCK. Phospho-LHK bound to a b/f complex, itself bound to plastoquinol, functions as the LHC II kinase. Inhibition of the LHC II kinase, but not the RC kinase, therefore results from displacement of PQH_2 from the b/f complex by PQ or by DBMIB.

and the 9 kDa phosphoprotein but not of other protein substrates [195].

(v) Sequence differences between phosphorylation sites of LHC II and of other substrates. LHC II is phosphorylated on threonine 6 or threonine/serine 4 (subsections II-A.1 and II-D.2) with basic residues on both sides, while all other PS II polypeptides are phosphorylated on threonine 2 with a neutral residue in position 1.

(vi) Different putative kinases with different apparent molecular weights [272–273,278]. The 64 kDa kinase of Coughlan and Hind [278] and Gal et al. [286] seems to be the genuine LHC II kinase (subsection II-D.2), though Forti et al. [280] report phosphorylated 64 and 62 kDa kinases in spinach. An incompletely denatured D1/D2 heterodimer has previously caused problems by showing up in this molecular weight range [294].

Comment on kinase(s)

The three observations appearing to support a single kinase would also be consistent with multiple kinases if the factors concerned (namely redox control, 64 kDa antibody and DBMIB sensitivity) were properties of all kinases, or, more likely, were properties of a kinase functioning at an early stage in a cascade and upon which subsequent kinase activations depend. This possibility is consistent with the scheme in Fig. 15.

Observations appearing to support a single thylakoid protein phosphatase.

None.

Observations appearing to support multiple thylakoid protein phosphatases

(i) Different kinetics of dephosphorylation, with LHC II being more rapidly dephosphorylated than the others [175,209,232], including the autophosphorylated LHC II kinase [280].

(ii) Mg^{2+} ion requirement for dephosphorylation. LHC II dephosphorylation occurs in the absence of Mg^{2+} whereas at least 0.5 mM Mg^{2+} is required for dephosphorylation of the 9 kDa and other PS II phosphoproteins [15]. On this basis we could expect the LHC II phosphatase(s) to be a PP1-type or PP2A-type phosphatase (and okadaic acid- and microcystin-sensitive) and the PS II phosphoprotein phosphatase(s) to be PP2C-type (and okadaic acid- and microcystin-*in*sensitive) [290,295]

(iii) Differential action of the phosphoproteins as substrates for alkaline phosphatase, which dephosphorylates PS II phosphoproteins but not phospho-LHC II [232].

(iv) Sequences differences of phosphorylation sites, as (v) above.

Comments on phosphatase(s)

The observations apparently favouring multiple phosphatases might reflect substrate differences between reactions catalyzed by a single enzyme.

General comment

Enumerating arguments for and against a hypothesis is no way to do science.

Those eager for elegant simplicity may by now have stopped reading. The lesson from regulation of glycogen metabolism [234] is that we could reasonably expect both multiple kinases and phosphatases, each with broad but distinct substrate specificity. We may also reasonably expect regulation of both kinases and phosphatases by protein phosphorylation, in which case the regulatory significance of some known phosphorylations may turn out to be activation or inactivation of other regulatory proteins, including any of the components already described. The dual-site redox control implied by the results of Gal et al. [286] may have multiple kinases as its explanation. A further possibility is the conversion of a single protein from a protein kinase to a protein phosphatase upon phosphorylation or dephosphorylation. It is possible to imagine that autophosphorylation of the 64 kDa LHC II kinase can alter its functional properties in this way.

Progress on the question of multiple kinases and multiple phosphatases should be expected from use of synthetic peptides as substrates in combination with purified enzymes. Bennett et al. [281] reported progress on synthesis of a peptide analogue of the phosphorylation site of the 9 kDa protein. It will be important to know if such a peptide acts as a substrate of the 64 kDa LHC II kinase [286], and, if so, what activation steps may be required.

Wollman and Lemaire [296] have made the suggestion that PS II reaction centre phosphorylation results from a phospho-LHC II-D1/D2 phosphotransferase activity, which is consistent with impairment of PS II phosphorylation by mutation or inhibition of factors (e.g., *b/f*) required for activation of LHC II phosphorylation. They propose as an alternative a sequence of substrate-induced modifications [296]. In either case they suggest that phosphorylation of PS II polypeptides depends upon the phosphorylation state of LHC II. A further possibility is selective control of phosphorylation of the different LHC II pools (subsection II-A.7). Wollman and Lemaire suggest sequential phosphorylation of LHCb, LHCa, and PS II. LHCa corresponds to the LHC II pool whose phosphorylation-dephosphorylation has the same kinetics as state transitions, and corresponds to the mobile LHC II pool of chloroplasts (subsection II-A.7) [296]. This suggestion is consistent with known data and the precedent set by control of glycogen metabolism. Selective phosphorylation of different LHC II pools may also turn out to be a function for the different intermediates in the activation pathway of Gal et al. [286] (Fig. 14). It would therefore be surprising if a regulatory chain or cascade of phosphorylations did not occur also in regulation of photosynthesis.

Wollman and Bulté [297] suggest that LHC II kinase activation may require formation of supercomplexes between the *b/f* complex and PS II, with ATP-induced dissociation of the *b/f* complex from PS I perhaps playing some part in regulation. Bulté and Wollman [298] have demonstrated stabilization of phosphorylation patterns and light-states by chemical cross-linking under conditions where LHC II mobility is unimpaired, suggesting an effect in suppressing structural changes in both kinases and phosphatases.

Insulin has the apparently paradoxical effect of promoting phosphorylation of some proteins while promoting dephosphorylation of others. The explanation [234] lies in the fact that certain of the phosphoproteins are themselves either protein kinases or phosphatases. Specifically, an insulin-stimulated protein kinase is shown to activate a protein phosphatase by serine phosphorylation of a regulatory subunit, with the effect therefore of promoting dephosphorylation of the substrate of the phosphatase. In this example, glycogen synthase is the substrate and it is activated by dephosphorylation.

With the same caveats otherwise reserved for the structural effects of phosphorylation (subsections IV-C and -D), I should therefore like to propose a tentative scheme for integrated redox control of LHC II and PS II reaction centre protein phosphorylations (Fig. 15). This scheme attempts to account for known data on this subject and makes a number of specific predictions. Specifically, it assumes the existence of at least two distinct kinase activities and three distinct phosphatase activities (Fig. 16). The number of enzymes catalysing these five reactions could be anything from one (for minimalists) to five or more. In view of the need for separate and specific control mechanisms, two kinases and two phosphatases might be a realistic expectation, even taking into account the likelihood of multiple substrates. Research councils and funding agencies should be warned.

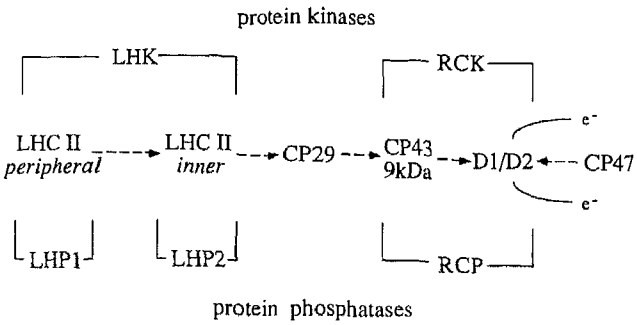

Fig. 16. Phosphorylation of LHC II and PS II core components. Broken arrows indicate the pathway of excitation energy transfer. Proposed protein kinase and phosphatase substrate specificities: LHK; light-harvesting kinase, LHP; light-harvesting phosphatase, RCK; reaction centre kinase, RCP; reaction centre phosphatase.

III. Phosphoproteins of photosynthetic prokaryotes

III-A. Cyanobacteria

III-A.1. Light-dependent phosphorylation in vivo and in vitro

Cyanobacteria and red algae carry out state 1–state 2 transitions just as effectively as chloroplasts, green algae and plants [31–32,141,299], and it has long been appreciated [27,130] that the absence of LHC II and the presence of phycobilisomes as peripheral light-harvesting complexes poses a problem in understanding the mechanism of all state transitions, phenomenologically and functionally fairly uniform, in terms of protein phosphorylation. Specifically, what might be the protein substrates phosphorylated in place of LHC II?

Such considerations prompted a search for cyanobacterial and red algal phosphoproteins in a number of laboratories. Biggins et al. [300] found twelve ^{32}P-labelled bands on SDS gels of samples from cells of the red alga *Porphyridium cruentum* grown on ^{32}P-P_i, but were unable to detect any differences in the intensity or pattern of labelling between cells illuminated with high intensity light 1 or light 2. They referred to similar results with the cyanobacterium *Synechococcus* 6301 (*Anacystis nidulans*) and concluded that the mechanism of state transitions in organisms containing phycobilisomes is radically different from that of green algae and higher plants [300]. Schuster et al. [301] studied protein phosphorylation in *Prochloron* and in the cyanobacterium *Calothrix* 7601 (*Fremyella diplosiphon*) and found labelling of thylakoid proteins in vitro, indicating a thylakoid protein kinase, but again the activity appeared to be light-independent [301].

Allen et al. [302] found light-dependent labelling of both membrane-bound and soluble proteins in *Synechococcus* 6301 cells grown in the presence of [^{32}P]P_i. The proteins whose labelling was most obviously light dependent had apparent relative molecular masses of 18.5 kDa and 15 kDa, the former predominating in the soluble fraction but also found in thylakoid preparations, and the latter located exclusively in the thylakoid fraction. From both fractions other labelled bands were seen but their labelling did not appear to be light-dependent. The illumination that produced labelling of the 18.5 and 15 kDa proteins in vivo also gave an shift in the cells' 77 K chlorophyll fluorescence emission spectra consistent with redistribution of excitation energy distribution in favour of PS I at the expense of PS II, indicating induction of state 2 [302].

Allen et al. also obtained phosphorylation of thylakoid membrane proteins in vitro using [γ-^{32}P]ATP, with light-dependent phosphorylation being observed in the case of a 15 kDa protein identified with that seen in the thylakoid fraction obtained from cells la-

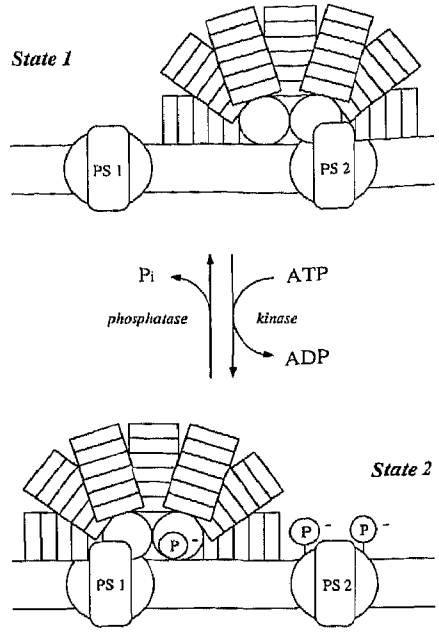

Fig. 17. Scheme for control of excitation energy distribution by protein phosphorylation in phycobilisome-containing organisms. The kinase catalyzing the protein phosphorylation(s) involved in the state 2 transition is assumed to be under redox control, analogous to the LHC II kinase. The identities of the proposed PS II core and phycobilisome phosphoproteins are discussed in the text. Redrawn from Ref. 302. See also Figs. 20.

belled in vivo [302]. Cyanobacterial thylakoid protein phosphorylation could be obtained in vitro in thylakoids isolated by osmotic lysis of sphaeroplasts, but not in thylakoids isolated by French press treatment, a finding attributed to different topological orientations of the two thylakoid preparations [303]. The illumination required for phosphorylation of the 15 kDa membrane protein in vitro also produced ATP-dependent alterations in 77 K fluorescence emission similar to those obtained in cells, indicating a state 2 transition [302]. Just as for in vitro phosphorylation, the ATP-induced increase in thylakoid F720/F685 at 77 K (taken to indicate redistribution of excitation energy to PS I from PS II) was absent from thylakoids made by French press treatment. Allen et al. [302] concluded that the state 2 transition in *Synechococcus* 6301 correlates with phosphorylation of the 18.5 kDa (soluble or weakly membrane-bound) and 15 kDa (thylakoid) proteins. They proposed that redox-controlled phosphorylation of these substrates was responsible for the state 2 transition in cyanobacteria [302–303]. The model proposed [302] was explicitly an attempt to translate the chloroplast LHC II model (section 2.1.4.) to cyanobacteria, and is depicted in Fig. 17. The 18.5 kDa protein was proposed as a phycobilisome component, the 15 kDa protein as a component of the PS II core [302].

This model [302] (Fig. 17) makes three specific predictions, concerning redox control, absorption cross-section changes, and phosphoproteins. All have proved controversial. The questions of redox control of excitation energy distribution and of absorption cross-section changes are considered in subsection III-A.5. As regards the identity of the phosphoproteins and their possible role in state transitions, attention has focussed on the question of regulation of phosphorylation and its correlation with fluorescence spectral changes in vitro. Biggins and Bruce [304] found that the 77 K fluorescence emission changes reported by Allen et al. for thylakoids [302] were not ATP-dependent, a finding confirmed by Sanders and Allen [305]. The reason for the inconsistency of the results of Allen et al. [302] with those of Biggins and Bruce [304] and Sanders and Allen [305] is not clear. It is conceivable that the fluorescence spectral changes observed [302] originated from a small population of unbroken cells or unlysed sphaeroplasts in the thylakoid preparation, in which case the absence of a requirement for added ATP does not disprove the involvement of protein phosphorylation in state transitions, the interpretation of Biggins and Bruce [304]. Further experiments suggesting the absence of a role for phycobilisome phosphorylation in state transitions is the observation of Bruce et al. [306] of the persistence of light-induced changes in 77 K fluorescence emission spectra in a phycobilisome-less mutant of *Synechococcus* 7002. Subsequent work (subsection III-A.2) suggests that modification of the 18.5 kDa protein is not activated in the same way as the 15 kDa protein [231] and so its involvement in state transitions may be unlikely. Bruce et al. did not report on the presence or absence of this specific component, or on its phosphorylation in the mutant used [306], so it is difficult to eliminate it purely on these grounds.

Subsequent studies by Sanders et al. showed a third protein, labelled exclusively under PS II light in cells of *Synechococcus* 6301 [307]. The identity of this 13 kDa soluble protein is now known and it is discussed in subsection III-A.3.

Mann et al. describe twelve phosphoproteins of the soluble fraction of *Anabaena* 7120 [308]. Redox control was apparent as effects of dithiothreitol on phosphorylation of three proteins, at 83, 78 and 34 kDa. Harrison has identified an 85 kDa protein that may be involved in regulation of excitation energy distribution in *Synechococcus* [231], which could be the *Anabaena* 83 kDa protein of Mann et al. [308]. Mann et al. also describe a 56 kDa protein whose phosphorylation is under metabolite control, specifically under conditions corresponding to altered ATP demand. The 56 kDa protein [308] appeared to co-purify with its kinase, which suggests the possibility of autophosphorylation in the manner of the 64 kDa LHC II kinase of chloroplasts. Phosphorylation of the 56 kDa phosphoprotein

seen by Mann et al. [308] had no discernible effect on glucose-6-phosphate dehydrogenase or other soluble enzyme activity. It is possible to imagine that this *Anabaena* 56 kDa phosphoprotein is an autophosphorylated protein kinase under redox control whose substrate is the 15 kDa or 85 kDa proteins implicated in control of excitation energy distribution between PS I and PS II (subsection III-A.4). In this case experiments seeking duroquinol activation and DBMIB inhibition would potentially be more important than ones with DTT, any cyanobacterial analogue of the LHC II kinase presumably sharing its specific requirement for the *b/f* complex and also possessing a quinol binding site.

