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Salt-induced redox-independent phosphorylation of light harvesting chlorophyll *a*/*b* proteins in *Dunaliella salina* thylakoid membranes

Xian-De Liu, Yun-Gang Shen*

Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 300 Fenglin Road, Shanghai 200032, China

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Abstract

This study investigated the regulation of the major light harvesting chlorophyll *a*/*b* protein (LHCII) phosphorylation in *Dunaliella salina* thylakoid membranes. We found that both light and NaCl could induce LHCII phosphorylation in *D. salina* thylakoid membranes. Treatments with oxidants (ferredoxin and NADP) or photosynthetic electron flow inhibitors (DCMU, DBMIB, and stigmatellin) inhibited LHCII phosphorylation induced by light but not that induced by NaCl. Furthermore, neither addition of $CuCl_2$, an inhibitor of cytochrome b_6f complex reduction, nor oxidizing treatment with ferricyanide inhibited light- or NaCl-induced LHCII phosphorylation, and both salts even induced LHCII phosphorylation in dark-adapted *D. salina* thylakoid membranes as other salts did. Together, these results indicate that the redox state of the cytochrome b_6f complex is likely involved in light- but not salt-induced LHCII phosphorylation in *D. salina* thylakoid membranes.

Keywords: *Dunaliella salina*; Thylakoid membrane; LHCII phosphorylation; NaCl; Redox state; Cytochrome b_6f complex

1. Introduction

Reversible protein phosphorylation is a general mechanism for signal transduction in eukaryotes and prokaryotes [1,2], and light-mediated phosphorylation of thylakoid membrane proteins was originally identified in pea chloroplasts [3]. The main thylakoid phosphoproteins in higher plants belong to the major light harvesting chlorophyll *a*/*b* proteins (LHCII) and photosystem II (PSII) core proteins (D1, D2, CP43 and PsbH), although phosphorylation of D1 protein does not occur in green algae [4]. The reversible phosphorylation of LHCII is generally considered an adaptation mechanism aimed at balancing energy distribution between photosystem I (PSI) and PSII; in the fresh water green alga, *Chlamydomonas*, it also serves as means to reroute the photosynthetic electron flow in response to cellular demands for ATP [5,6].

PSII protein phosphorylation is generally considered to be under redox-control, although different proteins follow distinct regulatory patterns in details [4–7]. Electron transfer inhibitor experiments and mutant plant studies have indicated that reduction of the plastoquinone pool is a key regulatory step for the phosphorylation of PSII core proteins, whereas LHCII phosphorylation additionally requires reduction of the cytochrome b_6f complex [8–13]. Specifically, plastoquinol occupation of the quinol oxidase (Qo) site of the cytochrome b_6f complex and transient structural changes of the Rieske iron–sulfur protein are directly related to kinase activation [14–19]. Additionally, light-induced exposure of the phosphorylation site of the chlorophyll-protein (LHCII, CP43) also modulates the thylakoid protein phosphorylation [20–22].

Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isoprophyl-p-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FeCy, ferricyanide; Fd, ferredoxin; LHCII, light harvesting chlorophyll *a*/*b* binding proteins; PSI, photosystem I; PSII, photosystem II

* Corresponding author. Tel.: +86 21 54924233; fax: +86 21 54924015.
E-mail address: ygshen@iris.sipp.ac.cn (Y.-G. Shen).

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Dunaliella salina is a unicellular green alga that lacks a rigid cell wall and is distinguished by its ability to adapt to a wide range of NaCl concentrations through accumulation of intracellular glycerol [23,24]. We have found that NaCl could induce LHCII phosphorylation in dark-adapted *D. salina* thylakoid membranes, but that in spinach thylakoid membranes, NaCl did not induce LHCII phosphorylation in the dark, and even inhibited light-induced LHCII phosphorylation [25]. Here, we studied the possible role of the cytochrome b_6f complex in NaCl-induced LHCII phosphorylation in *D. salina* thylakoid membranes by investigating the effects of oxidants and inhibitors of cytochrome b_6f complex reduction. New insights into this system could greatly improve our overall understanding of the LHCII phosphorylation mechanism.

2. Materials and methods

2.1. Plants

D. salina cells were grown in an artificial hypersaline medium containing 1.5 M NaCl, as previously described [26]. When the chlorophyll concentration was about 12 μg/ml, as determined by the method of Arnon [27], *D. salina* cells were dark-adapted for about 3 h, and then collected for experiments.

2.2. Phosphorylation of thylakoid proteins in vitro

Thylakoid membranes were isolated according to the method of Kim et al. [28]. The collected dark-adapted *D. salina* cells was suspended in sonication buffer (100 mM Tris–HCl, pH 6.8, 5 mM $MgCl_2$, 0.2% polyvinyl pyrrolidone K30, 3 mM aminocaproic acid, 1 mM aminobenzamidine and 0.2 mM phenylmethanesulfonyl fluoride), and then disrupted by sonication for 90 s. Unbroken cells and other large fragments were removed by centrifugation at 3000×*g* for 3 min at 4 °C. To avoid the aggregation of thylakoid membranes and to maintain the activity of LHCII kinase, the thylakoid membrane-containing supernatant (less than 0.5 ml) was diluted to 50 μg chlorophyll/ml with 20-ml phosphorylation reaction medium (50 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 10 mM NaF and 400 μM ATP) for thylakoid protein phosphorylation either in the light (about 200 μmol photons m^{-2} s^{-1}) or in darkness in the presence of 0.3 M NaCl [16,25]. These mixtures were incubated for 20 min at 25 °C in the presence of one of the following reagents: 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (Sigma), 5 μM 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB) (Sigma), 5 μM stigmatellin (Fluka), 50 μM $CuCl_2$, 10 mM ferricyanide (FeCy) or 5 μM ferredoxin (Fd) (Sigma) and 2 mM NADP. Samples were then centrifuged at 40 000×*g* for 20 min at 4 °C, and the pellets were resuspended in sonication buffer at 1 mg chlorophyll/ml.

