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Cyanobacterial thylakoid membrane proteins are reversibly phosphorylated under plastoquinone-reducing conditions in vitro

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Reversible, light-dependent protein phosphorylation was observed in isolated thylakoid membranes of the cyanobacterium *Synechococcus* 6301. A polypeptide of 15 kDa in particular was phosphorylated under plastoquinone-reducing conditions and was not phosphorylated under plastoquinone-oxidising conditions. Phosphorylation and dephosphorylation reactions involving this and several other membrane polypeptides showed sensitivity to inhibitors of protein kinases and phosphatases. Changes in phosphorylation state correlated with changes in low temperature fluorescence emission characteristic of changes in excitation energy distribution between the photosystems. The 15 kDa phosphopolypeptide is likely to be involved directly in light state adaptations in cyanobacteria.

Photosynthesis; Protein phosphorylation; State transition; Protein kinase; Phosphatase; Cyanobacterium

1. INTRODUCTION

Photosynthetic organisms are able to perform short-term adaptations known as State 1–State 2 transitions in response to changing spectral quality of incident light [1,2]. Transition to State 2 occurs under conditions giving preferential excitation of PSII and results in distribution of excitation energy away from PSII, in favour of PSI. Transition to State 1 occurs if PSI is preferentially excited, giving rise to increased excitation energy transfer to PSII. In this way, quantum efficiency of photosynthesis can be maintained despite variations in light regime (for review see [3]).

In higher plant chloroplast systems, transition to State 2 occurs under conditions in which preferential excitation of PSII induces a net reduction of the plastoquinone pool. Under these plastoquinone-reducing conditions, a redox-controlled protein kinase phosphorylates LHC-II [4–6], thereby inducing its dissociation from PSII [7]. This dissociation is manifested as a decrease in the absorption cross-section for PSII and decreased excitation energy transfer to the photosystem [4]. State 1 is achieved under oxidising conditions: the kinase is inactive and a dark-active phosphatase dephosphorylates LHC-II, permitting reassociation with PSII [8].

Despite a wealth of information concerning functional changes occurring during state transitions, considerable controversy remains surrounding the molecular mechanism by which phycobilisome-containing organisms, including both eukaryotic red algae and prokaryotic cyanobacteria, perform these adaptations. Recent advances have, however, provided evidence that state transitions in the cyanobacteria are also regulated by redox state of plastoquinone [9–11] and that the decrease in excitation energy transfer to PSII observed during adaptation to State 2 in these organisms occurs via a mechanism involving a decrease in PSII absorption cross-section [12] similar in character to that seen in chloroplasts. By analogy with the higher plant chloroplast system, the possibility therefore arises that redox-controlled phosphorylation of thylakoid membrane proteins may regulate state transitions in cyanobacteria, despite the widely differing nature of the light-harvesting apparatus in the two systems. A thylakoid membrane protein with a molecular mass of 15 kDa has indeed been observed to undergo light-dependent but irreversible phosphorylation in vitro in the cyanobacterium *Synechococcus* 6301 [13], leading to the proposal that this polypeptide functions in the dissociation of the phycobilisome from PSII during transition to State 2 [14].

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Abbreviations: DCMU, 3-(3′,4′dichlorophenyl)-1,1-dimethylurea; DQ, duroquinone; DQH_2, duroquinol; FSBA, 5′ *p*-fluorylsulphonylbenzoyl adenosine; HEPES, *N*-2-hydroxyethylpiperazine-*N*′2-ethanesulphonic acid; LHC-II, the chlorophyll *a*/*b*-binding light-harvesting complex of PSII; MV, methyl viologen; PSI, photosystem I; PSII, photosystem II; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis

In this study the influence of redox state of plastoquinone on the in vitro phosphorylation of thylakoid membrane polypeptides in *Synechococcus* 6301 is investigated in detail. In addition, the effects on observed phosphorylation and dephosphorylation reactions of established inhibitors of protein kinases and phosphatases are determined.