III-A.2. The 18.5 kDa phosphoprotein of the phycobilisome fraction

The 18.5 kDa protein of *Synechococcus* 6301 was found by Sanders and Allen in a purified phycobilisome fraction, where it was found to be labelled when isolated from cells illuminated for 20 min with PS II but not PS I light [309]. Though the cells showed alteration in chlorophyll fluorescence emission at 77 K, indicating adaptation to state 2 and state 1, respectively, there was no difference in 77 K fluorescence emission spectra between the labelled and unlabelled phycobilisome fractions, suggesting that any effect of modification of the 18.5 kDa protein on excitation energy transfer lay on the pathway between the phycobilisome and PS II rather than within the phycobilisome [309]. The labelling was also specific for PS II light in cells grown for 20 h in each light regime [307].

In a study of the requirements for activation of phosphorylation of *Synechococcus* proteins by [γ-^{32}P]ATP in thylakoids isolated from sphaeroplasts, Harrison and co-workers [310–311] confirmed the light-dependent labelling of the 18.5 kDa protein reported by Allen et al. [302], and extended the experiments to include an investigation of redox control and of the effects of protein kinase and phosphatase inhibitors. Harrison found that labelling of the 18.5 kDa protein was inhibited by DCMU but restored in darkness or in the presence of DCMU by duroquinol, indicating quinol activation. However, the labelling was not greatly inhibited in the light by duroquinone or methyl viologen [310–311], in contrast to the redox control exhibited by the 15 kDa membrane protein (subsection III-A.4). Also unlike labelling of the membrane protein and changes in 77 K fluorescence emission spectra (subsection III-B.1), labelling of the 18.5 kDa protein was insensitive to inhibition by FSBA [311], the protein kinase inhibitor introduced for chloroplast thylakoids by Farchaus et al. [144]. Furthermore, dephosphorylation of the 18.5 kDa cyanobacterial protein proceeded in darkness but was insensitive to inhibition by NaF, a protein phosphatase inhibitor shown to inhibit the state 1 transition in cyanobacteria by photoacoustic [312] and fluorescence emission spectroscopy [231]. These data [231,310–311] serve to call into question a direct role for the 18.5 kDa protein in regulation of excitation energy distribution, as originally proposed by Allen et al. [302], and consistent with the proposal of state transitions in a phycobilisome-less mutant [306]. Nevertheless, in vitro labelling of the 18.5 kDa protein responds to electron transport in such a way as to indicate a role in linking photosynthetic electron transport to some other process by means of redox control.

Harrison also carried out further purification of the 18.5 kDa protein and found the labelled amino acid to be phosphotyrosine [231]. By 2-D gel electrophoresis using isoelectric focussing and SDS-PAGE, the labelled protein from sucrose gradients was found to have the same apparent mobility in both dimensions as β-phycocyanin, with a slightly decreased isoelectric point consistent with phosphorylation [231]. This identification of the 18.5 kDa protein as β-phycocyanin is also consistent with results from microsequencing, though additional, minor sequences were present which allow the possibility that the label was associated with a low abundance but high specific activity contaminant [231].

As regards the possible function of phosphorylation of β-phycocyanin, Harrison [231] draws attention to the possibility of phosphorylation at tyrosine in position 119, a side chain which is believed to interact non-covalently with the chromophore [313]. Phosphorylation might thereby alter the absorption characteristics of the protein. Harrison suggests [231] that phosphorylation of β-phycocyanin produces a photochromic form of the pigment, that is, one showing a reversible light-induced spectral shift characteristic of photoreceptors such as phytochrome. The chromophore of phytochrome is, like the phycobilins, a linear tetrapyrrole, and the photochromic effect is responsible for the induction of a protein structural change that intiates signal transduction. Cyanobacteria show photomorphogenetic adaptations such as complementary chromatic adaptation, in which the photoreceptor is believed on the evidence of action spectra to be a modified phycobilin, showing a green/red reversibility resembling the red/far-red reversibility of phytochrome [314]. Phycobilin subpopulations exhibiting photochromic effects can be distinguished by isoelectric focussing [315]. Harrison's hypothesis [231] is that redox controlled phosphorylation of the β-subunit of phycocyanin is involved in a photoreceptor mechanism which senses changes in illumination conditions and brings about long-term alterations in the constitution of the photosynthetic apparatus. Such alterations must involve regulation of gene expression at the level of transcription, as in the case of cyanobacterial com-

plementary chromatic adaptation [316–317]. The possibility of redox control of transcription via protein phosphorylation is considered further in Section V.

III-A.3. The P_{II} protein or glnB gene product

The 13 kDa soluble protein labelled in vivo [307] was purified by Harrison et al. and an N-terminal sequence of 30 amino acids was obtained [318]. The sequence had an unexpected but strong resemblance to the P_{II} protein or *glnB* gene product of *E. coli*, a protein functioning in signal transduction and concerned with regulation of nitrogen assimilation at both post-translational and transcriptional levels of gene expression.

In *E. coli*, P_{II} becomes uridylylated by a uridylyltransferase which is activated in response to a decrease in the ratio of the concentration of glutamine to that of 2-ketoglutarate, which in turn results, under physiological conditions, from a decrease in ammonium ion concentration [319]. Uridylylated P_{II} then activates an adenylyltransferase which activates glutamine synthetase by catalysing its deadenylylation. Thus a signal of decreased ammonium ion concentration activates glutamine synthetase and lowers its K_m for NH_4^+, serving to maintain assimilation despite the altered substrate concentration. This is a post-translational mechanism of control analogous to the established role of protein phosphorylation in regulation of excitation energy distribution in photosynthesis. In *E. coli* the same uridylylation reaction also initiates a transcriptional level of control which results in de novo synthesis of glutamine synthetase [320]. In this pathway, uridylylation of P_{II} decreases its ability to activate a protein phosphatase which has as its substrate the protein NR_{II} (nitrogen regulator II) [321]. The resulting phosphorylation of NR_{II} converts it from a protein phosphatase to a protein kinase, and its substrate, NR_I (nitrogen regulator I) becomes phosphorylated. NR_I shows the helix-turn-helix motif of DNA binding proteins, and, in its phosphorylated form, it interacts with a σ^{54} RNA polymerase-promoter complex to initiate transcription of the *glnALG* operon at multiple sites [322]. *glnALG* contains the gene for glutamine synthetase (*glnA*) as well as those for the kinase, NR_{II}, and the effector, NR_I (*glnL* and *glnG*, respectively) [323].

Using probes constructed from the N-terminal sequence of Harrison et al. [318], Tsinoremas et al. have shown the presence of P_{II}-encoding *glnB* genes in representatives of a variety of cyanobacterial groups, and have cloned and sequenced the *glnB* gene of *Synechococcus* 7942 [324]. The deduced sequence of the cyanobacterial P_{II} protein shows over 60% identity with other P_{II}s, the best match being obtained with the P_{II} of the purple photosynthetic bacterium *Rhodobacter capsulatus* [325]. The predicted *Synechococcus* 7942

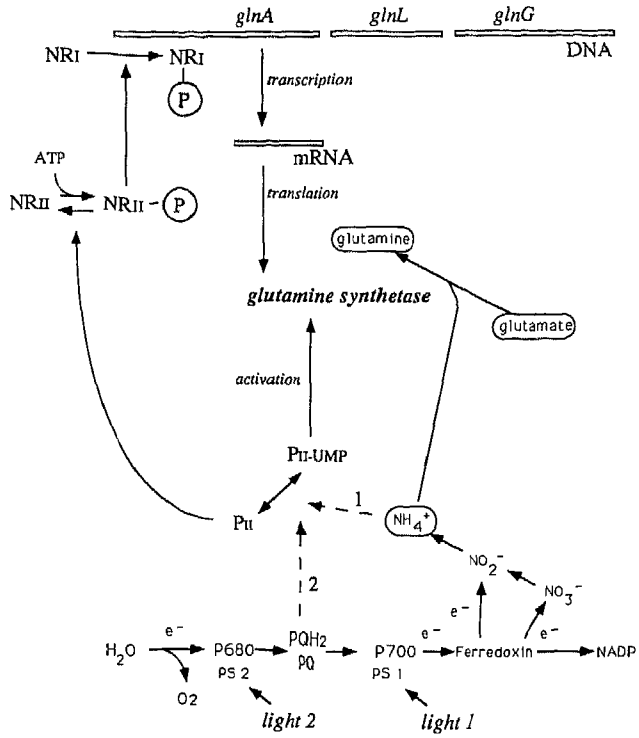

Fig. 18. The cyanobacterial P_{II} protein links regulation of nitrogen assimilation to photosynthetic electron transport. Glutamine synthetase is activated and its gene (*glnA*) is switched on when P_{II} is uridylylated to P_{II}-UMP. The effects of photosynthetic electron transport on modification of P_{II} might be explained by activation of P_{II} uridylylation by decreased NH_4^+ concentration (broken line 1) or by reduction of plastoquinone (broken line 2). NR_I, NR_{II} and their genes *glnL* and *glnG* are included by analogy with enteric bacteria, where the genes comprise an operon with *glnA*.

P_{II} [324] has 112 amino acids, including tyrosine at position 51, the site of uridylylation of P_{II} in *E. coli* [321]. Its predicted relative molecular mass is 12.3 kDa. Tsinoremas et al. also show that the ^{32}P-label of cyanobacterial P_{II} is easily removed by phosphodiesterase treatment, consistent with uridylylation, which involves a phosphodiester linkage [324].

The simplest interpretation of the identification of the redox-controlled 13 kDa cyanobacterial phosphoprotein as P_{II} is that P_{II} functions in cyanobacteria, as in *E. coli*, in regulation of assimilation of ammonia, but that in cyanobacteria its modification is regulated by photosynthetic electron transport in addition to ammonium ion concentration. Such a control pathway is outlined in Fig. 18. Inhibition of cyanobacterial P_{II} labelling in vivo by 2 mM ammonium sulphate [324] is consistent with its role in regulation of glutamine synthetase in the same way as in *E. coli*. However, for cyanobacteria there is no evidence that adenylylation regulates glutamine synthetase activity, and no labelling of glutamine synthetase subunits was seen by Tsinoremas et al. [324]. It is therefore possible that any

control of glutamine synthetase in cyanobacteria by means of P_{II} operates purely at a transcriptional level.

As regards the control of P_{II} uridylylation by photosynthetic electron transport, the evidence from labelling in vivo is that P_{II} is uridylylated in PS II light but not in PS I light [307,318,324]. As suggested by Tsinoremas et al. [324], this could result from the contribution of photosynthetic electron transport to reduction of nitrate and nitrite to ammonia, reactions requiring electrons from ferredoxin on the acceptor side of PS I. Thus uridylylation of P_{II} in PS II light but not in PS I light might be explained as follows. Under PS I light, PS I turnover is rapid, being supported by an effective donor system such as respiration, nitrate and nitrite reduction is therefore also rapid, and a high cellular ammonium ion concentration is maintained. The glutamine to 2-ketoglutarate ratio is high, and P_{II}, regulated as in *E. coli*, is not uridylylated. Under Light 2, PS I turnover is slow, ammonia production is therefore also slow, and P_{II} becomes uridylylated because intracellular ammonium ion concentration falls. This assumption is consistent with the inhibition of P_{II} uridylylation by ammonium ions [324]. An alternative mechanism for activation under PS II but not PS I light would be possible if P_{II} uridylylation were modified by the redox state of plastoquinone, in the manner of phosphorylation of chloroplast LHC II (subsection II-A.3). Reduction of plastoquinone by PS II light would then trigger uridylylation while the oxidation of plastoquinone by PS I light would cause its deuridylylation. Further experiments are required to distinguish between these possibilities. In principle a DBMIB titration should show complete inhibition of P_{II} uridylylation at 1 μM if regulation is exerted at the level of ammonia synthesis (1 in Fig. 18) or ferredoxin redox control, and show no inhibition until 20 μM or higher if regulation is at the level of plastoquinone (2 in Fig. 18).

Whatever the biochemical pathway leading to the modification of P_{II}, it appears that protein phosphorylation in cyanobacteria permits nitrogen metabolism to be controlled by imbalance in the relative rates of photosynthetic electron transport through Photosystem I and Photosystem II (Fig. 18). A comparable system may also operate in eukaryotic organisms, where expression of a nuclear gene for chloroplast glutamine synthetase may be switched on by light-induced changes in chloroplast metabolism [326].

It is also important to note that the light 1-light 2 control of uridylylation of cyanobacterial P_{II} is in principle a direct link between photosynthetic electron transport and control of gene expression at the level of transcription [327]. But which genes might be controlled? It has not yet been demonstrated that P_{II} regulates transcription of the *glnA* gene in photosynthetic organisms. It is interesting to consider the possibility that P_{II} itself controls expression of other genes such as those encoding components of the photosynthetic apparatus. Whether such a pathway underlies other instances of photocontrol of gene expression is discussed in subsection V-B.

III-A.4. The 15 kDa and other membrane phosphoproteins

The 15 kDa phosphoprotein of *Synechococcus* 6301 is labelled in vivo in a DCMU-sensitive reaction [302] and specifically under PS II light [307,310–311]. It is recovered from labelled cells exclusively in a thylakoid fraction [302,307] and can be labelled in vitro in thylakoids prepared from lysed sphaeroplasts and supplied with [γ-^{32}P]ATP [303,311], a property shared with the 18.5 kDa phosphoprotein but not with P_{II}.

Although the 15 kDa phosphoprotein is clearly membrane-bound, it does not seem to co-purify with PS I, with PS II, or with the cytochrome *b/f* complex [231]. Its identity is currently unknown. The conditions for labelling of the 15 kDa phosphoprotein in vitro coincide exactly with the conditions for redistribution of excitation energy from PS II to PS I, as judged by 77 K fluorescence emission spectroscopy of cells. Thus the 15 kDa protein is phosphorylated, and PS I/PS II fluorescence emission is increased, under conditions leading to reduction of plastoquinone. These include light 2 illumination and incubation with duroquinol in darkness or in light in the presence of DCMU [311]. In vitro phosphorylation and the increase in the 77 K PSI/PS II fluorescence ratio are inhibited by oxidising agents such as duroquinone as well as by DCMU. In addition, phosphorylation and the attendant fluorescence changes indicating a state 2 transition are inhibited by the protein kinase inhibitor FSBA, and dephosphorylation and the state 1 transition are inhibited by the protein phosphatase inhibitor, sodium fluoride [231,311]. The 15 kDa phosphoprotein is therefore almost certainly involved in cyanobacterial state transitions, its phosphorylation acting as a link between redox state of plastoquinone and excitation energy distribution between PS I and PS II. It is therefore likely to prove to be the cyanobacterial analogue, and perhaps homologue, of chloroplast LHC II.

However, as discussed in subsection II-D, state transitions in chloroplasts may involve multiple protein phosphorylations, and other phosphoproteins may therefore be expected to play a part in this process in cyanobacteria, too. Proteins at 85 and 55 kDa are conspicuously labelled in isolated thylakoids in reactions which are light dependent and which show sensitivity to fluoride and FSBA, though they are not apparently activated by duroquinol [311]. The same applies to minor bands at about 45, 35, and 25, 12 and 9 kDa. Duroquinol actually inhibits labelling of the 85 kDa protein and of two proteins of higher relative molecu-

lar mass. This could result from redox activation by phosphorylation of their protein phosphatase(s), as discussed for chloroplasts in subsection II-D.4. The question would then arise whether the 15 kDa-protein kinase and the phosphatase kinase were the same enzyme. The cyanobacterial thylakoid phosphoprotein nearest in apparent size to the spinach LHC II kinase (subsection II-D.2) is at 54 kDa. It would be interesting to see if antibodies to the LHC II kinase [286] reacted with this or other cyanobacterial membrane phosphoproteins and, if so, what effect they might have on cyanobacterial thylakoid protein phosphorylation and excitation energy distribution. It is also tempting to consider that the 85 kDa phosphoprotein might correspond to the phycobilisome LCM 'anchor' polypeptide [328–329], an identity compatible with redox control of its phosphorylation altering the interaction of the phycobilisome with PS II.

