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Protein phosphorylation and control of excitation energy transfer in photosynthetic purple bacteria and cyanobacteria

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Summary – The function of phosphorylation of light-harvesting polypeptides is well characterised in chloroplasts of green plants, but the prokaryotic cyanobacteria and purple photosynthetic bacteria have quite different light-harvesting polypeptides whose structure and function cannot be controlled in precisely the same way. Nevertheless, cyanobacteria show light-dependent phosphorylation of membrane polypeptides associated with photosystem II and with the light-harvesting phycobilisome, and purple bacteria show light-dependent phosphorylation of low molecular-weight chromatophore membrane polypeptides. In both cases membrane protein phosphorylation is associated with functional changes observed by chlorophyll fluorescence spectroscopy or chlorophyll fluorescence induction kinetics. Here we report on our recent protein sequence and other data concerning the identities of these phosphoproteins. We also discuss the significance of these findings for regulation by protein phosphorylation of photosynthesis in prokaryotes.

photosynthesis / protein phosphorylation / light-harvesting / photosystem / phycobilisome / chromatophore membrane

Introduction

The photosynthetic unit is an assembly of several hundred pigment molecules that must cooperate for the conversion of each quantum of light energy into electrochemical potential. This concept, originally developed to explain biophysical data on quantum yield of whole cells, now has the complete support of protein biochemistry and of structural studies of photosynthetic membranes and their components [1]. Of the several hundred pigment molecules or chromophores in each photosynthetic unit, almost all function as light-harvesting pigments that absorb light energy and then transfer that energy to neighbouring chromophores [2]. In most cases light-harvesting chromophores are molecules of chlorophyll, a cyclic tetrapyrrole containing magnesium that is non-covalently bound to protein. An important exception to this rule is found with the linear tetrapyrrole phycobilin light-harvesting pigments, covalently bound to light-harvesting proteins of cyanobacteria and red algae.

The primary event of energy conversion in photosynthesis is a photochemical reaction in which a proportion of the energy absorbed is conserved as electrochemical potential [3]. This photoelectrochemistry takes place in the reaction centre component of each photosynthetic unit where a specialized chlorophyll molecule is oxidized and a primary electron acceptor reduced, and where the resulting charge separation becomes stabilized thereby permitting secondary

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Abbreviations: **DCPIP,** 2,6-dichlorophenol-indophenol; **DCMU,** 3-(3,4-dichlorophenyl)-1,1-dimethylurea; **DPC,** diphenylcarbazide; **LHC,** light-harvesting chlorophyll complex; **PS,** photosystem.

electron transfer events on both the electron donor and acceptor sides of the reaction centre itself. In recent years 2 high-resolution structures of purple bacterial reaction centres have been solved from X-ray crystallographic data [4–5]. The first structure to be obtained was that by Deisenhofer *et al.* [4]. Their structure was of a protein complex from *Rhodopseudomonas viridis* that contained the *c*-type cytochrome electron donor on the periplasmic side of the structure in addition to the components of the reaction centre itself. The second structure was that of Allen *et al.* for the reaction centre preparation from *Rhodobacter sphaeroides* of Feher and co-workers [5].

Phosphoproteins were first implicated in regulation of photosynthesis in chloroplasts of green plants when Bennett described a number of chloroplast phosphoproteins spanning the range of molecular weight 7–70 kDa [6]. 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 2 polypeptides most conspicuously phosphorylated were a major thylakoid membrane component at about 26 kDa and a minor component (as judged by Coomassie blue staining) at about 9 kDa, both of which were phosphorylated on threonine residues [6]. Bennett identified the 26 kDa thylakoid phosphoprotein as a polypeptide of the light-harvesting chlorophyll *a*/*b* binding protein (LHC II), and suggested that thylakoid protein phosphorylation might be involved in regulation of the LHC II function.

