Volume 323, number 1,2, 35–39 FEBS 12465 May 1993

Identification of the *psb*H gene product as a 6 kDa phosphoprotein in the cyanobacterium *Synechocystis* 6803

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Received 12 February 1993; revised version received 26 March 1993

The product of the *psb*H gene has been identified in *Synechocystis* 6803 thylakoid membranes as a 6 kDa phosphoprotein. This protein becomes phosphorylated in vitro despite the fact that in cyanobacteria it is truncated at the N-terminus such that the phosphorylation site identified in the higher plant protein is missing. Phosphorylation occurred both in the light and in the dark but was inhibited by oxidising conditions, DCMU and zinc ions. The cyanobacterial 6 kDa phosphoprotein degrades when the membranes are subjected to high intensity illumination.

Protein phosphorylation; Cyanobacteria; *Synechocystis* 6803; *psb*H gene product; Photoinhibition

1. INTRODUCTION

Phosphorylation of chloroplast thylakoid membrane proteins has been implicated in the control of light energy distribution between the two photosystems. The reversible phosphorylation of the light harvesting chlorophyll *a/b* binding protein complex (LHC II) has been extensively studied and its role in the regulation of photosynthetic electron transport and state 1–state 2 transitions examined in detail (reviewed in [1] and [2]). The LHC II kinase system and its redox controlled activation have received considerable attention and much progress has been made recently in higher plants, algae and prochlorophytes [1,3–7]. In cyanobacteria, the control as well as the significance of protein phosphorylation has been a matter of dispute. Cyanobacteria lack LHC II but a number of other phosphorylated membrane bound proteins have been reported (reviewed in [1]).

Of the photosystem II (PS II) associated polypeptides the D2 reaction centre protein, the chlorophyll *a* binding 43 kDa protein and the product of the *psb*H gene undergo reversible phosphorylation in plants and algae [1,8,9]. The D1 reaction centre protein has also been shown to exist in a phosphorylated form in plants, but in algae this has been the subject of debate [9,10]. PS II phosphoproteins analogous to those described in plants and algae have not been unequivocally identified in cyanobacteria, although the D1, D2 and 43 kDa proteins are well conserved among the species and the potential phosphorylation sites maintained.

The significance of PS II core protein phosphorylation is unclear although a number of proposals have been made regarding the role of the *psb*H gene product, also referred to as the 9 kDa phosphoprotein, in PS II and the possible functional implications of its phosphorylation. It has thus been suggested that this protein may be involved in (i) regulation of excitation energy transfer [11], (ii) protection from photoinhibition [12–14], or (iii) function in a manner analogous to the H subunit of anoxygenic bacteria and affect electron transfer between Q_A and Q_B by virtue of its phosphorylation [15,16]. In addition, it has been speculated that the *psb*H gene product might be the LHC II phosphatase activated by phosphorylation, or that it might be involved as a redox sensor in the control of gene expression in photosynthesis [1].

All the above suggestions and proposals are based on the fact that this protein is phosphorylated in plants and algae. Sequencing of cyanobacterial *psb*H genes, however, has revealed that the equivalent genes have a deletion of 27 nucleotides at the 5′ end of their coding sequence [17–19]. Thus, the encoded protein is predicted to be about 6.5 kDa in *Synechocystis* 6803 and is missing the phosphorylation site of the corresponding chloroplast product; i.e. the threonine in position two [20,21]. The predicted C-terminal sequence of the *psb*H gene product is well conserved among different species [18,19]. We therefore made a synthetic decatetrapeptide corresponding to this region of the spinach *psb*H sequence and raised antibodies to it. We report the identification of a 6 kDa protein in *Synechocystis* 6803 thy-

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Abbreviations. PS II, photosystem II; LHC II, light harvesting chlorophyll *a/b* binding protein; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

lakoid membranes as the *psb*H gene product, which is phosphorylated despite the truncated N-terminus.

2. MATERIALS AND METHODS

A peptide with the sequence CYNSSVLLDGISMN was synthesised using Applied Biosystems 430A peptide synthesiser by Fmoc chemistry. This sequence corresponds to the C-terminal region of the *psb*H gene product in spinach and is well conserved in both higher plants and cyanobacteria [18,22]. A cysteine residue was placed at the N-terminus of the peptide for ease of conjugation to a purified protein derivative of tuberculin (PPD: Statens Seruminstitute, Denmark). The conjugation was carried out using the water soluble coupling reagent sulphosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC; Pierce Chemical Company, USA) as described previously [23]. Following purification the conjugated PPD-peptide was lyophilised prior to resuspension in distilled water. The peptide conjugate (1 mg in 800 μl) was combined in a 2 to 3 ratio (by volume) with Freund's incomplete adjuvant (IFA) emulsified and injected subcutaneously into a rabbit as a primary immunisation. Subsequent peptide boosts (100 μg protein in 800 μl IFA) were given every 14 days. Blood was collected and tested 7 days after each peptide boost. The final serum was stored at −20°C.