Of the minor thylakoid membrane phosphoproteins [231,311], the 35 kDa protein could in principle correspond to D1 or D2, and the 45 kDa protein to CP 43. As shown in Figs. 12 and 13, both D1 and D2 in cyanobacteria have potential phosphorylation sites near the N-terminus, and the same is true for cyanobacterial CP 43 [231,330]. However, Harrison [231] was unable to detect labelling of PS II core components in reaction centre preparations from labelled cells. The identities of the 25 kDa and high molecular mass bands [231,311] are unknown, though it is possible to imagine that the 25 kDa band corresponds to the 29 kDa band seen by van der Staay et al. in the prochlorophyte *Prochlorothrix hollandica* [331]. Representatives of other algal [332] and phycobilin-containing [333] groups show thylakoid protein phosphorylation, though it remains to be established whether this correlates with excitation energy distribution.

As described in subsection II-B.1., the cyanobacterial *psbH* gene encodes a protein that does not contain the phosphorylation site of the corresponding chloroplast gene product, the 9 kDa phosphoprotein [219–221]. Several labelled bands lower than 15 kDa were seen by Harrison and co-workers [231,311], so it cannot yet be determined that the cyanobacterial *psbH* gene product is not phosphorylated at another site. It could also turn out that the 15 kDa cyanobacterial is related to *psbH*, although until this and the other cyanobacterial membrane phosphoproteins are sequenced and identified the number of such guesses one can make is large and none can be sufficiently supported by currently available evidence.

III-A.5. Light-state transitions and control of protein phosphorylation in phycobilisome-containing systems

Redox control. Isolated chloroplasts incubated in the dark are usually in state 1 under aerobic conditions, since the plastoquinone pool is largely oxidized, and, without a PS I electron acceptor, any kind of light will act as light 2 [130]. In cyanobacterial and algal cells these conditions do not apply, however, and plastoquinone may be reduced in darkness by oxidation of respiratory substrates since photosynthetic and respiratory electron transport chains intersect and share a common plastoquinone pool [334–336]. Thus in cyanobacterial cells containing sufficient respiratory substrates the dark state may be state 2 even by a process of plastoquinone redox control. Thus light-versus-dark experiments may be state 2-versus-state 2 experiments, and absence of photocontrol of protein phosphorylation [300–301] is not necessarily evidence against a role for protein phosphorylation in the state 2 transition. Similarly, at high light intensity light absorbed by the chlorophyll antenna system of PS I may not function as light 1 if PS I acceptors are not regenerated quickly enough to keep pace with electron flow.

Mullineaux and Allen [337] used modulated chlorophyll fluorescence of *Synechococcus* 6301 cells at room temperature to demonstrate state 1–state 2 transitions by addition of a chlorophyll-specific blue PS I light to a weak, modulated, yellow light specific for phycobilin absorption. They found that unstarved cells reverted to state 2 when the PS I light was extinguished even in the presence of DCMU. This result is consistent with the transition to state 2 being driven by respiratory electron transport in the absence of electron transport into the plastoquinone pool from PS II [337]. Aeration of the cells in darkness was shown to decrease the rate of dark respiration, and it also decreased the rate and extent of the dark and DCMU-insensitive transition to state 2 [337]. They concluded [337] that excitation energy distribution was controlled, as in chloroplasts, by the redox state of plastoquinone or of a closely associated component of the electron transport chain located between PS I and PS II. Similar results, extended by the use of additional electron transport and energy transfer inhibitors, were reported by Dominy and Williams who also reached the conclusion that cyanobacterial state transitions were the result of redox control at the level of plastoquinone [338–339]. These data [337–339] are consistent with the model of redox-controlled protein kinase proposed by Allen et al. [302].

Mullineaux and Allen subsequently employed a variety of chemical and illumination treatments on cyanobacterial cells, using 77 K fluorescence emission spectroscopy to measure excitation energy distribution between PS I and PS II [340]. In all cases treatments which lead to oxidation of the plastoquinone pool induced a shift towards state 1 whereas treatments which lead to reduction of the plastoquinone pool induced a shift towards state 2. These experiments [340] were designed to distinguish between the model of redox control [302,337] and competing models for

the mechanism controlling distribution of excitation energy in state transitions.

Thus the model of Biggins et al. [300,341] is one by which state transitions are induced by localised electrochemical gradients around PS I and PS II such that turnover of PS I induces state 1 while turnover of PS II induces state 2. This is difficult to reconcile with the finding of Mullineaux and Allen that duroquinol induces state 2 [340], since duroquinol should act as an electron donor to PS I and induce state 1 according to the model of Biggins et al. [300]. In contrast, induction of state 2 by duroquinol is readily understood in terms of redox control at the level of plastoquinone. The alternative model of Satoh and Fork [342–343] states that the state 1 transition is induced by cyclic electron flow around PS 1. Methyl viologen, which inhibits cyclic electron transport by providing a rapid non-cyclic pathway to oxygen, should therefore induce state 2. In fact addition of methyl viologen was found to induce state 1 [340], which again is more easily seen to be consistent with the redox control hypothesis since methyl viologen will tend to promote electron transport on the acceptor side of PS I, thereby leading to net oxidation of the plastoquinone pool.

Besides intrinsic concern for the factor triggering state transitions, the relevance of these results [337–340] for the functional role of protein phosphorylation in cyanobacteria is that precisely those factors which control state transitions via redox state of plastoquinone also induce thylakoid protein phosphorylation both in vivo and in vitro. Additional observations supporting a mechanism involving redox-controlled protein phosphorylation are that the state 1 transition is inhibited by the protein phosphatase inhibitor sodium fluoride [311–312], while the state 2 transition is inhibited by the protein kinase inhibitor FSBA [311]. In view of the data reviewed in this section it now seems very difficult to sustain the view of Biggins and Bruce [341] that protein phosphorylation plays no part in regulation of excitation energy transfer in organisms containing phycobilins. In the writer's opinion the chloroplast LHC II model (Fig. 7) is therefore a satisfactory precedent for cyanobacterial and red algal state transitions (Fig. 17), even though the protein substrates concerned have not yet been identified with certainty nor their kinase(s) isolated for activation studies and sequence and structural comparison with the chloroplast counterpart [286]. The model of redox-controlled protein phosphorylation in cyanobacteria is testable, since it makes clear predictions about the outcome of these investigations.

Spillover and absorption cross-section (Part II: cyanobacteria). Another implication of redox-controlled protein phosphorylation [302] is that in cyanobacteria and red algae the state 2 transition is primarily a decrease in absorption cross-section of PS II for light absorbed through the phycobilisome. The view otherwise held is that the state 2 transition is primarily an increase in spillover of excitation energy from PS II to PS I [300,341]. The recognition that chloroplast LHC II phosphorylation alters absorption cross-section rather than spillover (subsection II-A.2) was crucial in pointing to the significance of lateral heterogeneity and mobility of phospho-LHC II (subsection II-A.6), and the same question is important if we are to understand the mechanism by which cyanobacterial photosynthetic unit function may be controlled by a redox-sensitive protein kinase. The question of spillover or absorption cross-section is also relevant in evaluating the various mechanisms that are proposed for structural and function effects of membrane protein phosphorylation, as described in Section IV. Thus, the conceptual associations of protein phosphorylation with redox control and absorption cross-section, as compared with localised high-energy states with photosystem turnover and spillover, demand that attention be paid to the nature of the alteration in the pathway of excitation energy transfer. Any reader not already aware that the writer favours the former package of associations is advised of this now. The latter package has been favoured in reviews by Biggins and Bruce [341] and by Fork and Satoh [31]. It should also be noted that some groups toe neither party line. Vernotte and co-workers, for example, favour redox control of spillover [344].

Mullineaux et al. [345] addressed the question of absorption cross-section versus spillover using analysis of fluorescence induction transients in the presence of DCMU, and found a decrease in both F_m and F_o, consistent with a decrease in PS II absorption cross-section during the cyanobacterial state 2 transition in vivo, as determined for chloroplast thylakoids by Horton and Black [82] amongst others and for green algal cells by Hodges et al. [115]. The basis on which this analysis is made is described in subsection II-A.2. Mullineaux et al. [345] also found that the kinetics of the two state transitions were similar to those for green plants [83,142], and observed a decrease in sigmoidicity in the fluorescence rise after the state 2 transition which is consistent with a decrease in cooperativity of PS II units (subsection II-A.2). These experiments were subsequently extended to distinguish between effects with excitation wavelengths favouring the phycobilisome from those attributable primarily to chlorophyll excitation [346]. The effects appeared to derive from the same process, both having a half-time for state 2-induced changes of around 45 s. The decrease in absorption cross-section was specific for light absorbed by the phycobilisome, a result taken to indicate functional decoupling of the phycobilisome from the PS II core [346], consistent with the state transition model of Allen et al. [302] and also resembling effects of decreased temperature in partially decoupling phycobili-

somes from PS II and decreasing PS II cooperativity [347]. Also using analysis of fluorescence induction, Mullineaux and Holzwarth found that only a proportion of PS II traps could be closed by a brief exposure to phycocyanin-absorbed light, suggesting the existence of a population of PS IIs unconnected to phycobilisomes [348]. This population was a smaller proportion of total PS IIs in state 1 than in state 2 [348]. In contrast, Vernotte et al. [344] identify state transitions with spillover changes on the basis of fluorescence induction analysis, and note that it is difficult to reconcile their results with those of Mullineaux and coworkers [345–346,348] except on the basis of species differences.

The problem of the nature of the alteration in excitation energy distribution has also been addressed by picosecond time-resolved fluorescence emission spectroscopy [349–350]. The basis of this approach is that a change in spillover is, by definition, a change in the rate-constant for energy transfer between PS II and PS I (k_t in Eqn. 1) which will affect the yield (Φ_f) of their respective fluorescence decay components differently at different rates of photochemical trapping ($k_p[Q]$), these in turn determined by the proportion of open PS II traps ([Q]). In contrast, a change in absorption cross-section should be seen only as a change in fluorescence amplitude of the respective decay components. Bruce et al. interpret their results in terms of a decoupling of PS II chlorophyll *a* from subsequent energy transfer to PS I upon transition to state 1 [349]. Using single-photon timing and global data analysis, Mullineaux et al. [350] found that the the state 2 transition decreased the amplitude of the PS II component with phycocyanin excitation (620 nm) by about 60% irrespective of whether the cells were at F_o (when $\tau = 370$ ps) or F_m (when $\tau = 1400$ ps), with the lifetime, τ, being unaffected by the transition itself. They further found a corresponding increase in the amplitude of the component attributed to the phycobilisome terminal emitter, ($\tau = 150$ ps at F_o, 200 ps at F_m), this

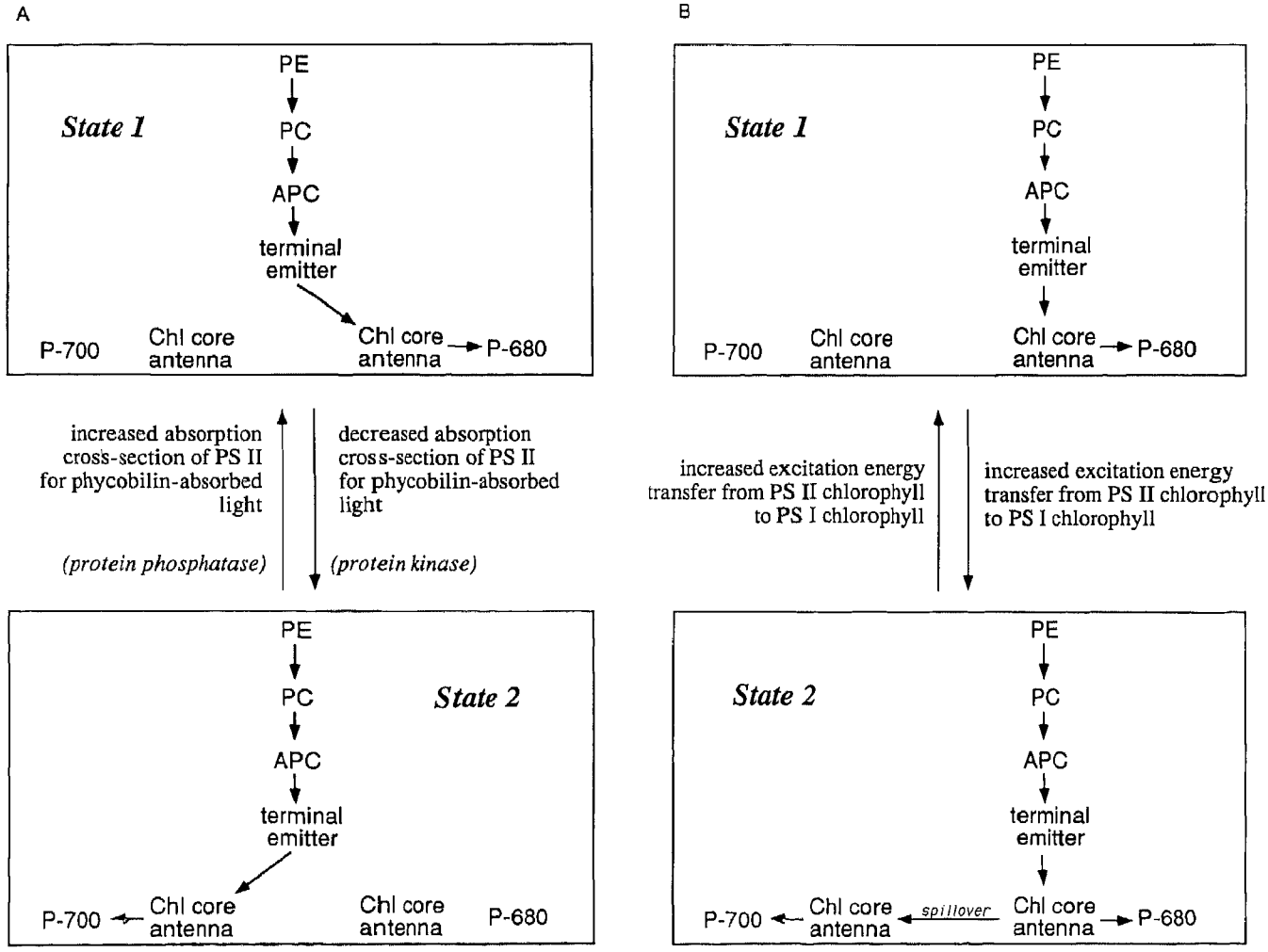

Fig. 19. Two models for redistribution of excitation energy between PS I and PS II during state transitions in phycobilisome-containing organisms. A: Control of absorption cross-section. Switching of phycobilin-absorbed excitation energy between PS I and PS II is independent of PS II photochemistry. B: Control of spillover. Switching of excitation energy to PS I occurs by increased excitation energy transfer from PS II, which competes with and is dependent upon PS II photochemistry. See also Fig. 17.

proportional increase also being independent of photochemistry. They therefore found that state transitions had no effect on the lifetime of PS II fluorescence, a result inconsistent with alterations in energy transfer, that is, spillover. Fluorescence amplitude is directly proportional to absorption cross-section and the results of Mullineaux et al. therefore decidedly favour the model depicted in Fig. 19A, with the decrease in PS II fluorescence amplitude accompanying an increase in phycobilisome terminal emitter fluorescence amplitude, indicating complementary changes in exciton density in the two pigment systems consistent with their decoupling. The contrasting interpretations of the state 2 transition in cyanobacteria are illustrated in Fig. 19.