Bennett subsequently confirmed that the 26 kDa phosphoprotein was LHC II [7], and showed that phosphorylation of both thylakoid proteins is light-dependent *in vitro* even where [γ-^{32}P]ATP is used to label proteins of isolated thylakoids in the presence of an uncoupler of ATP synthesis [8]. 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 itself is light-dependent. In further experiments Bennett showed that both the protein kinase [8] and protein phosphatase [9] activities are present, like their protein substrates, in isolated thylakoid membranes.

The most obvious consequence of phosphorylation of LHC II in isolated thylakoids is a change in their chlorophyll fluorescence emission properties. Phosphorylation is accompanied by a decrease in total chlorophyll fluorescence yield at room temperature which is consistent with decreased emission from PS II, and fluorescence spectroscopy at 77 K shows that LHC II phosphorylation produces decrease in yield in the PS II/LHC II emission bands relative to that of the PS I band [13, 14]. It can be inferred that excitation energy from light absorbed by LHC II becomes diverted away from PS II as a result of phosphorylation, and the possibility also then exists that all or part of that energy reaches PS I instead.

The discovery of the redox control of the chloroplast LHC II kinase [15, 16] at the level of plastoquinone led to the proposal of a protein phosphorylation mechanism for autoregulation of relative delivery of excitation energy transfer to PS I and PS II [15, 16]. That this mechanism accounts for the adaptations described as state 1-state 2 transitions [17] is now widely accepted in broad principle [18–25]. The fact that photosynthetic prokaryotes such as cyanobacteria exhibit state 1-state 2 transitions but lack LHC II was recognized as an anomaly in this story at its inception [15].

In general, there are 4 main types of light-harvesting antenna systems in photosynthesis that in principle could be modified by phosphorylation. 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 cryptophytes. Regulation of light-harvesting function by protein phosphorylation has been most completely described for group (ii). Here we describe evidence for analogous reactions in groups (i) and (iv). There has been no report so far on protein phosphorylation in green bacteria (iii), though this may simply be because it has yet to be investigated.

Photosynthetic purple bacteria

The photosynthetic apparatus of the purple non-sulphur bacterium *Rhodospirillum rubrum*

consists of 2 types of pigment-protein complex: the photochemical reaction centre and the light-harvesting B880 complex. Each molecule of B880 consists of 2 membrane-spanning polypeptides, B880-α and B880-β, together with 2 molecules of bacteriochlorophyll and one of carotenoid [26]. *R. rubrum* therefore has a minimal photosynthetic unit consisting of one reaction centre together with its associated molecules of B880.

Phosphorylation of the B880 light-harvesting pigment-protein complex of *Rhodospirillum rubrum* was first suggested by the work of Loach *et al.* [27], who incubated cells of *R. rubrum* in the light in the presence of Mg^{2+} and in the dark in the absence of Mg^{2+} and monitored the connectivity of the photosynthetic units by measuring reaction centre absorbance changes after flash excitation. They concluded that the photosynthetic units of cells incubated in the dark without Mg^{2+} functioned independently of one another whereas those of cells incubated in the light with Mg^{2+} showed significant connectivity. Using organic solvent extraction and Sephadex LH60 chromatography, they purified B880-α from cells incubated in the presence of ^{32}P-P_i and showed that more ^{32}P-P_i was associated with the B880-α prepared from cells incubated in the light with Mg^{2+} (showing connectivity of photosynthetic units) than with B880-α prepared from cells incubated in the dark without Mg^{2+} (with photosynthetic units functioning independently). Chemical determination of phosphate associated with B880-α led to a similar conclusion.

Holmes and Allen also correlated B880 phosphorylation and photosynthetic unit connectivity in *R. rubrum* cells under similar incubation conditions to those used in [27]. We used bacteriochlorophyll fluorescence *in vivo* to monitor connectivity and SDS–PAGE of ^{32}P-P_i-incubated cells to monitor B880 phosphorylation [28]. We found that phosphorylation of a polypeptide of 13 kDa, identified with B880-α, correlated with the photosynthetic units showing connectivity in agreement with [27]. We also found that under conditions where the photosynthetic units were functioning independently a second polypeptide, at 10.5 kDa and identified with B880-β, was phosphorylated. We concluded that phosphorylation might regulate photosynthetic unit connectivity.