Synechocystis 6803 cells were grown at 32°C in BG11 medium [24] supplemented with 10 mM TES, pH 8.2, and 5 mM glucose. Cultures were stirred continuously, bubbled with air and illuminated with white light (350 $\mu E \cdot m^{-2} \cdot s^{-1}$). Cells in the logarithmic growth phase were harvested by sedimentation at 5,000 × *g* for 10 min at 25°C. The pellet was resuspended in 50 mM HEPES, pH 7.5, 30 mM $CaCl_2$ and resedimented as above The final pellet was resuspended on ice in 50 mM HEPES, pH 7.5, 800 mM sorbitol and maintained in the dark for 30 min prior to disruption. The cells were broken by passing them three times through a chilled French press at 18,000 psi. The suspension was centrifuged at 1,000 × *g* for 5 min at 4°C to remove unbroken cells and the resultant supernatant spun at 15,000 × *g* for 15 min. The pellet derived from the latter centrifugation and containing the thylakoids was resuspended in 50 mM HEPES, pH 7.5, 800 mM sorbitol and either used directly or stored at −80°C for later use.

Prior to in vitro phosphorylation the isolated membranes were washed twice in 50 mM HEPES, pH 7.5, before resuspension in the same buffer, containing 10 mM NaF, to give a protein concentration of 1 $mg \cdot ml^{-1}$. ATP (10 μM) and, when indicated, divalent cations at a concentration of 10 mM were added Finally, [γ-^{32}P]ATP at a concentration of 1.7 $\mu Ci \cdot \mu g$ protein was added and the samples were incubated at 30°C for 60 min either in the light or in the dark as indicated. DCMU was added to the samples 5 min before the addition of ATP and used at a concentration of 20 μM. Reducing conditions were generated by the addition of freshly prepared sodium dithionite (1 $mg \cdot ml^{-1}$) and oxidising conditions by potassium ferricyanide (1 mM). These compounds were again added to the samples 5 min prior to ATP addition The reaction was terminated by the addition of trichloroacetic acid (final concentration 5%) followed by incubation on ice for 15 min. The samples were centrifuged at 13,500 × *g* for 5 min and resuspended in solubilisation buffer for polyacrylamide gel electrophoresis as described [25]. The gels used were either 17% polyacrylamide {16.5% (w/v) acrylamide and 0.5% (w/v) bisacrylamide} or 13.5% polyacrylamide/4 M urea [26], and Western blotting was carried out essentially as previously described [27] with the incorporation of a biotin/extravidin-alkaline phosphatase step. The former gel type was used unless otherwise indicated. Autoradiography of gels was carried out at −80°C using Fuji RX X-ray film and high-speed intensifying screens Protein concentration was estimated by the bicinchoninic acid method [28].

Photoinhibitory treatment, when indicated, was carried out at 20°C using white light from a Gallex LS15 source at 5,000 $\mu E \cdot m^{-2} \cdot s^{-1}$ for 20 min; the membranes were suspended in 0.3 M sucrose, 5 mM $MgCl_2$, 10 mM NaCl, 10 mM NaF, 20 mM tricine, pH 8.0 at a chlorophyll concentration of 0.05 $mg \cdot ml^{-1}$. Chlorophyll concentration was estimated as described [29].

3. RESULTS AND DISCUSSION

When thylakoid membranes isolated from the cyanobacterium *Synechocystis* 6803 are subjected to in vitro phosphorylation at least six polypeptides are found to be labelled as shown in Fig. 1B. We have used a gel that resolves proteins of low molecular weight, and the apparent masses of these phosphoproteins were estimated to be of 52, 25, 15, 9, 6 and 4 kDa. We did not, however, observe the same pattern of protein phosphorylation in every experiment and have not been able to ascribe this observation to any particular parameter that we could control. As shown in Fig. 1C protein phosphorylation is occasionally restricted to polypeptides with molecular weights of 25, 15, 6 and 4 kDa and these proteins are reproducibly phosphorylated. In some experiments another phosphoprotein with an estimated molecular weight of 103 kDa could also be resolved (data not shown). It is worth noting that our data demonstrate that cyanobacterial membranes can be phosphorylated following French press treatment in contrast to previous reports [30].

Light-dependent phosphorylation of proteins with molecular weights of 15 and 18.5 kDa have previously been reported in *Synechococcus* 6301 thylakoids [31,32]. The latter protein has been proposed to be a component of the phycobilisome, while the 15 kDa protein has been suggested to be involved in light state adaptations. Minor phosphoproteins at 9, 12, 25, 35, 45, 55, 85 and 120 kDa were also reported [32] but their identities have remained obscure.