The question of the ability of PS I to do photochemistry with the energy redirected from PS II in state 2 (section 2.1.2) also extends to cyanobacteria. Tsinoremas et al. [351] concluded from measurements of P-700$^+$ flash yield in two cyanobacterial species at chlorophyll-specific and phycobilin-specific wavelengths that in all cases the state 2 transition results in diversion of excitation energy to PS I. Similar results were obtained from steady-state measurements on cryptophyte cells by Snyder and Biggins [333]. Malkin et al. [352] reached similar conclusions from photoacoustic spectroscopy of a red alga and favour a direct interaction of the phycobilisome with PS I in state 2. These and other regulatory mechanisms may serve to control the effective antenna size and quantum yield of PS I in phycobilisome-containing organisms [353]. Clearly spillover from PS II to PS I might occur under some circumstances in addition to the complementary changes in absorption cross-section that could be viewed as a primary event. PS II centres decoupled by protein phosphorylation from the phycobilisome might have a higher probability of interacting functionally with PS I. Alternatively, the phycobilisome might under some circumstances act as bridge for excitation energy transfer between PS I and PS II.

Structural effects of phosphorylation. Structural effects of thylakoid membrane protein phosphorylation should be expected to determine the physiological adaptations they produce. Alterations in chromophore orientation [354–355] are in principle consistent with either absorption cross-section or spillover changes, but can most easily be envisaged in terms of the model depicted in Fig. 19A, and might be expected to arise from protein structural changes of the kind discussed in subsection IV-C.

Changes in membrane architecture upon state transitions have been reported by Olive et al. [356] and Vernotte et al. [344]. These are consistent with the effect of the state 2 transition in decreasing cooperativity of PS II units by decreasing their linear connectivity in the thylakoid membrane. The significance of linear arrays of phycobilisomes and coupled PS II cores in providing a structure within which excitation energy

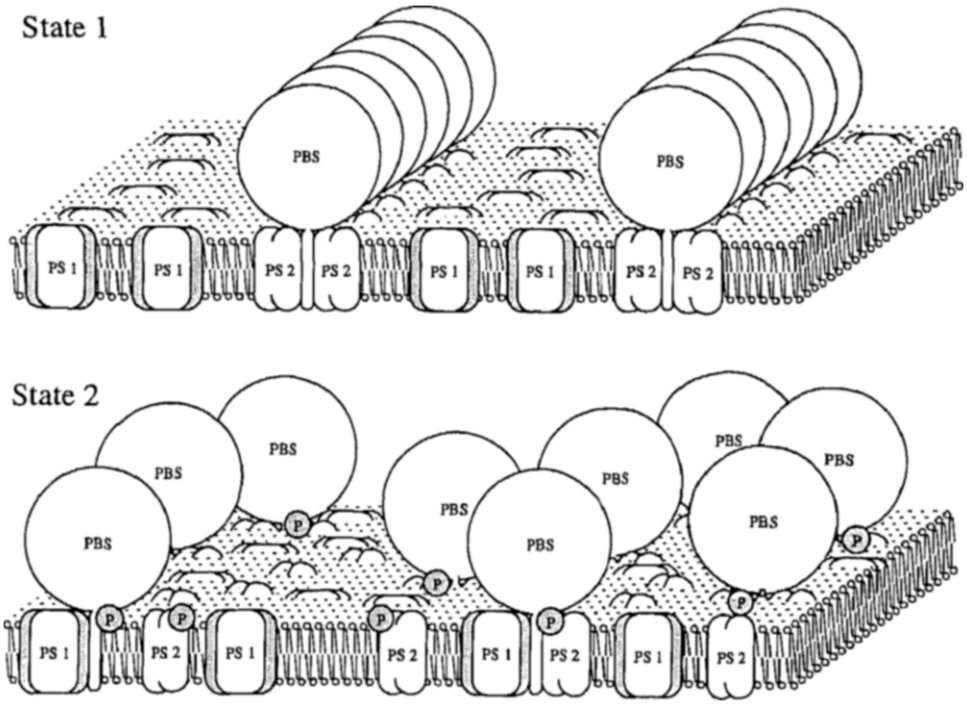

Fig. 20. A model for states 1 and 2 in phycobilisome-containing organisms. With respect to state 1, state 2 is characterised by the following: phosphorylation of proteins involved in docking of the PS II core with the phycobilisome (PBS); decreased absorption cross-section of PS II for PBS-absorbed light; increased absorption cross-section of PS I for PBS-absorbed light; decreased energy transfer between PS II cores (decreased PS II cooperativity). Increased energy transfer from PS II to PS I cores (spillover) may also occur. See also Figs. 17 and 19.

can migrate has been noted by Lange et al. [357]. In view of the strong correlation of protein phosphorylation with the state 2 transition (subsection III-A.4) and its ability selectively to control protein structure and protein-protein interactions (subsection IV-C), it seems likely that all the primary components and secondary effects of the state transition phenomenon have membrane protein phosphorylation as their underlying cause.

A general model depicting these events for phycobilisome-containing organisms is shown in Fig. 20. This model is intended to account for redox-controlled and protein phosphorylation-driven dissociation of PS II units otherwise connected for excitation energy transfer by virtue of their linkage with phycobilisome anchoring complexes [357], a reaction analogous to the conversion of PS IIα to PS IIβ in chloroplasts (subsection II-A.2). The decoupling of the phycobilisome from a proportion of PS II cores then decreases the absorption cross-section of PS II for phycobilisome-absorbed light while increasing that of PS I, the complementary change in absorption cross-section characteristic of state transitions. Increased energy transfer from PS II to PS I (spillover) is not excluded, though if it occurs it results from the decoupling of the phycobilisome from PS II since this might allow PS I to compete for energy transfer otherwise taking place between PS II units Phosphorylation of the 15 kDa thylakoid protein is specifically concerned with altered protein-protein interactions that alter chromophore orientation and energy transfer pathways, thereby controlling PS II cooperativity and essentially toggling energy from the phycobilisome between core chlorophylls of PS II and PS I. Phosphorylation of the 85 kDa protein might equally be expected to contribute to these changes. Retention of an anchor subpopulation in phycobilisome-free thylakoids has been shown [328] and is consistent with a role for the anchor polypeptide in PS II cooperativity and with the operation of phosphorylation-driven state transitions as depicted in Fig. 20.

Mutants will undoubtedly be crucial in determining the roles of the different membrane protein phosphorylations. The persistence of state transitions in phycocyanin-deficient mutants [306,344] suggests that regulation of excitation energy transfer by protein phosphorylation is a property of the PS II core antenna system and its interaction with the membrane anchor or core system of the phycobilisome. Mutants also establish that the PS II core of phycobilisome-containing systems closely resembles that of LHC II-containing systems even as regards cooperativity [358], and, presumably, its control. The protein phosphorylation model therefore predicts that the 15 kDa and 85 kDa phosphoproteins in particular (subsection III-A.4) are components of the PS II core and of the phycobilisome anchoring and core system, with phosphorylation of peripheral components such as β-phycocyanin effecting some different regulatory process (subsection III-A.2). Characterisation of polypeptide composition and protein phosphorylation in phycobilisome-deficient mutants [306,344,358] should therefore be given high priority.

III-B. Purple photosynthetic bacteria

III-B.1. Chromatophore phosphoproteins

The purple non-sulphur bacteria, or rhodospirillaceae, have only one type of photosynthetic unit, with a reaction centre resembling that of PS II (subsection I-C) and a light-harvesting system consisting typically of two membrane-intrinsic bacteriochlorophyll-protein complexes designated B800–850 and B880 in accordance with their long-wavelength absorption maxima, though the precise numbers vary slightly according to species and laboratory [359]. Most species have both light-harvesting complexes, each encoded by separate genes, with B880 genes sharing an operon (the *puf* operon) with genes for the reaction centre polypeptides L and M [360]. An exception is *Rhodospirillum rubrum* has which has only B880 [361]. Both types of complex are built of higher-order trimers or hexamers of a heterodimer containing α and β polypeptide chains [361]. Each constituent polypeptide has one hydrophobic α-helical domain which separates the N-terminal domain at the cytoplasmic surface from the C-terminal domain at the periplasmic surface [362]. The N-terminal domain is therefore the one exposed to the reaction medium of isolated chromatophores.

Protein phosphorylation was first implicated in regulation of purple bacterial photosynthesis by Loach et al. [363] who found increased phosphate composition of B880-α isolated from cells of *R. rubrum* incubated under conditions giving increased photosynthetic unit cooperativity. Holluigue et al. [364] demonstrated phosphorylation of polypeptides at 90, 86, 64, 31, 13 and 11 kDa in *R. rubrum* cell-free extracts incubated with [γ-^{32}P]ATP, of which the 86 and 11 kDa polypeptides were phosphorylated also in a purified chromatophore fraction [364,365]. Turner and Mann found a similar range of phosphoproteins in cells of *Rhodomicrobium vanniellii* labelled in vivo [366] and in cell-free extracts labelled with [γ-^{32}P]ATP [367]. Holmes et al. [368] reported a range of phosphoproteins in cells of *Rhodobacter sphaeroides* and in isolated chromatophores incubated with [γ-^{32}P]ATP and showing redox-dependent phosphorylation in vitro. Holmes and Allen also used *R. rubrum* cells [369,370] and chromatophores [371] to investigate redox control of phosphorylation and to probe associated functional changes. Distribution of purple bacterial phosphoproteins and the conditions under which they become labelled have been discussed elsewhere [372,373]. Fur-

ther work on identification of *R. rubrum* chromatophore phosphoproteins [374] and on analysis of redox control and characterization of a putative B880-β kinase [375] has also been carried out.

Holmes and Allen [369] investigated the correlation between protein phosphorylation and changes in cooperativity proposed by Loach et al. [363], using ^{32}P-labelling and SDS-PAGE of whole cell extracts with analysis of bacteriochlorophyll fluorescence induction transients. They observed reciprocal labelling of two low molecular mass polypeptides running at positions corresponding to 13 and 11.5 kDa by reference to mobility of marker proteins. In cells with higher cooperativity, indicated by sigmoidicity in fluorescence induction, the 13 kDa protein was labelled and the 11.5 kDa protein unlabelled. In cells with lower cooperativity, the 13 kDa protein was unlabelled and the 11.5 kDa protein labelled. They suggested therefore [369] that the 13 kDa phosphoprotein corresponds to phospho-B880-α, since it became labelled under the conditions described by Loach et al., and that the 11.5 kDa protein was B880-β [369]. These assumptions allowed a model to be proposed for control of cooperativity [370]. However, Holuigue et al. found the *R. rubrum* 13 kDa phosphoprotein to be a soluble protein [364], a result confirmed by Cox and Allen [374]. Ghosh et al. [375] nevertheless obtained labelling with [γ-^{32}P]ATP of two bands in isolated *R. rubrum* chromatophores which they identified with the 13 and 11.5 kDa phosphoproteins seen by Holmes and Allen [369] in labelled cells. Further work (Cox, A. and Allen, J.F., unpublished) suggests that B880-α and -β (apparently at 10 and 6–8 kDa respectively) may be phosphorylated in *R. rubrum* chromatophores, but at low specific activity, while amino acid sequencing of LDAO-solubilized bands coincident on SDS-PAGE with membrane phosphoproteins from *Rhodobacter sphaeroides* shows that they are B870-β and B800–850-β, but with no radioactivity detectable by a monitor connected on-line to the HPLC used for sequencing. In support of B880 as a substrate, Ghosh et al. [375] described an *R rubrum* soluble protein kinase activity that gave phosphorylation of the β-chain of purified B880 and of a band from chromatophores with approximately the same mobility. A soluble kinase activity was also detected by Holmes and Allen [371]. Ghosh et al. [375] found that the kinase had a molecular weight of about 18 kDa, and was able to phosphorylate added histones. All groups nevertheless seem in agreement that a kinase activity is also associated with chromatophore membranes themselves [364,371,374–375].

Thus, with the possible exception of B880-β, identities of the phosphoproteins are largely a matter of conjecture. The existence of P_{II} (subsection III-A.3) as a 12.3 kDa protein in *Rhodobacter capsulatus* [325] suggests that this may be the soluble 13 kDa protein seen to be labelled in *R. rubrum* cells [369], though why it might be labelled specifically under conditions of low photosynthetic unit cooperativity is not clear. Low cooperativity was induced by a 2 h incubation of cells in darkness, so labelling may simply reflect a control by light, perhaps via generation of reducing power for nitrate reduction, as suggested for cyanobacteria (section III-A.3) though with the added requirement for respiratory substrates for reverse electron flow to NAD^+. Labelling would then be a response to lowered NH_4^+ concentration.

Holmes and Allen [371] concluded that phosphoproteins of 60, 57, 50, 30, 16, 13 and 11 kDa were common to a number of studies with different species. It is tempting to imagine that the 57 kDa polypeptide is a redox-controlled autophosphorylating kinase, as suggested for cyanobacteria (subsection III-A.4). The 30 kDa phosphoprotein could be the reaction centre subunit H, with a possible homology with the 9 kDa phosphoprotein as suggested by Packham [226] (subsection II-B.2). A possible phosphorylation site is indicted in Fig. 11. The N-terminus of H is exposed to the periplasmic face of the bacterial membrane [39–40], while the N-terminus which carries the phosphorylation site of the 9 kDa protein is on the cytoplasmic (stromal) side in the chloroplast thylakoid [238]. A phosphorylation site located towards the C-terminal side of the H membrane-spanning domain would be topologically equivalent to the phosphorylation site of the 9 kDa protein, as indicated in Fig. 11, and would occupy a position consistent with an effect on electron transport on the acceptor side of the reaction centre. Alternative roles for phosphorylation of H would be to influence the binding and interaction of the L/M/H RC complexes with each other or with B880. Potential phosphorylation sites of L and M are indicated in Figs. 12 and 13. L lacks the N-terminal threonine know to be phosphorylated in D1 (section 2.3.2.), but has a cluster of basic amino acids at a similar distance from serine in position 4 (Fig. 12). M has a threonine in position 8 which is separated from an arginine by four non-polar amino acids, just as for the threonine phosphorylation site of D2 (Fig. 13). Phosphorylation of light-harvesting polypeptides in purple bacteria would present the possibility of regulation of absorption cross-section and photosynthetic unit cooperativity, while phosphorylation of reaction centre polypeptides might allow regulation of cooperativity of reaction centres themselves as regards electron transport on the acceptor side, as suggested also for PS II (subsection IV-E).

Such functional effects of chromatophore membrane protein phosphorylation might serve to maintain high quantum yield by fine-tuning primary electron acceptor characteristics and excitation energy transfer so that the two are optimally coupled. The increased quantum yield of a 'lake' arrangement of photo-

synthetic units over the corresponding number of 'puddles' [376] is greatest when the proportion of open traps is 0.5, and interconversion of the two arrangements by protein phosphorylation would in principle be able to maximize light-harvesting efficiency at low or intermediate light intensities while decreasing it at high light intensities. Such effects may be responsible for slow variations in bacteriochlorophyll fluorescence yield described by Setlik et al. [377] and for increased capacity for electron transport at high light intensity in high light-grown cells [378].