Allen and Holmes [29] have suggested that regulation of light-harvesting function in purple bacteria, cyanobacteria and in photosynthetic eukaryotes might operate by similar mechanisms, stressing the importance of electrostatic effects directed parallel to the membrane plane in modulating the interactions between light-harvesting complexes. The correlation of phosphorylation of B880-β with non-connectivity would fit well with such a mechanism but the correlation of phosphorylation of B880-α with connectivity is more problematical. However, it may be that the sites for phosphorylation on B880-α and on B880-β are spatially separated for example on opposite sides of the membrane and therefore that phosphorylation of B880-α serves to neutralize an existing positive charge on the polypeptide, leading to a local decrease in charge and allowing the photosynthetic units to move closer together.

Phosphoproteins have also been identified in cells of *Rhodomicrobium vannielii* [30] and *Rhodobacter sphaeroides* [31] including phosphopolypeptides with mobility on SDS–PAGE consistent with their being light-harvesting polypeptides. In *R. sphaeroides* a phosphopolypeptide of 12 kDa was seen [31]. In *R. vannielii* a polypeptide of 12.7 kDa was phosphorylated, although not apparently under conditions where the culture was light-limited [30].

Phosphorylated B880 polypeptides have been reported, together with other phosphopolypeptides, from *in vitro* studies using chromatophore membranes [31–33]. Holuigue *et al.* [32] using [γ-^{32}P]ATP reported the phosphorylation of several polypeptides in chromatophores of *R. rubrum*, including 2 with mobilities on SDS–PAGE corresponding to 13 kDa and 11 kDa. Phosphorylation of the 11 kDa polypeptide required only the chromatophore fraction but phosphorylation of the 13 kDa polypeptide required the presence of both chromatophore and soluble fractions. We have also observed phosphorylation of polypeptides of 13 kDa and 10 kDa *in vitro* with [γ-^{32}P]ATP [33]. Phosphorylation of the 13 kDa polypeptide required only the presence of chromatophores but phosphorylation of the 11 kDa polypeptide required both chromatophores and soluble fraction [33] in contrast to the conditions reported in [32]. This discrepancy may reflect uncertainties in estimates of polypeptide molecular masses by SDS–PAGE.

Control of B880 phosphorylation in *R. rubrum*

We have examined the conditions for phospho-

rylation of these putative B880-α (13 kDa) and B880-β (11 kDa) polypeptides. Light stimulated the phosphorylation of the 13 kDa polypeptide. Phosphorylation of this polypeptide was also stimulated in the presence of 2 mM potassium ferricyanide in the dark but was inhibited in the presence of 5 mM sodium dithionite in the dark. This effect of ambient redox potential suggested the possibility of regulation of the protein kinase by an electron transport component, possibly the ubiquinone pool. Regulation of phosphorylation would then be analogous to regulation of LHC II phosphorylation in chloroplasts [15, 34]. Consistent with this suggestion, the electron transport inhibitor DBMIB, which inhibits at a site between the reaction centre and the cytochrome *bc* complex, also inhibits the phosphorylation of the 13 kDa polypeptide in the light [33].

The migration on SDS–PAGE of the low molecular weight phosphopolypeptides seen in both cells and cell-free systems of *R. rubrum* is consistent with their idendification as B880-α (13 kDa polypeptide) and B880-β (10–11 kDa polypeptide). We have suggested [33] that connectivity changes may serve to compensate for metabolic or environmental conditions that lead to a build-up of electrons between the reaction centre and the cytochrome *bc* complex. The reduction of the ubiquinone pool would then lead to an activation of the kinase, followed by phosphorylation of B880-β and a decrease in connectivity of the photosynthetic units. Such a separation could relieve the over-reduction of the ubiquinone pool by lowering the probability of an absorbed quantum of light reaching an open photochemical trap in a reaction centre. It has been suggested (for discussion see [3]) that, in the chromatophore membrane, reaction centres cooperate to generate a stably reduced ubiquinol (QH_2 species). A separation of photosynthetic units might be expected to lower this cooperation which could decrease the rate of electron transfer and so also lower of reduction of the ubiquinone pool.