2. MATERIALS AND METHODS

Synechococcus 6301 was cultured as previously described [10]. Cells were harvested by centrifugation at 6500 × *g* for 10 min and resuspended in 20% sucrose, 50 mM HEPES-NaOH, 10 mM $MgCl_2$, pH 7.8. This and all subsequent buffers contained 0.5 mM phenylmethylsulphonyl fluoride and 1 mM benzamidine as protease inhibitors. Sphaeroplasts, prepared by incubating for 2 h at 37°C with lysozyme at 3 mg·ml^{-1}, were subsequently harvested by centrifugation at 10000 × *g* for 15 min. Thylakoid membranes were produced by lysis of the sphaeroplasts in 10 mM HEPES-NaHO, 10 mM $MgCl_2$, pH 7.8 with vigorous homogenisation. Deoxyribonuclease (3 μg·ml^{-1}) was added to degrade nucleic acids. Unbroken cells and cell debris were removed by centrifugation at 6500 × *g* for 15 min, prior to harvesting of the thylakoid membranes by centrifugation at 15000 × *g* for 30 min. Thylakoids were washed a further 3 times with the lysis buffer to ensure complete removal of soluble-phase proteins. Thylakoids were finally suspended to a chlorophyll concentration of 0.5 mg·ml^{-1} in 10% glycerol, 25 mM HEPES-NaOH, 10 mM $MgCl_2$, pH 7.6 and were stored on ice in the dark.

Aliquots of thylakoid membrane suspension (200 μl) were incubated under the conditions specified in Table I in the presence of [γ^{32}P]ATP at a specific activity of 750 Ci·mol^{-1} and a final ATP concentration of 40 μM. Reactions were terminated by the addition of 4 vols of acetone at −20°C after which protein material was pelleted by centrifugation. Samples were solubilised in electrophoresis buffer and analysed by SDS-PAGE on 15–25% acrylamide gradient minigels essentially as previously described [13]. Stained dried gels were subjected to autoradiography as described [13]. Stained dried gels were subjected to autoradiography as described [13] and the resulting autoradiographs were scanned with an LKB Ultrascan XL laser densitometer.

For low temperature fluorescence emission spectroscopy, thylakoid membranes were incubated at a chlorophyll concentration of 200 μg·ml^{-1} with non-radioactive ATP at a concentration of 40 μM under the specified conditions prior to being rapidly diluted 10-fold with thylakoid resuspension buffer and frozen at 77K. Fluorescence spectroscopy was performed with excitation at 435 nm, with fluorescence emission detected in the range 630 nm to 750 nm as in [13].

Table I

Incubation conditions for in vitro phosphorylation of *Synechococcus* 6301 thylakoid membranes. Left column refers to lanes of SDS-PAGE gels subjected to autoradiography (see Fig. 1). Incubations, preincubations and postincubations were for 15 min each. MV, methyl viologen; DQ, duroquinone; DQH_2, duroquinol

	Preincubation	Incubation	Post-incubation
1	Dark	Dark	.....
2	Dark	Light	.....
3	Dark	Light	Dark
4	Dark	Light	.....
5	Dark	Light + 10 mM NaF	.....
6	Dark	Light + 10 mM NaF	Dark
7	Light + 40 μM FSBA	Light	.....
8	Dark	Dark	.....
9	Light + 20 μM DCMU	Light	.....
10	Light + 20 μM DCMU	Light + 0.5 mM DQH_2	.....
11	Dark	Light + 50 μM MV	.....
12	Dark	Light + 0.5 mM DQ	.....
13	Dark	Dark	.....
14	Dark	Dark + 0.5 mM DQH_2	.....

3. RESULTS AND DISCUSSION

Autoradiographs obtained subsequent to SDS-PAGE analysis of *Synechococcus* 6301 thylakoid membranes radiolabelled with [γ^{32}P]ATP are shown in Fig. 1. The conditions under which each incubation was performed are detailed in Table I. From Fig. 1 it is evident that phosphorylation of a number of species of polypeptide is induced in this system, and comparison of lanes 1 and 2 indicates clearly that these phosphorylation reactions show a degree of light