III-B.2. Control and function of phosphorylation

Despite an absence of hard information about identities of chromatophore phosphoproteins, several of the phosphorylations are regulated in such a way as strongly to implicate them in regulation of photosynthesis. The correlation of labelling in vivo with altered cooperativity is particularly striking [363,369], though the treatments used were incubation for 2 h in the light in the presence of 20 mM Mg^{2+} for cooperating units and in the dark in the absence of Mg^{2+} for non-cooperating units. These treatments should be expected to have multiple effects at almost every level of photosynthesis. Simpler light vs. dark experiments with whole cells reveal marked differences in labelling but the prolonged incubations again permit many alternative interpretations [374].

Holmes and Allen [371] found marked effects of redox reagents and electron transport inhibitors on protein phosphorylation during 15 min incubations of *R. rubrum* chromatophores with [γ-^{32}P]ATP. The strong reductant sodium dithionite suppressed labelling of all bands except one at about 70 kDa, while labelling was activated in darkness by potassium ferricyanide. This is precisely the opposite of the effects of dithionite and ferricyanide on chloroplast thylakoid protein phosphorylation [130], and suggests the possibility of activation of the chromatophore protein kinase by the oxidised form of some component of the electron transport chain. Similar results were obtained with *Rhodobacter sphaeroides* chromatophores [368], where both the more specific reducing agent duroquinol and the physiological donor succinate were found to have inhibitory effects similar to that of dithionite. Holmes and Allen [371] found labelled bands at about 6 and 10 kDa after light-incubation, both of whose labelling was inhibited by DBMIB, the quinol oxidase site inhibitor also inhibitory to LHC II kinase reactions. Antimycin A, the quinone reductase inhibitor without effect on phosphorylation in chloroplasts, inhibited phosphorylation in chromatophores of the 6 kDa band but not the 10 kDa band [371]. These data could be taken to suggest that the chromatophore 6 kDa-protein kinase has a requirement for the reduced form of a component of the *b*/*c* complex, that it is itself DBMIB-sensitive perhaps by virtue of displacement of a quinone required for activation. Alternatively, the 6 kDa-protein kinase could be dependent on the redox state of the ubiquinone pool, with activation requiring predominance of the oxidised form, ubiquinone. An equally plausible alternative would be a redox-controlled chromatophore protein phosphatase, active with ubiquinol but inactive with ubiquinone. At present there is no data which might distinguish between these possibilities.

The only certain conclusion to date is that a range of purple bacterial chromatophore proteins is phosphorylated, some of them under conditions indicating redox control. Unfortunately, none of them has been identified unambiguously. Their potential regulatory significance is considerable, however, and it is important that further work on this topic should be carried out. In subsection V-B it is suggested that a membrane phosphoprotein associated with the *b*/*c* complex should be expected to act as a redox sensor in two-component redox control of gene expression at the level of transcription. Besides the important questions of identification and location of phosphorylation sites, dissection of their various regulatory roles will come from use of antibodies to chloroplast analogues, from selective phosphorylation of different proteins [232], and from further investigation of inhibitor action (fluoride, FSBA, okadaic acid) and redox control. A redox titration of chromatophore membrane protein phosphorylation comparable to those carried out on thylakoids [133–134] would help to identify those reactions involved in control of photosynthetic unit function in photosynthetic bacteria.

IV. How does protein phosphorylation control photosynthetic unit function?

IV-A. Inter-membrane electrostatic forces: the surface charge hypothesis

One proposal for the mechanism by which phosphorylation of chloroplast LHC II exerts its functional effects is that put forward by Barber [30,379–380] and by Arntzen and co-workers [23,116]. According to this view, the primary effect of phosphorylation is an increase in the negative charge on the outer (cytoplasmic) surface of the appressed domain of the thylakoid membrane, and the magnitude of the change is sufficient to overcome the attractive forces otherwise holding together LHC IIs on adjacent, appressed thylakoid domains, thereby causing them to move apart. In this hypothesis, the electrostatic forces controlling protein-protein interactions act in a direction perpendicular to the membrane plane, and the initial event following phosphorylation is electrostatic repulsion between opposing phospho-LHC II complexes. The com-

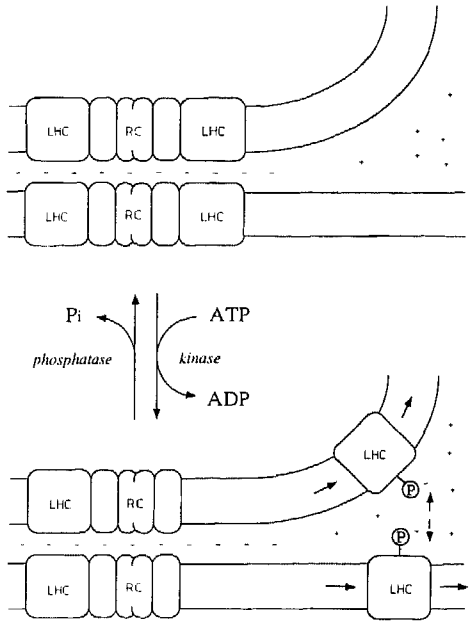

Fig. 21. The surface charge hypothesis. Phosphorylated light-harvesting complexes (LHC) are driven from appressed to unappressed thylakoid domains by intermolecular, intermembrane forces. Electrostatic repulsion between phosphate groups (broken arrow) acts perpendicular to the membrane plane. Cations in the stroma serve to screen the phosphate groups and other fixed negative charges.

plexes then migrate laterally into unappressed thylakoid domains, where increased distance and screening cations (chiefly Mg^{2+}) in the aqueous phase (the chloroplast stroma) serve to decrease the repulsive forces acting between them. Since LHC II is the primary contact point for membrane appression, phosphorylation also causes a degree of unstacking. This model is summarized in Fig. 21.

The surface charge hypothesis integrates protein phosphorylation with effects of altered free cation concentration on thylakoid structure and function, since increasing negative surface charge would be expected to have much the same effects as decreasing the concentration of cations screening the charges. One of many effects of decreasing cation concentration is to unstack thylakoids and increase spillover of excitation energy from PS II to PS I, a result of randomization of the distribution of PS I and PS II [381–382], and before the advent of protein phosphorylation this phenomenon was proposed as the physiological mechanism regulating excitation energy distribution [381–383]. Control by protein phosphorylation came initially to be viewed in the manner of an extension of control by cation concentration [30,81,109,379–380,384], and effects of protein phosphorylation on spillover were therefore favoured until fluorescence induction and other studies suggested a specific detachment of LHC II from PS II (subsections II-A.2 and III-A.5). Thus, the surface charge hypothesis seems to have been formulated to account for similarities between effects of phosphorylation and effects of decreased cation concentration, and does not obviously explain their differences. The surface charge model also seems to provide a basis for lateral mobility (subsection II-A.6) and phosphorylation-induced unstacking of thylakoids.

Problems with the surface charge hypothesis can be listed as follows:

(i) Specificity. If protein phosphorylation works by altering electrostatic potential throughout a membrane domain, how can it avoid altering interactions between each protein and all others in its domain, whether phosphorylated or not? It is likely that electrostatic coupling between membrane proteins governs many of their structural and functional interactions [385] and general perturbation of electrostatic coupling would be likely to change all protein-protein and protein-lipid interactions. Decreased cation concentration has very wide-ranging effects, and can increase energy transfer from LHC II to PS I even in a purified pigment-protein complex [231,386].

(ii) Coherence. If lateral heterogeneity is maintained by charge distribution between appressed and unappressed regions of membrane, how can increased negative charge on LHC II cause it to move into unappressed domains relatively rich in protein complexes already excluded from appressed domains by their more negative surface charge? In general, the surface charge model may or may not be a valid explanation of the static organization of laterally heterogeneous thylakoids, but as a sufficient explanation of protein phosphorylation in thylakoid dynamics it seems to resemble the paradox of picking oneself up by the bootstraps.

(iii) Dependence on lateral heterogeneity. Movement of pigment-protein complexes between discrete domains cannot occur in laterally homogeneous membranes, since they have no such domains. Miller and Lyon [63] suggest that protein phosphorylation evolved in photosynthesis as a means for lateral migration of LHC II "to overcome the difficulties introduced by the stacked membrane system". Yet membrane protein phosphorylation regulates photosynthesis in laterally homogeneous, unstacked prokaryotic membranes (section 3) [387] as well as in LHC II-containing algae such as *Euglena* [388] and *Chlamydomonas*. [128,100,208,] that show no distinct thylakoid organisation into grana and stroma [389]. Many green and brown algae have thylakoids that are closely appressed over their whole surface [390]. Both have phosphorylatable light-harvesting complexes [391] and exhibit state transitions [392]. In red algae and cyanobacteria thylakoids often lie parallel to each other but are separated by up to 100 nm of aqueous phase accommodating phycobilisome rows [390]. The familiar division of thylakoids into grana and stroma is thus a fairly specialised evolutionary development. It would be extraordinary for

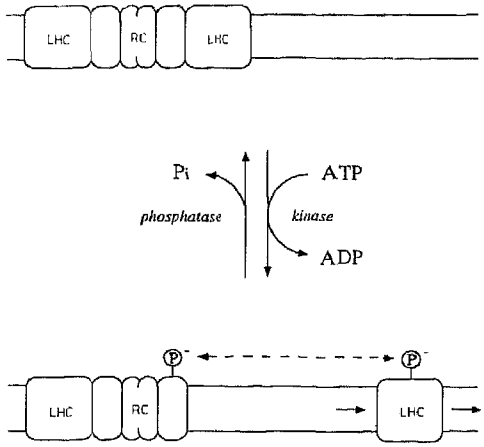

Fig. 22. The local charge hypothesis. Phosphorylated light-harvesting complexes (LHC) become separated from neighbouring complexes of the photosynthetic membrane by intermolecular, intramembrane forces. Electrostatic repulsion between phosphate groups (broken arrow) acts parallel to the membrane plane.

regulation by thylakoid protein phosphorylation to be confined to such systems.

(vi) Effects of LHC II phosphorylation on thylakoid stacking in vitro require 1–2 mM Mg^{2+} (109,384), while the average concentration of Mg^{2+} in the chloroplast is generally thought to be higher. It is therefore difficult to see how LHC II phosphorylation could have an appreciable effect on thylakoid surface charge in vivo.

IV-B. Intra-membrane, intermolecular forces: the local charge hypothesis

Allen and Holmes [393] put forward an alternative hypothesis to account for effects of protein phosphorylation in terms of intermolecular electrostatic repulsion. They suggest that the electrostatic forces which are induced by phosphorylation and which control protein–protein interactions act in a direction parallel to the membrane plane. If true, this proposal removes the requirement for distinct domains and replaces surface charge with more localised charge on individual protein complexes. This model can therefore accommodate prokaryotes and unstacked chloroplast thylakoids, circumventing problem (iii) of the surface charge model. This 'mutual electrostatic repulsion' model is depicted in Fig. 22.

With qualification, the local charge model of Allen and Holmes [393] also circumvents problem (i), that of the non-specificity of surface charge. This is because individual complexes rather than whole domains would have their charge altered by phosphorylation. However, the requirement for this process to have specific effects on protein-protein interactions introduces the additional constraint that all participating complexes should be phosphorylated, since otherwise phosphorylation of a single protein would alter its interaction, non-specifically, with all its neighbours. This constraint is consistent with the multiple phosphorylations observed in chloroplasts, cyanobacteria and purple bacteria, and in fact provides the basis for proposed identities and functions of purple bacteria chromatophore [393–394], cyanobacterial [302], and chloroplast [185, 209,237–238] thylakoid phosphoproteins.

In purple bacteria it was suggested [393] that polypeptides of both B880 and B800–850 would be phosphorylated, thereby decoupling the latter from excitation energy transfer to the reaction centre via B880. This is consistent with recent findings, which suggests that both β-chains may be phosphorylated (subsection III-B). For cyanobacteria the 18.5 kDa phosphoprotein was initially proposed as a phycobilisome component functionally analogous to LHC II, with the 15 kDa phosphoprotein acting as the phosphorylated intermediate light-harvesting complex from which the 18.5 kDa phosphoprotein was repelled [302,393]. As described in subsection III-A.2, the 18.5 kDa protein is indeed likely to be a phycobilisome component, but its phosphorylation does not exhibit the appropriate redox control nor sensitivity to kinase and phosphatase inhibitors. Nevertheless, as pointed out in the context of differential FSBA inhibition of 9 kDa and LHC II phosphorylation (subsection II-B.3), if two proteins must be phosphorylated for their repulsion to occur, then control of phosphorylation of either of them will be sufficient to bring about control of their mutual interaction and all subsequent functional changes. The 18.5 kDa protein cannot therefore be excluded purely on grounds of absence of redox control, though on balance it may be more likely that the 85 kDa phosphoprotein carries out this role. The 15 kDa phosphoprotein is still strongly implicated in control of excitation energy transfer, and could be viewed as a partner in electrostatic repulsion along the lines proposed by Allen and Holmes [393].

For chloroplast thylakoids the local charge model raises questions concerning the identity of the component(s) of the PS II core acting as phosphorylated intermediates on the pathway of excitation energy transfer and repelling phospho-LHC II to cause its decoupling. Allen and Holmes [393] and Allen and Findlay [209] suggested this role for the 9 kDa phosphoprotein (subsection II-B), and predicted on this basis its sequence similarity with LHC II [216]. Ikeuchi et al. [237] and Michel et al. [238] proposed in the context of this model [393] that CP 43 functions as the phosphorylated PS II component responsible for the repulsion of phospho-LHC II. Both these candidates (9 kDa, CP 43) seem to be excluded from participation in this kind of mechanism by the experiment of Harrison [231–232] in which the functional effects of LHC II phosphorylation are retained even when reaction cen-

tre proteins are unphosphorylated (subsection II-B.3.). Larsson et al. [185] made the specific proposal that the outer, mobile and inner, immobile LHC II pools repel each other when phosphorylated, with the unphosphorylated CP 29 acting as a kind of spacer to minimize direct effects between phospho-LHC II and the reaction centre. In view of Harrison's results [232], the proposal of Larsson et al. [185] is the most securely founded version of the model as it applies to chloroplast thylakoids, with the mobile and immobile pools of LHC II functioning as the peripheral and intermediate phosphorylated light-harvesting complexes in a way analogous to that proposed for B800–850 and B880, respectively, in purple bacteria [393].

Mutual electrostatic repulsion in the local charge model also solves the bootstrap paradox, problem (ii), since it does not require heterogeneity in surface charge among membrane domains. It does not, however, exclude heterogeneity in charge of microdomains surrounding the phosphorylatable complexes themselves. Certainly chloroplast thylakoids show heterogeneity in lateral distribution of photosynthetic units (subsection II-A.6). It can be argued that PS-I- and PS-II-enriched linear domains may exist in cyanobacteria, at least in state 1 (subsection III-A.5). Light-harvesting complexes may also act as points of membrane adhesion in stacking of purple photosynthetic bacterial chromatophores [394–395].

The model of Allen and Holmes [393] proposes a function for protein phosphorylation in regulation of cooperativity of photosynthetic units in single-photosystem bacteria (subsection III-B.2). The evolutionary development of two photosystems could then be utilized by the system controlling cooperativity to bring about additional changes, namely complementary changes in antenna size which satisfy energetic requirements for the adaptations seen experimentally as state transitions (subsections II-A.4 and III-A.5). Lateral heterogeneity as seen in chloroplasts would then do no more than introduce an additional requirement for migration of the phosphorylated light-harvesting complex between appressed and non-appressed domains.

IV-C. Intramolecular charge compensation and protein structural changes: molecular recognition

There is a third possibility, here proposed, namely that the electrostatic forces perturbed by phosphorylation are entirely intramolecular, and lead ultimately to major structural changes that alter the interactions of two or more proteins through their effects on the complementarity of their respective docking surfaces.