Isolation of B880-α and B880-β

We have isolated B880 polypeptides from a phosphorylated chromatophore preparation by the following method. A mixture of chromatophores and 144,000 *g* supernatant (soluble fraction) was incubated with the ATP analogue [^{35}S]adenosine-5′-[γthio]triphosphate in the presence of 2 mM potassium ferricyanide as in [31]. At this stage an aliquot of the preparation was analyzed by SDS–PAGE and autoradiography to ensure phosphorylation of the 13 kDa and 10 kDa polypeptides. After being freeze-dried, the preparation was extracted in 1:1 chloroform-methanol containing acetic acid and ammonium acetate and the extract chromatographed on Sephadex LH–60 [36]. The elution profile is shown in Figure 1. The second peak collected contained only 2 polypeptides, as indicated by SDS–PAGE, at 13 kDa and 10 kDa — B880-α and B880-β. The B880 polypeptides were purified by SDS–PAGE followed by electroelution of the bands. The purity of the B880-α and B880-β was confirmed by SDS–PAGE with the gels stained with Coomassie blue.

Although some ^{35}S was seen to follow the B880 peak on chromatography (Fig. 1) the overall stoichiometries measured after SDS–PAGE and electroelution were low. The estimated stoichiometries (mol phosphate bound per mol polypeptide) were 1:3000 for B880-α and 1:9000 for B880-β with protein estimated with fluorescamine [36].

There are a number of possible explanations for such an apparently low stoichiometry. It may be that the phosphorylated bands seen on SDS–PAGE are not in fact B880 polypeptides but are polypeptides having a similar mobility. Such a double coincidence seems improbable. The phosphate may be bound to the B880 polypeptides under the conditions of SDS–PAGE but not under our conditions of organic solvent extraction — this may imply a non-covalent association or a labile covalent bond. A third possibility is that the chromatophores were isolated with the B880 polypeptides already phosphorylated. A fourth possibility is that optimal phosphorylation of B880 may require components lost in chromatophore preparation. However, this last possibility seems unlikely since autoradiography of gels from whole cells and from chromatophores having similar amounts of B880 polypeptide indicate that the B880 polypeptides are radiolabelled to a similar extent.

Cyanobacterial membrane phosphoproteins

The cyanobacterial thylakoid membrane differs markedly from that of the chloroplast in that LHC II, the principal chlorophyll *a*/*b*-binding protein which is the antenna of chloroplast pho-