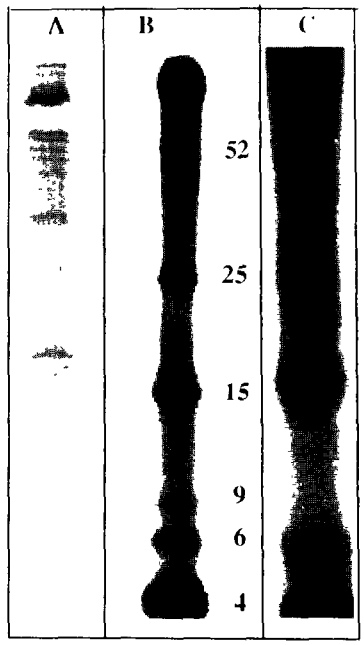

Fig. 1. Phosphorylation of thylakoid membrane proteins in *Synechocystis* 6803. Coomassie blue stained gel (A) and autoradiographs (B,C) of [γ-^{32}P]ATP labelled thylakoid membranes. Numbers indicate the apparent molecular weights of the major phosphoproteins.

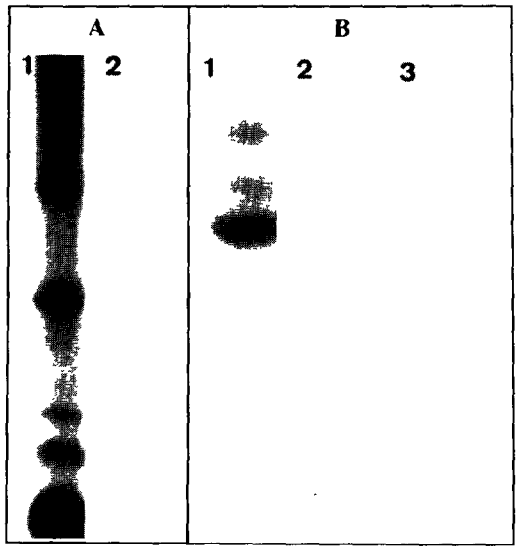

Fig. 2. Identification of the 6 kDa phosphoprotein in *Synechocystis* 6803 thylakoid membranes as the product of the *psb*H gene. (A) Autoradiograph of a gel of phosphorylated *Synechocystis* 6803 thylakoid membranes (1) and Western blot of a similar gel using an antibody raised against a synthetic decatetrapeptide corresponding to the C-terminus of the spinach *psb*H gene sequence (2); (B) autoradiograph of a 13.5% polyacrylamide gel of phosphorylated spinach thylakoid membranes (1) and Western blot of a similar gel probed with the above antibody (2) or with the antibody to the plant 9 kDa phosphoprotein (3).

We used antibodies raised against a synthetic decatetrapeptide corresponding to the C-terminal sequence of the spinach *psb*H gene product, which is highly conserved between species. We probed in vitro phosphorylated thylakoid membranes isolated from *Synechocystis* 6803 as well as spinach chloroplasts, and the results obtained are shown in Fig. 2. The antibody reacts with a protein corresponding to the 6 kDa phosphoprotein in the cyanobacterial membranes (Fig. 2A, lane 2). As shown in Fig. 2B (lane 2), the reaction of this antibody with plant thylakoids corresponds to the 9 kDa phosphoprotein. We also used an antibody raised against the plant 9 kDa phosphoprotein (a kind gift from Dr. P. Böger) but we found it to give very poor reactions with the cyanobacterial membranes. As seen in Fig. 2B (lane 3), however, the reactions of the two antibodies correspond to the same phosphoprotein in plant thylakoids. We therefore assign the 6 kDa phosphoprotein in *Synechocystis* 6803 as the product of the *psb*H gene. The predicted N-terminal sequence of this protein in cyanobacteria [18,19] contains a threonine residue at position five and a serine residue at position sixteen and it is possible that either, or both, of these residues are phosphorylated. Alignment of all known *psb*H sequences to the predicted transmembrane region indicate that both the above residues are conserved among the species.

The product of the chloroplast *psb*H gene has been reported to be selectively lost upon high intensity illumination of spinach membranes [12]. We subjected in vitro phosphorylated membranes to photoinhibitory treatment and the results are shown in Fig. 3. It is apparent from the autoradiograph and the Western blot that there is a loss of this phosphoprotein in cyanobacteria analogous to that reported in plants under high light treatment, therefore the proposals that have been put forward for the involvement of this gene product in protection from photoinhibition in plants [12–14] could be extrapolated to include cyanobacteria.