This hypothesis is described here as the 'molecular recognition' hypothesis, a term intended to convey succinctly the proposed link between phosphorylation and its functional effects. 'Charge compensation' would be appropriate but would not distinguish it from the other models without the qualification 'intramolecular'. 'Structural change' is imprecise and 'conformational change' has other connotations. In preliminary form the hypothesis has been described elsewhere [396].

The hypothesis envisages nine sequential events in control of photosynthetic unit function by phosphorylation of membrane proteins, as follows.

(i) Phosphorylation of membrane proteins reversibly increases fixed negative charge at the phosphorylation site.

(ii) The increased negative charge alters electrostatic interactions between the side chain of the phosphorylated amino acid and other amino acid side chains located in its immediate vicinity, within about 5 Å. This distance corresponds to no more than four residues where the side chains are within the same polypeptide segment.

(iii) The negative charge of the phosphate group compensates for fixed positive charges on basic side chains which would otherwise repel each other if brought close together (10–12 Å) in an α-helix.

(iv) Charge compensation usually occurs within one polypeptide segment, but may occur between polypeptides where a phosphorylation site is close to the N-terminus of one of them.

(v) Charge compensation permits a change in secondary structure of the polypeptide segment containing the phosphorylation site. In LHC II this change is formation of an α-helix. Rotation of the C-N bond of the phosphorylated amino acid (Fig. 2) may contribute to altered secondary structure. In the helix of phospho-LHC II, the phosphate group neutralizes the interaction of positive charges located on side chains 3–4 residues away on each side of the phosphorylation site, at about 5–6 Å (one helix turn) from the phosphorylation site and therefore 10–12 Å from each other.

(vi) The local change in secondary structure perturbs long-range interactions between side chains, and this perturbation causes a change in the tertiary structure of the hydrophilic domain.

(vii) The tertiary structural change may involve further electrostatic interaction between the phosphorylation site and basic side chains located many residues away or on an adjacent polypeptide, bringing the nitrogen atoms involved to within 3.5 Å of oxygen atoms of the phosphate group.

(vi) Tertiary structural changes alter the surface topology of the phosphoprotein in such a way as to decrease the complementarity of this surface with that of a neighbouring protein, a process equivalent to a change in quaternary structure.

(vii) The decrease in complementarity decreases the sum of the various interactions holding the two proteins together. Their hydrophilic domains cease to bind together, and the two proteins then become free to

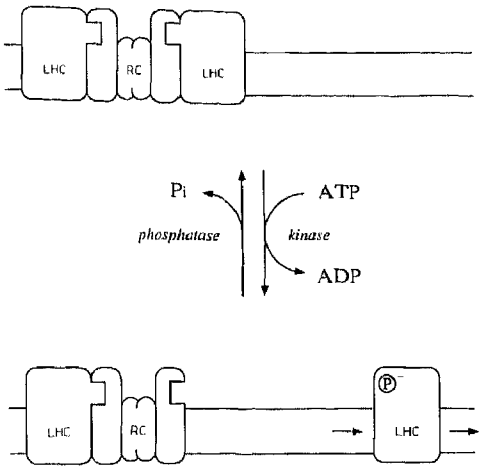

Fig. 23. The molecular recognition hypothesis. Phosphorylated light-harvesting complexes (LHC) become separated from neighbouring complexes of the photosynthetic membrane by intramolecular, intramembrane forces. The phosphate group neutralizes electrostatic repulsion between fixed positive charges on basic side chains flanking the phosphorylation site. This causes a structural change which perturbs the complementarity of docking surfaces required for functional interaction with neighbouring complexes. Molecular recognition is disrupted by an alteration in the sum of a number of weak forces holding neighbouring complexes together, and the dissociated phospho-LHC becomes free to diffuse within the membrane plane.

diffuse independently of each other within the membrane.

(viii) The two proteins become separated by lateral diffusion, and their functional interaction is prevented. For light-harvesting proteins, intermolecular excitation energy transfer is prevented.

(ix) The structural change described in (vi) may create a new surface topology that is complementary to that of a third protein complex. This third protein complex may therefore bind and interact functionally with the phosphorylated but not the dephosphorylated form of the original protein complex.

The overall process of control of membrane protein-protein interactions by a phosphorylation-induced structural change is depicted in Fig. 23.

The molecular recognition hypothesis suffers none of the problems of the surface charge hypothesis (subsection IV-A), since it does not depend on heterogeneity of membrane domains. In chloroplast thylakoids, the adhesion of proteins responsible for stacking can also be imagined to be under the control of phosphorylation-induced structural changes, so there is no conflict with limited unstacking upon phosphorylation. A particular advantage of the molecular recognition hypothesis is that it accounts for the specificity of protein phosphorylation-induced changes, without the additional requirement of the local charge hypothesis (subsection IV-B) that both proteins must become phosphorylated in order for their interaction to be controlled. The molecular recognition hypothesis also shares the advantages of the local charge hypothesis in accounting for control of photosynthetic unit cooperativity as a fundamental event in regulation, and one on which changes in absorption cross-section could have been superimposed during the evolution of two light-reaction photosynthesis. Where the local electrostatic interaction occurs between side chains of different polypeptides, the molecular recognition hypothesis differs from the local charge hypothesis on the basis of the requirement of the former for structural changes, the question 'how local is the local charge?' being one merely of semantics.

The molecular recognition hypothesis is put forward with the precedent of the structural change known to occur upon phosphorylation of serine 14 of the soluble enzyme glycogen phosphorylase [2,397]. This phosphorylation site is bounded by basic side chains, as shown in Fig. 24. Upon phosphorylation (steps (i)–(ii)), charge compensation occurs between the basic side chains in positions 9–11 and 16 (step (iii)). This triggers the formation of a helix by residues 5–16 (steps (iv)–(v)), and the serine phosphate becomes linked by salt bridges to arginine 69 of the same chain (step (vi)) and to arginine 43 of the opposite chain of the dimer (step (vii)) [2,397].

LHC II has a phosphorylation site that is also bounded by basic amino acid side chains (Fig. 24). The following specific sequence of steps is proposed by direct analogy with glycogen phosphorylase. Residues are numbered with accordance with the pea sequence of Bürgi et al. [70] shown in Fig. 3A.

Upon phosphorylation of threonine 6 of pea LHC IIβ (steps (i)–(ii)), charge compensation occurs between the basic residues 2–3 and 8–9 (step (iii)), enabling the N-terminal segment of phospho-LHC II to form a helix approximately between residues 1 and 10

```
                1       5         10
                M R K S A T*T K K V A S S        LHC II

S R P L S D Q E K R K Q I S*V R G L A G          glycogen phosphorylase
    5        10        15        20

---------- helix ------
```

Fig. 24. N-terminal segments of pea LHC IIβ and glycogen phosphorylase, showing phosphorylated amino acid residues (*) and basic amino acid residues (bold) flanking the phosphorylation sites. *Helix* shows the extent of the α-helix induced by phosphorylation of glycogen phosphorylase.

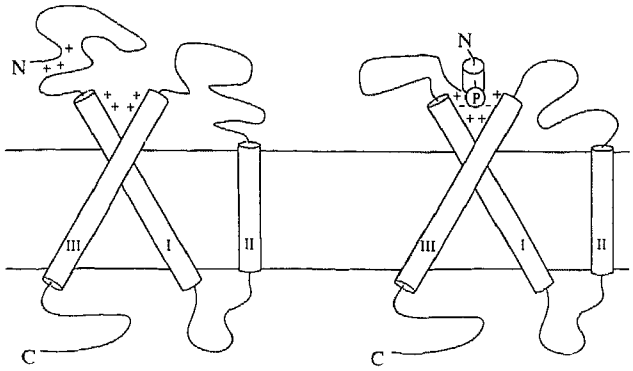

Fig. 25. Outline of a possible structural change upon phosphorylation of pea LHC IIβ, proposed by analogy with glycogen phosphorylase. Left: unphosphorylated LHC II. Right: phospho-LHC II. The N-terminal segment of phospho-LHC II is assumed to form a helix in the region of residues 1 to 10, and the threonine phosphate (in position 6) to form salt bridges with lysine 61 or arginine 63 close to the top of membrane helix I, and with lysines 178, 180 or 183 close to the top of membrane helix III. This would be expected to induce a large change in tertiary structure in the cytoplasmic (stromal) surface-exposed domain of LHC II.

(steps (iv)–(v)). The threonine phosphate could then form salt bridges with lysine 61 or arginine 63 close to the top of membrane helix I, or with lysines 178, 180 or 183 close to the top of membrane helix III (step (vi)). This would be expected to induce a large change in tertiary structure in the whole cytoplasmic surface-exposed domain of LHC II. It is significant that membrane helices I and III and the segments closest to them on the membrane surface and which contain the basic side chains have conserved sequences [398]. These correspond to the helical regions extending beyond the membrane and to which the hook-like features are attached [79] (Fig. 3B). The structural change envisaged for the surface-exposed domain is depicted diagrammatically in Fig. 25. Such a structural change could provide the basis for alterations in complementarity of a docking surface of the outer, mobile, and inner, immobile LHC II complexes, causing their dissociation. It could also provide the basis for alteration in complementarity of opposing faces of LHC II located on adjacent membranes, thereby initiating the process of unstacking. The model of Thornber and co-workers [65–66] places chlorophyll-binding sites within the conserved segments on top of helices I and III that also contain the basic side chains that are here suggested to be binding sites of the N-terminal threonine phosphate. The structure of Kühlbrandt and Wang [79] indeed shows chlorophylls close to the extended regions of helices I and III. This allows the possibility that the phosphorylation-induced structural change could block a pathway of excitation energy transfer between LHC IIs on opposing, appressed thylakoids, thereby preventing energy transfer between PS II units and hence decreasing their cooperativity. This possibility is considered further in subsection IV-E.

For the PS II core phosphoproteins of chloroplasts, alteration of molecular recognition by phosphorylation could involve charge compensation between basic side chains on the phosphoprotein and those on neighbouring polypeptides. Indeed, the extreme N-terminal position of the phosphothreonine in these cases suggests a role in the introduction of negative charge at another site, for example to control Q_A to Q_B electron transfer. This could be facilitated by additional, structural changes in the phosphoproteins themselves. Altered interactions between threonine 6 of LHC II and side chains of neighbouring polypeptides is also possible. In the cases of cyanobacteria and purple bacteria it is too early to predict whether charge compensation is intra- or intermolecular, since the membrane protein phosphorylation sites are not yet known.

Fig. 26 shows a computer prediction of the secondary structure of pea LHC II*b*, with a similar prediction for the same peptide when the phosphorylation site (thr 6) has been replaced with glutamate, a change comparable to the introduction of a fixed negative charge by threonine phosphorylation. It is seen that the effect of the substitution is to increase the helix probability for positions 1 to 12 by at least a factor of two (Fig. 26A) and to decrease the β-strand probability between positions 4 to 11 (Fig. 26B). Substitution of aspartate has a similar effect, and the increased helix and decreased β-strand probabilities occur with other LHC II sequences, including those with the phosphorylation site in position 4 (not shown). Similar effects occur with similar substitutions for the phosphorylation site of glycogen phosphorylase (Fig. 27), with an extension of helix probability of the segment 11 to 21 upon increased negative charge at position 14 (Fig. 26A). From X-ray crystallography the helix formed upon phosphorylation of glycogen phosphorylase is seen to extend from residues 5 to 16 [2]. These computer predictions (Figs. 26 and 27) are thus consistent with the proposed change in secondary structure of LHC II proposed here and with the known change in secondary structure of glycogen phosphorylase.

IV-D. Prospects for high-resolution models of structural change

The structures from X-ray crystallography of the *a* and *b* forms of glycogen phosphorylase [2,397] set the standard by which work on phosphoproteins should be compared. The best characterised effects of phosphorylation in photosynthesis are those of LHC II, but LHC II has not yielded large, stable three-dimensional crystals [75–77,398]. Work on electron diffraction of two-dimensional crystals of unphosphorylated LHC II has given a two-dimensional electron density map with

a resolution of 3.7 Å in projection (Fig. 4) [77], and this should be sufficient to resolve the major tertiary structural change proposed here (subsection IV-C, step (vii)). The 6 Å-resolution LHC II structure from three-dimensional reconstruction [79] shows little detail outside the hydrophobic domain. If the model proposed here is correct, then phospho-LHC II should show a different structure in this region. The unphosphorylated complex should perhaps not differ greatly in structure from a complex from which the phosphorylation site has been cleaved by proteolysis, since the model suggests that the unphosphorylated N-terminal segment has few if any specific interactions determining tertiary structure, in contrast with the salt bridges envisaged for the threonine phosphate. Further refinement of the technique [399] is expected to give higher resolution [79], 3.4 Å resolution having been obtained with bacteriorhodopsin [400]. Separation of phosphorylated and dephosphorylated LHC IIs can be achieved, but neither gives three-dimensional crystals [401].

In the absence of three-dimensional crystals of the whole complex, three-dimensional crystallization of an isolated N-terminal domain obtained by proteolytic cleavage might be a feasible route for testing the molecular recognition hypothesis by X-ray crystallography, though it is doubtful whether this domain would retain its native conformation, particularly in view of the interactions to be expected (Fig. 25) between this domain and the surface-exposed segment between helices II and III. High-field NMR spectroscopy might also be a possible route with a smaller isolated domain, the complete N-terminal surface-exposed segment being roughly 65 residues long.