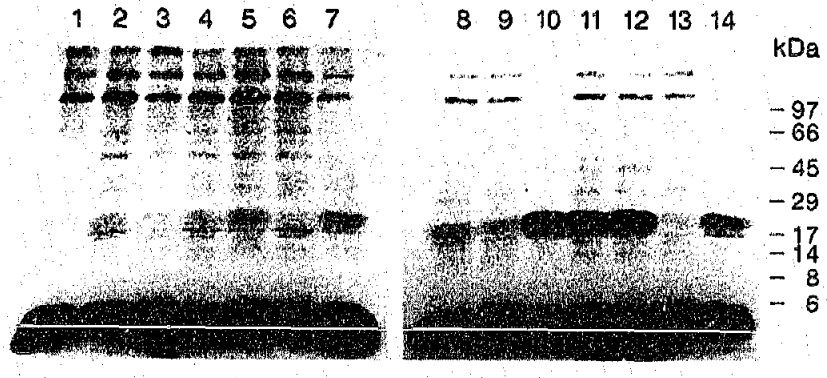

Fig. 1. Autoradiographs subsequent to SDS-PAGE analysis of [γ^{32}P]ATP-radiolabelled *Synechococcus* 6301 thylakoid membranes. Each gel track (gels not shown) was loaded with thylakoid membrane proteins equivalent to 2 μg chlorophyll *a*. Incubation conditions were as detailed in Table I. Positions and M_r of SDS-PAGE standard proteins are indicated.

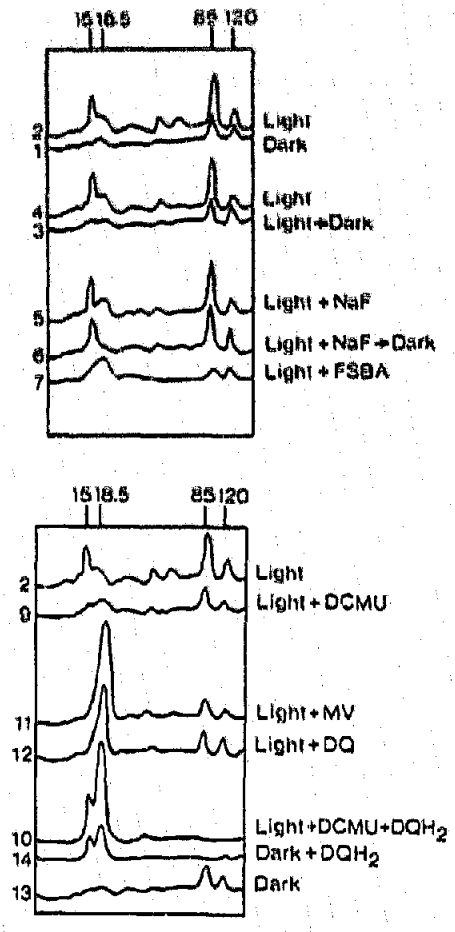

Fig. 2. Densitometric scans of the autoradiographs shown in Fig. 1. Positions and M_rs of principal phosphoproteins are indicated. SDS-PAGE lane number and incubation conditions (as in Table I) are indicated for each profile. Superimposed scans are plotted to the same scale.

dependence, in contrast with an earlier report which suggested that only light-independent phosphorylation occurred in vitro [15]. To permit a more throrough appraisal of conditions influencing in vitro phosphorylation, the autoradiographs shown in Fig. 1 were subjected to scanning densitometry, the results of which are shown in Fig. 2. From Fig. 2, top, (lanes 1 and 2) it is clear that phosphorylation of polypeptides of molecular masses 15 and 18.5 kDa occur specifically in the light. Phosphorylation of two polypeptides of molecular mass 85 and 120 kDa occurs to some extent in the dark (Fig. 2, top, lanes 1 and 3), and phosphorylation of these species is also significantly increased in the light. Minor species of phosphoprotein with masses of 9, 12, 25, 35, 45 and 55 kDa are also evident.