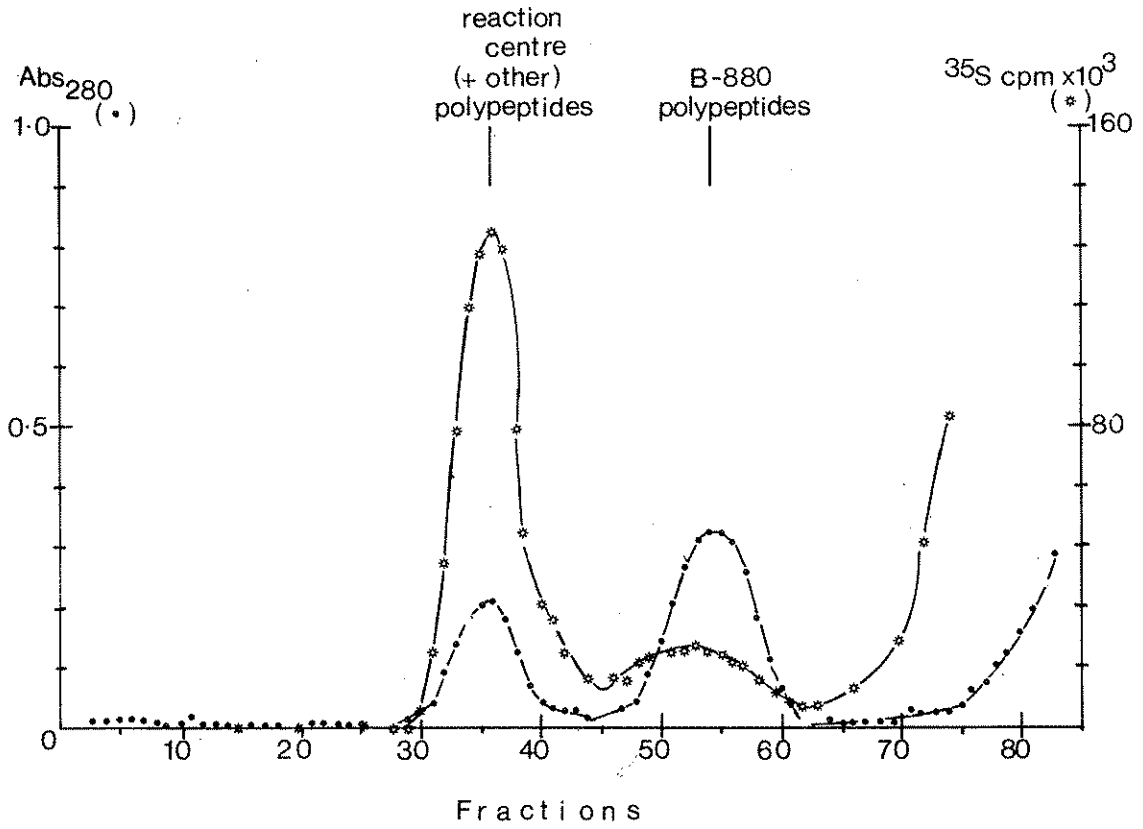

Fig. 1. Elution profile for the solvent extract of phosphorylated chromatophores of *Rhodospirillum rubrum*, as described in the text. Abs_{280} was measured with a 10 mm light path.

tosystem II, is replaced in cyanobacteria by the phycobilisome. This macromolecular complex is an association of water-soluble polypeptides containing covalently-bound phycobilin pigments [11].

The 2 systems are, however, similar in that they are able to adapt the light-harvesting capacity of each photosystem in response to the spectral quality of incident light in order that the quantum yield or efficiency of photosynthesis is maximized [37, 38]. Thus, light preferentially absorbed by PS II (light 2) gives rise to an increase in excitation energy distribution to PS I, whereas light preferentially absorbed by PS I (light 1) prevents this redistribution. States induced by light 1 and light 2 are referred to as state 1 and state 2 respectively, and the transitions between each state as state 1-state 2 transitions. For a review of this subject see [17].

The molecular mechanism of state 1-state 2 transitions in chloroplast thylakoids rests on the phosphorylation of LHC II by a protein kinase [6, 7], the activity of which is regulated by the redox state of plastoquinone or of another electron transport intermediate situated between the photosystems [15, 16]. Incident light favouring excitation of PS II gives net reduction of plastoquinone, resulting in kinase activation with subsequent phosphorylation of LHC II. This results in its dissociation from PS II [39]. This transition to state 2 gives increased fluorescence from PS I at 77 K, implying a possible reassociation of LHC II with this photosystem. Transition to state 1 is effected by net oxidation of plastoquinone: kinase activity is reduced and dephosphorylation of LHC II occurs, this reaction being catalysed by a phosphatase postulated to be continually active [9] and causing reassociation of LHC II with PS II.

The existence of state transitions in cyanobacteria implied a system closely analogous to that in chloroplasts [18]. Initial experiments, how-

ever, showed only light-independent phosphorylation of a number of polypeptides in red algae and cyanobacteria [40, 41]. No apparent effect of light 1 or light 2 could be detected. Subsequently, however, Allen and co-workers were able to demonstrate phosphorylation of three polypeptides of molecular mass 18.5 kDa, 15 kDa and 13 kDa, in *Synechococcus* 6301 under conditions shown to correspond to state 2 [42]. The model for regulation of state transitions in cyanobacteria by protein phosphorylation was further supported by the observation that a phosphatase inhibitor, sodium fluoride, inhibits transition to state 1 [43].