The structure of the purple bacterial reaction centre [39–42] should be expected to include a docking site for B880. The flat structure formed by the RC chloro-

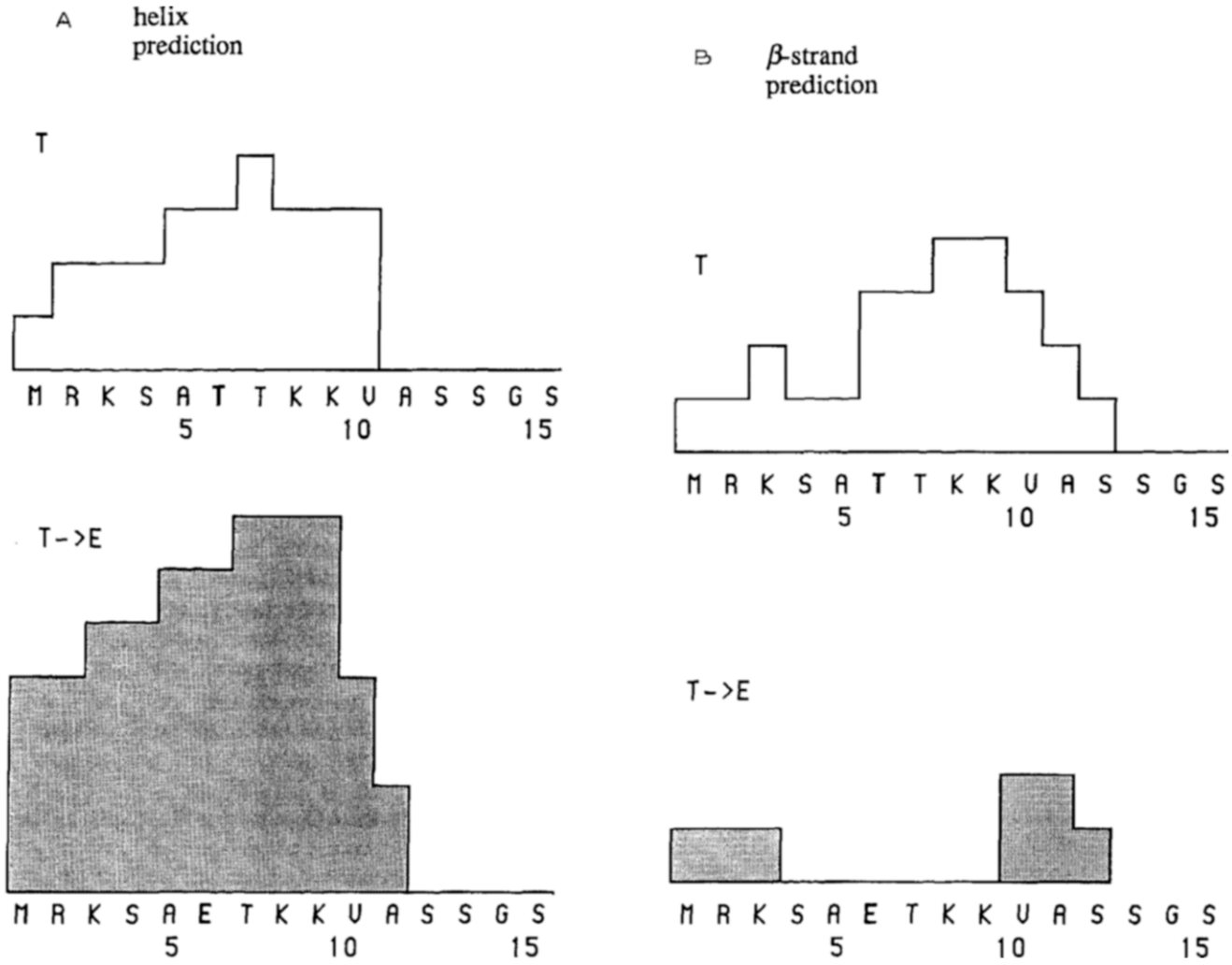

Fig. 26. Computer predictions of secondary structure of the phosphorylated N-terminal segment of pea LHC IIβ, with threonine (above) and glutamate (below) in position 6. The substitution increases helix probability (A) and decreases β-strand probability (B). The y-axis is a measure of probability of helix or β-strand secondary structure at each position in the sequence. Predictions were made using the program *Predict* of the Leeds University-Birkbeck College ISIS sequence and structure database [440] (*Serpent* from Oxford Molecular Ltd., Oxford).

phylls and the L/M heterodimer and from which the carotenoid protrudes presumably gives a large surface area for energy transfer from adjacent light-harvesting chlorophylls with chlorin rings plane parallel to those of the special pair. Chemical cross-linking suggests a role for the H subunit in interactions with the light-harvesting system [44], and the single membrane helix of H is located above one face of the L/M heterodimer. Thus the possibility of phosphorylation of H itself is particularly interesting (subsection III-B), while phosphorylation of B880-β (subsection III-B) could also induce the predicted structural changes. The eventual solution of structures for B880, B800–850 and for a complete photosynthetic unit should make it possible to determine the surfaces whose complementarity might be controlled by protein phosphorylation.

In the meantime, indirect measurements on any of these systems might be expected to show evidence of altered pigment orientation [402–403]. Chemical cross-linking indicates changes in protein topography upon phosphorylation of chloroplast thylakoids [404] and in cyanobacterial membranes undergoing state transitions [405]. Light-induced Fourier transform infrared difference spectroscopy has been used to demonstrate protein conformational changes in isolated reaction centres [406] and could be applicable to phosphorylated and dephosphorylated light-harvesting complexes and photosynthetic units. A further route for probing structure-function relationships is of course site-directed mutagenesis (reviewed for PS II in [407]), in view of which there is an additional need to characterize and identify the thylakoid phosphoproteins in transformable cyanobacteria (subsection III-A).

IV.E. A model for control of PS II cooperativity and antenna size

Fig. 28 is a model for the changes in organization of PS II that occur upon phosphorylation of LHC II and core polypeptides. It is consistent with available data on phosphorylation of the individual polypeptides, its control and resulting functional effects (subsections II-A.7–II-C).

In this model, phosphorylation of LHC II by its

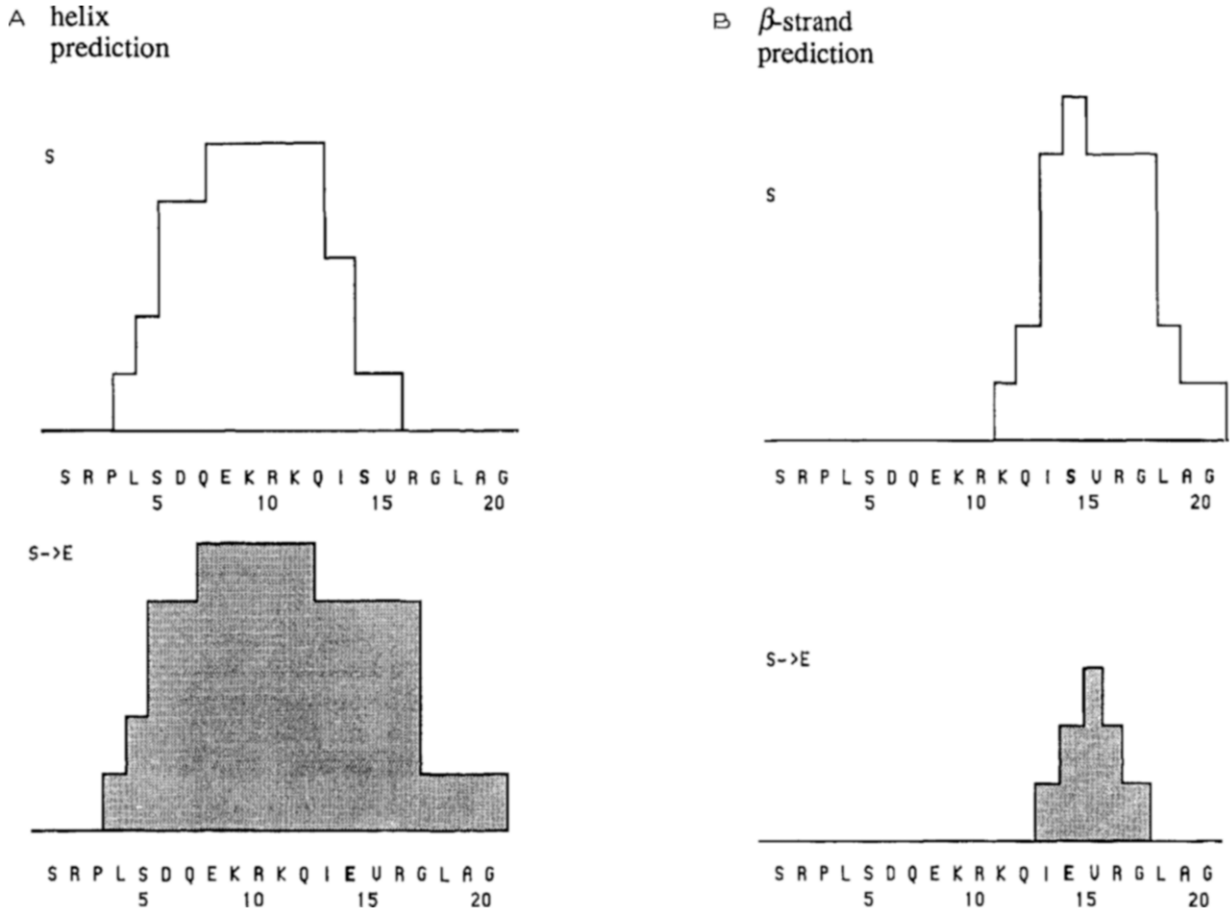

Fig. 27. Computer predictions of secondary structure of the phosphorylated N-terminal segment of glycogen phosphorylase, with serine (above) and glutamate (below) in position 14. The substitution extends helix probability (A) and decreases β-strand probability (B). Predictions were made as for Fig. 26.

kinase is assumed to cause secondary and tertiary structural changes as described in subsection IV-C. The effect of these changes is to dissociate the outer and inner pools of LHC II, and to dissociate LHC IIs on opposing, appressed thylakoids. The first dissociation decreases excitation energy transfer between the outer and inner LHC IIs, causing a decrease in the absorption cross-section or antenna size of PS II. The second dissociation decreases energy transfer between opposing LHC IIs, and thus decreases the cooperativity of the photosynthetic units which is assumed to rely in part on energy transfer perpendicular to the membrane plane (see also Fig. 16). Diffusion of the phosphorylated outer LHC II pool then becomes independent of that of the inner pool and associated PS II core. The phosphorylated outer LHC II is thus free to diffuse until it makes contact with an LHC I complex, with which it is able to bind by virtue of possessing a docking surface complementary to that of LHC I. This lateral migration is merely a result of diffusion, and may be suppressed by decreased temperature. Complete lateral migration of phospho-LHC II decreases PS II cooperativity and causes the complementary changes in the antenna sizes of PS II and PS I that are characteristic of the state 2 transition. Incomplete lateral migration may decrease PS II cooperativity and antenna size without having any effect on the antenna size of PS I.

Conversely, dephosphorylation of LHC II returns it to a conformation in which its binding with PS I is disrupted. Under normal circumstances it is then free to diffuse until it meets with the inner LHC II pool, to which it binds, increasing PS II absorption cross-section and cooperativity in the transition to state 1. If diffusion is restricted by sub-optimal temperature or if the LHC II (inner) docking surface is still in its phosphorylated conformation, LHC II may be able to associate with neither PS I nor PS II, and the LHC (outer) phosphorylation may again have no observable effects on PS I. Incomplete state transitions of either kind may

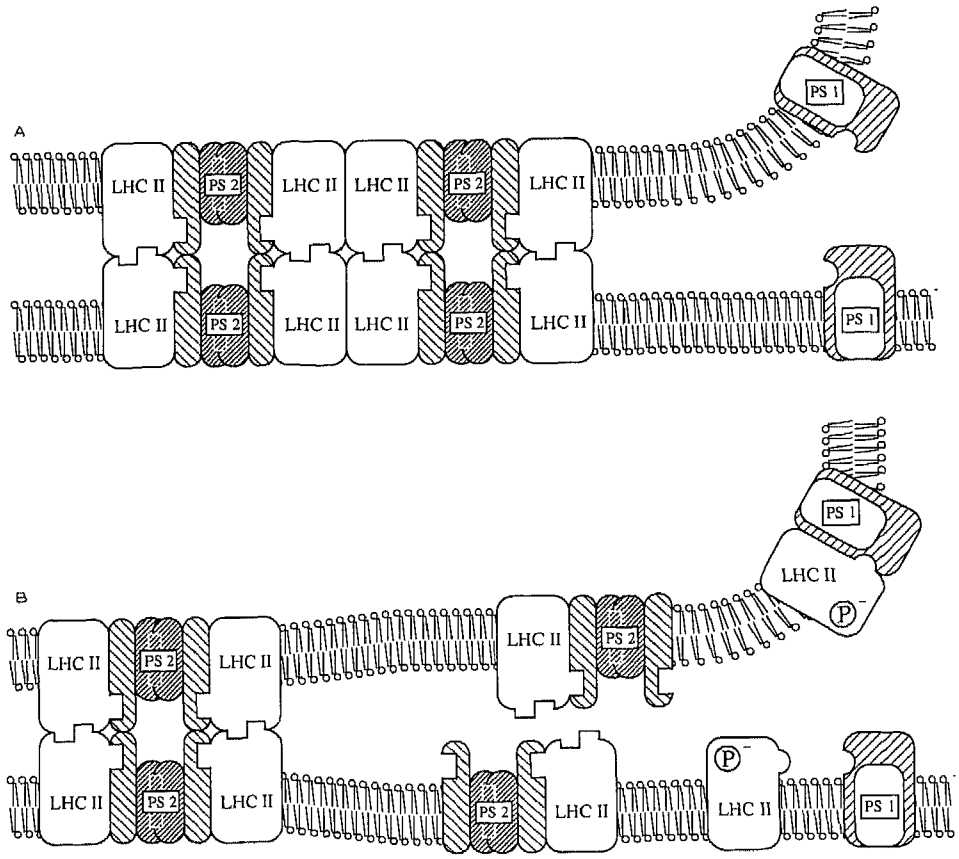

Fig. 28. (A) A model for the organisation of the chloroplast thylakoid. PS II-α centres are connected laterally and tranversely for excitation energy transfer by docking of LHC II complexes with the PS II core antenna system (diagonally hatched) and with each other. This brings the acceptor side of PS II reaction centres into opposition. (B) The proposed effect of LHC II phosphorylation on this organisation. Phospho-LHC II has a decreased affinity for the PS II core (lateral protein-protein interactions) and for itself (transverse protein-protein interactions), and therefore becomes free to diffuse independently within the membrane, eventually to dock instead with the PS I core antenna system. The connectivity and antenna size of PS II units is decreased, and the loss of adhesion contact surfaces causes some transverse separation of adjacent thylakoids of the grana stack. A proportion of PS II reaction centres cease to be in opposition, with effects on acceptor-side electron transfer that may be mitigated by phosphorylation of reaction centre core polypeptides. A is state 1. B is state 2.

account for the different results obtained in different laboratories concerning the presence or absence of effects of phosphorylation on antenna size of PS I (subsection II-A.2).

In the model of Fig. 28 it is assumed that opposing PS II centres may be connected for efficient electron or proton transport on the acceptor side when membranes are appressed as a result of binding of dephosphorylated, opposing LHC IIs. Decreased antenna size and cooperativity of PS II will both serve to decrease the rate of PS II electron transfer at limiting light intensity. In addition, the unstacking caused by LHC II phosphorylation will tend to destroy the cooperativity of opposing PS II centres for electron transport on the acceptor side. The function of phosphorylation of PS II core polypeptides may then be to retard release of Q_B from its binding site on D1.

Thus phosphorylation of PS II core polypeptides, specifically the 9 kDa phosphoprotein, could be a response to unstacking by which PS II electron flow into the plastoquinone pool is maintained. This would account for the effect of PS II phosphorylation in increasing the rate of PS II electron transport independently of effects of phosphorylation of LHC II [232]. A further possibility is that PS II phosphorylation at high light intensity may direct electrons back across the membrane to the donor side, perhaps via cyt b_{559}, hence serving to protect PS II from photoinhibition. If free $Q_B \cdot -$ reduces oxygen univalently to generate superoxide and subsequent oxygen free radicals implicated in photoinhibitory breakdown of D1 [253], then retardation of its release by PS II core protein phosphorylation would also be expected to provide protection from photoinhibition [126].

Alteration of PS II acceptor-side kinetics in this way could also be consistent with decreased herbicide binding, a reported effect of protein phosphorylation [125]. Fully phosphorylated PS II cores, uncoupled from each other and from the mobile LHC II pool, would have many of the properties of PS II-β, and may be free to migrate into unappressed thylakoid domains. Phosphorylation of CP 43 has no central role in this model, but it is possible that it may further disrupt energy transfer between opposed PS IIs, decreasing PS II cooperativity at the level of the PS II core antenna.

In the model of Fig. 28, the LHC II kinase is assumed to be located in or close to the cytochrome b/f complex, located in the partition region. Rotation of the complete PS II unit about an axis perpendicular to the membrane would then be required for phosphorylation of all peripheral LHC IIs, some of which will located on the other side of the reaction centre from the kinase. Movement of phosphorylated LHC IIs within the appressed domain is therefore required, as proposed by Albertsson et al. [161]. In view of the oligomeric structure of LHC II, it is possible to imagine a number of steps of degree of LHC II phosphorylation stretching laterally over the grana membranes. Fully dephosphorylated PS II-α (PS II-α_1) would be found in the centre of the appressed domain, with the degree of phosphorylation increasing with distance towards the grana margin. These steps may correspond to the heterogeneity within PS II-α proposed by Albertsson et al. [161], and Fig. 28 is proposed as a schematic outline of events occurring in such a partition region [161] (Fig. 10). The proposed location of the different PS II-α centres is consistent with the idea of cooperativity within PS II-α by means of energy transfer between thylakoids, since PS II-α_1 has the largest antenna size and is located in the most tightly appressed domain.