Removal of light-incubated thylakoid membranes to dark conditions (Fig. 2, top, lanes 3 and 4) gave decreased levels of radiolabeling for all observed species of phosphoprotein, indicating reversibility of the phosphorylation reactions and implying the presence of a dark-active phosphatase. This implication was tested by removing phosphorylated membranes to the dark in the presence of the non-specific phosphatase inhibitor sodium fluoride (Fig. 2, top, lanes 5 and 6), which prevented dephosphorylation in the dark of all polypeptides with the exception of the 18.5 kDa species. Presence of sodium fluoride has been demonstrated to fix cyanobacterial cells in State 2 [16], consistent with a phosphatase requirement for transition to State 1. The phosphorylation reactions of all polypeptides with the exception of the 18.5 kDa species were sensitive to the kinase inhibitor FSBA: preincubation of thylakoid membranes with this compound significantly decreased or abolished light-dependent phosphorylation (Fig. 2, top, lane 7). The resistance of the reactions involving the 18.5 kDa polypeptide to established kinase and phosphatase inhibitors does suggest that radiolabeling of this species occurs by a phosphate transfer mechanism other than kinase activity, perhaps via some form of pyrophosphate intermediate.

Incubation in the light, which gave rise to phosphorylation, would be expected to induce plastoquinone reduction in this isolated membrane system depleted of electron acceptors. The influence of redox state of plastoquinone on phosphorylation of *Synechococcus* 6301 thylakoid membrane polypeptides in vitro was further investigated by inclusion of electron transport mediators in membrane incubations (Fig. 2, bottom). The herbicide DCMU blocks electron transfer from the reducing side of PSII to plastoquinone, leading to plastoquinone oxidation and State 1 [9]. Preincubation with DCMU gave significant decreases in the levels of phosphorylation of the 15, 85 and 120 kDa polypeptides (Fig. 2, bottom, lanes 2 and 9), with phosphorylation of the 18.5 kDa species affected to a lesser extent.

The implication that phosphorylation of all polypeptides with the exception of the 18.5 kDa species is dependent on net reduction of the plastoquinone pool is tested by inclusion of the mediators methyl viologen, duroquinone or duroquinol. Methyl viologen may act as an electron acceptor from PSI, whereas duroquinone will accept electrons directly from the reducing side of PSII. In either case the net effect would be oxidation of the plastoquinone pool and both give functional State 1 in vivo [10]. Duroquinol may compete with plastoquinol for sites at the oxidising side of the cytochrome b6/f complex [17] and would therefore be expected to give rise to increased reduction of plastoquinone. Duroquinol gives rise to functional State 2 in whole cells [10]. The observation (Fig. 2, bottom, lanes 11 and 12) that both methyl viologen and duroquinone decrease the light-dependent phosphorylation of all polypeptides except the 18.5 kDa species supports the conclusion that the kinase activity requires the reduction of plastoquinone or reduction of a component close to plasto-

quinone in the electron transport chain, as is the case in the chloroplast system [6]. Anomalously, incubation under these conditions gave markedly elevated levels of phosphorylation of the 18.5 kDa polypeptide. Incubation with duroquinol gave rise to high levels of phosphorylation of both the 15 kDa and 18.5 kDa polypeptides (Fig. 2, bottom, lanes 10 and 14), but abolished completely phosphorylation of all other species, suggesting the possibility of a redox-activated phosphatase. These effects of duroquinol were observed irrespective of preincubation with DCMU or incubation in the dark, suggesting that reduction of plastoquinone or of a component of the cytochrome b6/f complex is required for kinase activity.

Phosphorylation of the 18.5 kDa polypeptide occurs to the greatest extent under conditions in which electron transport is promoted by the presence of electron donors or acceptors, rather than specifically under conditions of plastoquinone reduction. Methyl viologen and duroquinone will promote electron transport through PSI and PSII respectively, whereas duroquinol could promote electron transport through the cytochrome b6/f complex even in the dark via the respiratory electron transport system [18]. It is conceivable therefore that the mechanism giving rise to phosphorylation of the 18.5 kDa polypeptide is sensitive to some other factor, for example any ΔpH gradient established as a result of electron transport.