The 18.5 kDa phosphoprotein is a water-soluble protein and has been shown to co-purify with the phycobilisome [44]. The 2 remaining polypeptides are integral membrane proteins, or proteins tightly bound to the membrane. The 15 kDa was shown to be the principal species phosphorylated *in vitro* in thylakoids with $\gamma[^{32}P]$ATP [42].

Current work in our laboratory consistently shows the 13 kDa polypeptide to be the predominant species undergoing light-dependent phosphorylation *in vivo*, and this polypeptide has been purified by chromatographic methods and provisional N-terminal sequence data obtained (M.A. Harrison, unpublished data). The provisional N-terminal sequence is :

XVAIEVIIRP

where X is an unidentified residue. This sequence is apparently novel, no strong database matches having been found.

The 13 kDa phosphoprotein does not appear to be associated strongly with either photosystem, as judged by its failure to sediment with either of these during sucrose-density centrifugation following solubilisation of membranes with the non-ionic detergent *n*-dodecyl maltoside. The phosphoprotein always remains at the top of the gradient. This fractionation method readily separates the photosystems, yielding as PS II fraction incompetent for O_2-evolution, but capable of catalysing electron transport from DPC to DCPIP. No phosphorylation was observed in this PS II material which could be considered to arise from reaction centre components [45]. Additionally, immunoblotting experiments have shown that this material does not contain any polypeptides which bind strongly an antibody raised against a peptide comprising the N-terminal region of the 9 kDa phosphoprotein of chloroplast thylakoids, perhaps indicating the absence of a polypeptide in cyanobacteria having identity with that in chloroplasts.

It is reasonable to speculate that the membrane phosphoproteins of cyanobacteria are involved in the regulation of light-state adaptations. Failure to isolate them in association with major pigment-protein complexes does not preclude the possibility that they are novel regulatory proteins involved in implementing the dissociation of the phycobilisome from PS II during transition to state 2 [42, 46]. This could be postulated to occur by localised conformational changes induced by phosphorylation, or by electrostatic repulsion as suggested by Allen and Holmes [29]. Indeed, it might be suggested that it is necessary for a protein to be peripheral to the major pigment-protein complexes in order to fulfill such a role.

Conclusion

Each of the 4 main types of light-harvesting system described in the *Introduction* is associated with a particular membrane architecture. Purple bacteria have chromatophores, which are specialised invaginations of the cell membrane, chloroplasts have topologically discrete thylakoids which are usually appressed to neighbouring thylakoids for at least part of their surface area, green bacteria have light-harvesting chlorosomes or "chlorobium vesicles" attached to the cytoplasmic surface of the cell membrane, and cyanobacteria have unstacked thylakoids with attached phycobilisomes, hemispherical or hemidiscoidal objects typically 50 nm in diameter and containing 500 or more phycobilin chromophores. The reasoning that cyanobacteria and chloroplasts may have an underlying similarity in their mechanism of state 1-state 2 transitions has prompted work in our laboratory that demonstrates redox control of excitation energy distribution in cyanobacteria [47] and that the cyanobacterial state 2 transition is a decrease in the absorption cross-section of PS II for phycobilisome absorbed light [48].

The concentration of attention on phosphorylation of chloroplast light-harvesting proteins has led to a view [49] that protein phosphorylation is a plausible mechanism for regulation of light-harvesting function only in chloroplast-type membrane systems, that is, in thylakoids stacked to form discrete appressed (grana) and non-appressed (stroma) regions. Lateral movement of LHC II upon its phosphorylation obviously

depends on the existence of discrete lateral domains between which it may migrate. It is our view, however, [50] that the evidence from purple bacteria and cyanobacteria makes protein phosphorylation a plausible mechanism of control even in membrane systems devoid of lateral heterogeneity in distribution of protein complexes in the photosynthetic membrane. These control mechanisms and their components are now under active investigation.

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