V. Protein phosphorylation in control of gene expression

V-A. Protein phosphorylation and redox control in developmental adaptation

Photosynthetic organisms generally possess a broad repertory of adaptive responses to alterations in light quality and quantity. As in the case of the direct post-translational alteration of structure and function of light-harvesting and reaction centre proteins, regulation at other levels of gene expression can serve to match light energy conversion to availability of substrates for coupled assimilation and other energy-requiring processes.

Developmental responses to changes in the light regime [29] can be regarded as long-term counterparts of the short-term, physiological adaptations that result directly from phosphorylation of mature, functional proteins. Examples in green plants are control of photosynthetic unit size by changes in relative abundance of LHCPs [408–409], control of the stoichiometry of PS IIα and PS IIβ [410], and control of the stoichiometry of reaction centres of PS I and PS II [28,167,411], apart from the differences between sun and shade leaves and plants at macro-physiological and morphological levels. Cyanobacteria and red algae are able in this way to control photosystem stoichiometry [412], antenna size by selective control of phycobilisome structure [413], and relative chromophore composition in complementary chromatic adaptation [316–317]. Purple bacteria carry out many similar adaptations, including alteration of composition and stoichiometry of antenna proteins according to growth conditions [414].

Control of cyanobacterial photosystem stoichiometry by redox state of plastoquinone has been proposed by Fujita et al. [415], specifically by regulation of PS I formation [416]. This makes sense for the same reasons as plastoquinone-control of state transitions, since the

imbalance and energetic losses corrected by adjustment of photosystem stoichiometry will result from unequal rates of energy utilization by PS I and PS II. A feedback control correcting the imbalance should therefore be expected to include redox state of a component located between PS I and PS II. Melis [417] has suggested that photosystem stoichiometry adjustment is a response to imbalance in the relative rates of electron transport in PS I and PS II. In support of this idea, Melis presents evidence suggesting that all known cyanobacterial and higher plant mutants deficient in PS II antenna size possess elevated PS II/PS I stoichiometries and are unable to carry out long-term adjustments to photosystem stoichiometry as a pleitropic effect of the mutation [417]. Such mutants would also be expected to retain an oxidised plastoquinone pool regardless of light regime and be locked in state 1, consistent with the proposal of control of gene expression by plastoquinone redox state.

In green algae, LHC II phosphorylation has been proposed as a factor in long-term adaptation of antenna size [418–419]. In cyanobacteria, protein phosphorylation has been implicated in long-term adjustment of photosystem stoichiometry [307,420] which itself has effects on short-term state transitions [421]. Parallel regulation of membrane protein phosphorylation and of photosystem biosynthesis has been proposed by Melis et al. [422]. Analysis of membrane protein phosphorylation in LHC II and phycobilisome-deficient mutants [417] should help to distinguish those phosphoproteins involved in control of long-term adaptation.

It therefore seems useful to consider whether redox-controlled protein phosphorylation functions in signal transduction to initiate a response at the level of transcriptional or translational control of gene expression. The mechanism by which this could occur is not yet clear, though there are several thylakoid proteins whose redox-controlled phosphorylation has no clearly defined function, the most obvious one in chloroplasts being the 9 kDa phosphoprotein (subsection II-B). The 9 kDa phosphoprotein could therefore be considered as a candidate for a redox sensor in photosynthetic control of gene expression. It also is possible that a thylakoid GTPase [423] could function in such a process, GTPases being involved in wide range of signal transduction processes [424].

Fig. 29 outlines a possible feedback control. It cannot yet be determined whether control is exerted at the level of translation, of transcription, or of both. Protein phosphorylation may be involved directly in regulation at both levels, with phosphorylation of ribosomal proteins [425–427] and DNA-binding proteins [428] being established in plants. The peptide elongation factor involved in protein synthesis, itself a GTPase [424], is phosphorylated at multiple sites [427]. Phosphorylation of nuclear DNA-binding proteins is implicated in photocontrol of *cab* gene expression [428], a control ascribed to phytochrome in non-photosynthetic cells [429–430]. Red/far-red reversibility of gene expression

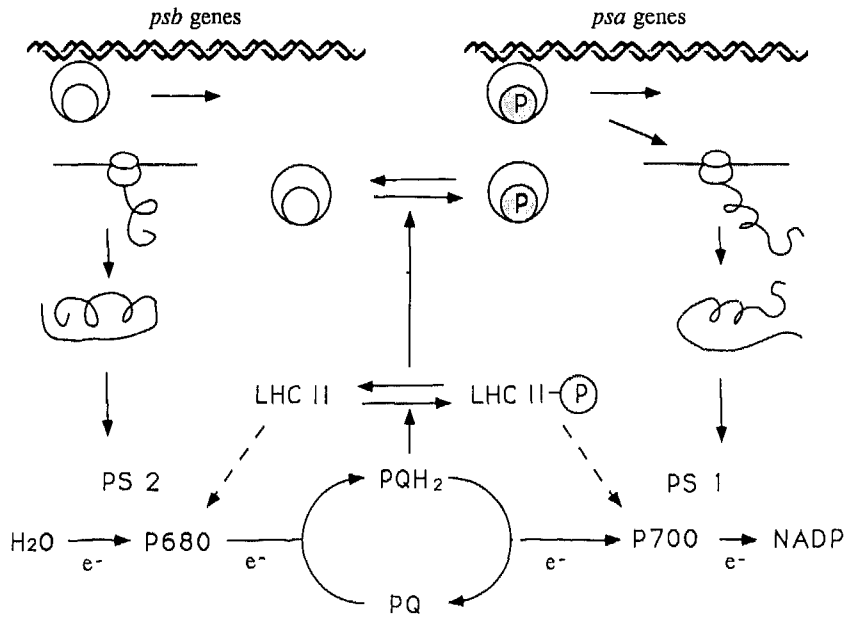

Fig. 29. Outline of a possible redox control of photosystem stoichiometry by effects on expression of genes for components of the photosystems. Control of transcription may involve phosphorylation of DNA-binding proteins. The depiction of transcriptional control is purely schematic, and may involve either activation or inactivation of proteins involved in either positive (activator) or negative (repressor) control. These proteins would function as specific redox effectors, with their corresponding redox sensors responding to the redox state of plastoquinone or of the *b/f* complex. The LHC II kinase is therefore a possible redox sensor. See also Fig. 30.

in mature, photosynthetically-active systems could in principle result from redox control at the level of plastoquinone. Determination of action spectra [138,431] and dose-response relationships should serve to distinguish between redox and phytochrome control. In Fig. 29 it is proposed that *psa* genes are switched on and *psb* genes switched off when plastoquinone becomes reduced, and that *psa* genes are switched off and *psb* genes switched on when plastoquinone becomes oxidised.

In view of structural similarities between chloroplast and cyanobacterial DNA-binding proteins [432], it seems reasonable to consider the possibility that one reason for the evolutionary persistence of the chloroplast genetic system is to provide a direct and rapid means of control of gene expression by chloroplast function, primarily by photosynthesis. In this case, one could view the common feature of chloroplast-encoded proteins of the photosynthetic apparatus as a need for a rapidly-responding biosynthetic machinery that can be regulated on-site, if necessary without immediate reference to nuclear control. Perturbation of redox poise is likely to be an important signal to which the cell must respond more rapidly than can be achieved by control of nuclear gene expression. Thus protein phosphorylation in control of gene expression within the chloroplast might be a fairly direct process. In addition, a possible route for redox control of replication of chloroplast DNA is suggested by the report of Wu et al. [433] that an 18 kDa protein that binds to the origin of replication of chloroplast DNA is an iron-sulphur protein and related to a subunit of NADH dehydrogenase.

In cyanobacteria, any change in spectral composition leading to decreased light-harvesting ability with a given phycobilisome composition would be expected to cause oxidation of the plastoquinone pool, signalling the requirement for alteration in light-harvesting capacity at a number of different levels, the first response being a state 1 transition. Complementary chromatic adaptation [314,316–317] could also be initiated by this change, but the biosynthetic machinery could not 'know' merely from plastoquinone oxidation whether the problem lay in too little red light or too little green light, that is, whether more or less phycoerythrin was required relative to phycocyanin. For this response a specific photoreceptor would be required. Thus the proposal of Harrison [231] that β-phycocyanin may convert to a photoreceptor by redox-controlled phosphorylation is particularly interesting, and could represent a double level of control of effective PS II antenna size.

V-B. Photosynthesis and two-component regulation

The modification of the signal-transducing *glnB* gene product by selective excitation of PS II (subsection III-A.3) is an important precedent for the multiple levels of control by protein phosphorylation proposed here and outlined in Fig. 30, for it links photosynthetic electron transport with a known pathway for both post-translational and transcriptional control. Detailed studies of repressors and activators of transcription have usually concentrated on DNA-binding proteins that themselves require, as co-factors, the small molecules such as lactose, methionine or tryptophan whose altered intracellular concentration is both the signal and end-product of control. Thus atomic-resolu-

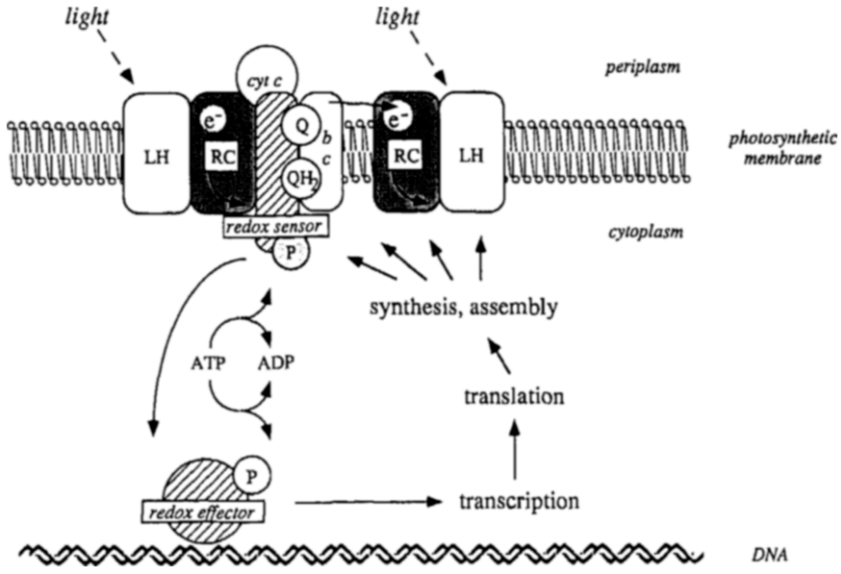

Fig. 30. A scheme for protein phosphorylation in two-component regulation of photosynthesis, consisting of a redox sensor and redox effector (cross-hatched shapes). The redox sensor is assumed to be a redox active membrane phosphoprotein and a component of one of the complexes of the photosynthetic electron transport chain. The redox effector is a DNA-binding protein whose phosphorylation by the sensor is required for the initiation of transcription of genes encoding components of the photosynthetic apparatus.

tion structures are known for the *lac*, *trp*, and *met* repressors, for example, and it is seen that reversibility of control arises from non-covalent binding of the substrate to the repressor. In contrast, in transcriptional control responding to intracellular NH_4^+ concentration and mediated by P_{II}, covalent modification of NR_I occurs by phosphorylation, the NR_I kinase (NR_{II}) itself being controlled by phosphorylation.

Wherever cells must respond by transcriptional control to changes that cannot be communicated by direct, non-covalent binding of substrate to a DNA-binding protein, it seems likely that a process such as protein phosphorylation will be involved instead. The nitrogen-regulatory system which includes P_{II}, NR_I and NR_{II} [320,322] exemplifies this kind of control, and has many features in common with systems controlling diverse factors such as osmotic pressure, phosphate assimilation, virulence, transport, chemotaxis, motility and sporulation [434–435]. Several of these appear to share a common mechanism of transcriptional activation [436]. In general, such systems have two components, sensors and regulators. Though NR_{II} is soluble, sensors are typically membrane proteins which undergo histidine phosphorylation at a conserved site, and regulators are DNA-binding proteins [437] phosphorylated on arginine by a phosphotransferase [434]. The known communication of the signal from the sensor NR_{II} to the regulator NR_I by protein phosphorylation has been suggested by Ronson et al. [437] as a precedent for the mechanism of control in all such two-component regulatory systems. In passing it should be noted that the H-chain of the purple bacterial reaction centre has the same membrane orientation as many sensors in two-component systems.

In Fig. 30 the existence of specific redox sensors and redox effectors is proposed. The redox sensor may be a component of cytochrome *b/c* complexes, since it would there be most favourably placed to respond to perturbation in redox poise, the signal initiating the control. The ideal *b/c* redox sensor would be a membrane phosphoprotein with a redox-active prosthetic group such as haem, and should have protein kinase or transferase activity using the redox effector as substrate. The LHC II kinase may be an example.

The oxygen sensor controlling expression of nitrogen-fixation genes in *Rhizobium meliloti* is a membrane haemoprotein with protein kinase and phosphotransferase activity, termed FixL [438]. FixL catalyzes its own phosphorylation, and is a protein kinase for FixJ, the oxygen effector. The membrane redox sensor proposed here (Fig. 30) for two-component regulation of bioenergetic systems such as photosynthesis might also be a haemoprotein, redox sensors in general standing in relation to oxygen sensors as cytochromes do to haemoglobins and myoglobins.

It is therefore proposed that photosynthesis is under two-component regulation, with membrane phosphoproteins functioning as redox sensors, communicating the redox state of the photosynthetic electron transport chain to the redox effector of the transcriptional apparatus (Fig. 30). By this reasoning the phenomenon of state 1– 2 transitions can be viewed as just the post-translational arm of an integrated control system serving to maintain energetic homeostasis in phototrophic organisms. The precedents of two-component control, and the ntr and FIx systems in particular, strongly implicate protein phosphorylation in sensor and effector mechanisms.

It may be that changes in the quality of the light environment were an example from early in evolution of a signal that can play no direct part in protein–DNA interactions, unlike the substrate availability of comparable concern to chemotrophic organisms. If so, photosynthesis may be an archetype for two-component signal transduction. As with other areas of photosynthesis, regulation by protein phosphorylation is not just interesting, it is fundamental, and likely to operate by common principles elaborated by many different biological processes.

Acknowledgements

I acknowledge the influence of many colleagues and collaborators, particularly my partners in the Leeds Photosynthesis Group whose work is discussed here. I thank Bertil Andersson, Alma Gal, Werner Kühlbrandt, Nick Mann, Itzhak Ohad, Claudie Vernotte and Engelbert Weis for manuscripts prior to publication, and Alma Gal and Itzhak Ohad for permission to include their unpublished scheme in Fig. 14. I thank John Bennett for introducing me to chloroplast protein phosphorylation. I thank members of the Departments of Biochemistry and Molecular Biology and of Genetics in Leeds for practical help, use of computer facilities, and discussions, and the latter Department for a Visiting Professorship. I thank Carol Allen and Beston Nore for interest and support, and for taking on many difficult tasks that threatened the completion of this review. Financial support for the Leeds group came from SERC, The Nuffield Foundation, The Royal Society, and NERC. In Oslo support for this work comes from NAVF and the Nordisk Energiforsknings Program.

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