The influence of incubation conditions on distribution of excitation energy in the isolated thylakoid membrane system was studied by low temperature fluorescence emission spectroscopy (Fig. 3). At 77K, PSII gives rise to fluorescence emission at 685 and 695 nm, whereas PSI gives emission at 720 nm. Variations in the emission ratio F720/F695 have consistently been used to demonstrate changes in excitation energy distribution after light state adaptation [1,10,19,20], and from Fig. 3 the decrease in this ratio observed upon incubation in the light in the presence of ATP is consistent with decreased energy transfer to PSII or increased spillover of energy from PSII to PSI. Previously observed light-induced changes in the ratio F720/F695 occurring in isolated membrane systems have been interpreted as due to reversible photooxidation of chlorophyll [13,15]. This interpretation is not possible for the effects observed here, since the increase in the ratio F720/F695 observed upon transition from light to dark was abolished by the inclusion of sodium fluoride, suggesting that this redistribution of excitation energy requires the activity of a membrane-bound phosphatase. Preincubation with FSBA partly inhibited the increase in F720/F695 observed upon incubation in the light, implying the requirement for a kinase activity in the light-induced redistribution of excitation energy.

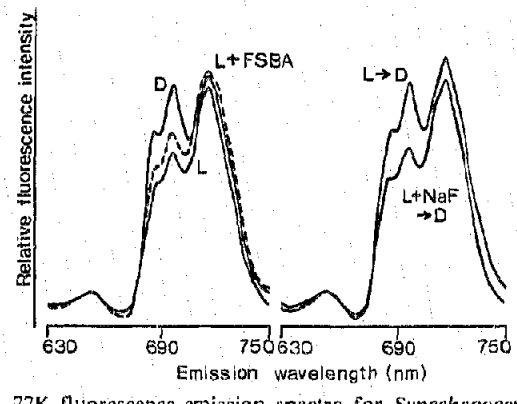

Fig. 3. 77K fluorescence emission spectra for *Synechococcus* 6301 thylakoid membranes incubated under varying conditions with 40 μM ATP. Fluorescence emission maxima were at 685 nm, 695 nm and 720 nm. Spectra were normalized to the emission in the range 630–650 nm. Incubation conditions were as defined in Table I.

From this study it seems evident that reversible phosphorylation of at least one polypeptide of mass 15 kDa occurs specifically under plastoquinone-reducing conditions known to give rise to State 2. The phosphorylation of all species of polypeptide with the exception of that with mass 18.5 kDa was inhibited by a kinase inhibitor and phosphorylation was maintained under otherwise dephosphorylating conditions by a non-specific phosphatase inhibitor. Reversible changes in PSII fluorescence yield at 77K were also abolished by these inhibitors. A strong correlation between phosphorylation of thylakoid membrane proteins and redox state of plastoquinone is therefore suggested, with these phosphorylation events occurring concurrently with observed changes in excitation energy distribution diagnostic for state transitions.

Phosphorylation of the 18.5 kDa protein in vivo has previously been described [21,22], and this polypeptide has been proposed to be a component of the phycobilisome [21]. This work does demonstrate that this species undergoes reversible light-dependent phosphorylation in vitro, but this phosphorylation reaction is not coupled to reduction of plastoquinone since duroquinone, methyl viologen and duroquinol all induced high levels of phosphorylation of this protein. The phosphorylation and dephosphorylation reactions involving this polypeptide were not sensitive to inhibitors and are not correlated with changes in excitation energy distribution, contrary to the proposal of Allen et al. [13].

Recent work has suggested that redox-controlled transition to State 2 in the cyanobacteria involves dissociation of phycobilisome-PSII units and decreased PSII absorption cross-section [23,24]. Increased energy transfer to PSI [19] and increased PSI photochemistry in State 2 [25] may result from association of PSI with the phycobilisome [25] or by spillover from the chlorophyll antenna of PSII [19,26]. Increased association of these complexes with PSI is implied, suggesting a randomization of membrane complexes upon transition to State 2 which is consistent with observed ultrastructural changes [27].

We therefore propose that phosphorylation of thylakoid membrane polypeptides, in particular the 15 kDa species, is involved in inducing dissociation of the phycobilisome from PSII in State 2 by a redox-controlled mechanism similar to that dissociating LHC-II from PSII in higher plants. Identification of the phosphorylated peptides is required fully to test this hypothesis.

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