We examined the cation requirements for the phosphorylation of this and the other proteins (Fig. 4) and found that there is no strict requirement. Minor differences were detectable in the sense that Mg^{2+} enhanced the level of phosphorylation of the 15 and 6 kDa proteins, as did the presence of Ca^{2+}, while the latter cation appears to cause a slight decrease in the phosphorylation level of the 9 kDa protein. Complete inhibition of phosphorylation was, however, obtained in the presence of Zn^{2+} in a manner similar to that previously reported for other membrane systems [33,34].

We investigated whether the observed phosphorylation is redox controlled and the data we obtained are shown in Fig. 5. It can be seen that protein phosphorylation occurs both in the light and in the dark (Fig. 5B and D, respectively), and thus it appears that the process is light independent. Such a conclusion has been reached before in studies of cyanobacterial phosphorylation [35]. It was however observed that phosphorylation was inhibited by DCMU (Fig. 5A) and by oxidising conditions generated by ferricyanide (Fig. 5C). Reducing conditions imposed by the addition of dithionite in the dark resulted in protein phosphorylation in a manner indistinguishable from that induced by light (data not shown). It therefore seems that the redox state of

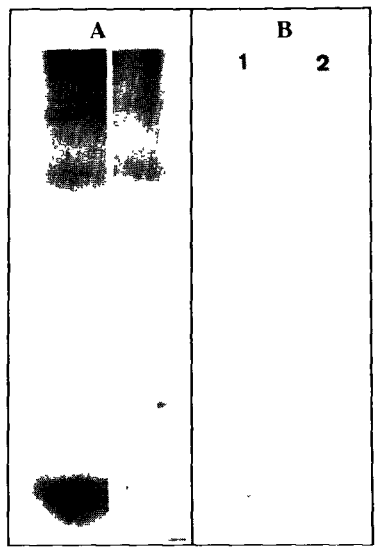

Fig. 3. The effect of strong illumination on the 6 kDa phosphoprotein of *Synechocystis* 6803 thylakoid membranes. Autoradiograph (A) and Western blot (B) of control (1) and illuminated (2) membranes.

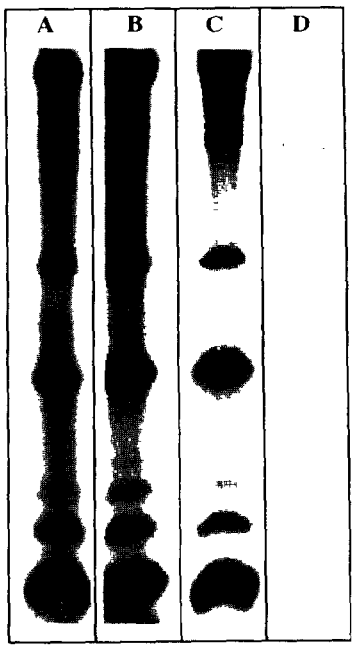

Fig. 4. The effect of divalent cations on the phosphorylation of thylakoid membrane proteins from *Synechocystis* 6803. Phosphorylation was carried out in the presence of (A) no added cations, (B) magnesium, (C) calcium or (D) zinc ions. All cations were used at a concentration of 10 mM.

plastoquinone influences protein phosphorylation in these membranes as previously determined in plants and the 15 kDa membrane protein in *Synechococcus*

Fig. 5. Autoradiographs of thylakoid membranes of *Synechocystis* 6803 labelled with [γ-^{32}P]ATP in different conditions (A) light in the presence of DCMU; (B) light; (C) light in the presence of ferricyanide; (D) dark.

6301 [32]. The absence of control by illumination could be explained by the fact that the plastoquinone pool in cyanobacteria can be reduced in the dark by respiratory enzymes, as discussed by Allen [1].

In conclusion, we have identified a 6 kDa phosphoprotein as the *psb*H gene product in the cyanobacterium *Synechocystis* 6803. Considering the truncation of this protein at the N-terminus and the lack of the plant phosphorylation site at threonine two we suggest that either threonine at position five and/or serine at position sixteen are phosphorylated in the cyanobacterial protein. Work is currently in progress to resolve this issue.

Acknowledgements We thank Dr. P.A Millner for help with the conjugation of the synthetic peptide and Dr. M.E Selkirk for advice on the production of antibodies to the conjugated peptide. An antibody to the higher plant '9 kDa phosphoprotein' was a generous gift of Dr. P. Böger The *Synechocystis* 6803 cells were a gift from Professor J Barber whom we also thank for the use of his culture facilities. Financial support was provided from an Agricultural and Food Research Council grant to K.G. H.L.R acknowledges an Agricultural and Food Research Council studentship

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