2.3. Thylakoid membrane protein analysis and immunoblotting

Thylakoid membranes were solubilized in 0.5 M Tris–HCl (pH 6.8), 7% SDS, 20% glycerol and 2 M urea, and then incubated at 50 °C for 30 min. Unsolubilized materials were removed by centrifugation at 3000×*g* for 5 min [28]. Thylakoid membrane proteins were resolved by SDS-PAGE (15% acrylamide, 0.5% bisacrylamide and 4 M urea [28]) using 0.75×6×8-cm slabs on a miniprotein three-cell system (Bio-Rad), with each sample containing 2.5-μg chlorophyll. The separated polypeptides were electrophoretically transferred to Hybond™ ECL™ nitrocellulose membranes (Amersham Pharmacia) with a semi-dry transfer cell (Amersham Pharmacia) for immunoblot analysis. Phosphorylated thylakoid membrane proteins were detected with rabbit polyclonal phosphothreonine (P-Thr) antibody (Zymed), since PSII core phosphoproteins and LHCII proteins are known to be phosphorylated at an N-terminal threonine residue [29].

3. Results

3.1. Both light and NaCl induce LHCII phosphorylation in D. salina thylakoid membranes

Previous reports indicated that dark-adapted *D. salina* [26,30] and *Chlamydomonas* [12,18,19,31,32] cells retain substantial LHCII phosphorylation levels, probably due to the plastoquinone pool, which is partially reduced by chlororespiration in green algae [33,34]. In this work, we observed the presence of LHCII phosphorylation in dark-adapted *D. salina* thylakoid membranes (Fig. 1, lane 1). There was no increase in LHCII phosphorylation levels in *D. salina* membranes following the addition of ATP to the protein phosphorylation medium in darkness (Fig. 1, lane 2), indicating that LHCII phosphorylation was inactive in dark-adapted *D. salina* thylakoid membranes. Consistent with our previous observation, not only light but also NaCl could induce LHCII phosphorylation in isolated *D. salina* thylakoid membranes (Fig. 1, lanes 3 and 4). This

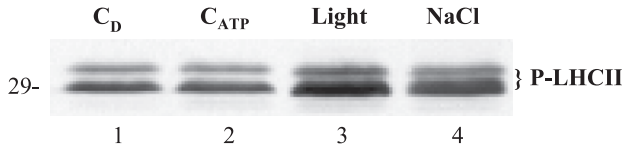

Fig. 1. Light and NaCl induce LHCII phosphorylation in *D. salina* thylakoid membranes. Thylakoid membrane proteins from dark-adapted *D. salina* were phosphorylated at 25 °C for 20 min in the dark in the absence (C_D, lane 1) or presence of 400 μM ATP (C_{ATP}, lane 2), or incubated in the presence of ATP either in the light (about 200 μmol photons m^{-2} s^{-1}) (Light, lane 3) or in darkness with the addition of 0.3 M NaCl (NaCl, lane 4). The positions of phosphorylated LHCII proteins recognized by the P-Thr antibody are indicated.

phenomenon is interesting, since LHCII phosphorylation in vitro is generally considered to be light-dependent.

3.2. Effects of photosynthetic electron flow inhibitors on light- or NaCl-induced LHCII phosphorylation in D. salina thylakoid membranes

The Q_B site-specific inhibitor DCMU blocks light-dependent plastoquinone reduction by PSII [9,10]. The quinone analog DBMIB binds the Qo pocket of the cytochrome b_6f complex and competes with plastoquinone, thus preventing reduction of the cytochrome b_6f complex [35–37]. Stigmatellin binds at the Qo site near the Rieske center and blocks electron transfer by fixing the iron–sulfur protein in an inflexible conformation [35,37–40]. To investigate the possible role of intersystem electron carriers in the observed light- and NaCl-induced phosphorylations, we examined the effects of these inhibitors. As observed in isolated thylakoid membranes from pea [8,9], spinach [13,16] or *Acetabularia* [14], these inhibitors could abolish light-induced LHCII phosphorylation in *D. salina* thylakoid membranes (Fig. 2A). However, in terms of NaCl-induced LHCII phosphorylation in *D. salina* thylakoid membranes, they had no inhibitory effect (Fig. 2B). These results suggest that light and NaCl affect LHCII phosphorylation in *D. salina* thylakoid membranes via different pathways and/or mechanisms.

Recently, Cu^{2+} was found to bind at a site distant from the Qo site and inhibit plastoquinol binding by restraining the hydrophilic head domain of the iron–sulfur protein of the cytochrome b_6f complex [40,41]. To further investigate NaCl-induced LHCII phosphorylation, protein phosphorylation was carried out in the presence of 50 μM $CuCl_2$. Interestingly, this cytochrome b_6f complex reduction inhibitor induced LHCII phosphorylation efficiently in dark-adapted *D. salina* thylakoid membranes (Fig. 3, lanes 1 and 2). Furthermore, $CuCl_2$ even enhanced both light- (Fig. 3, lanes 3 and 4) and NaCl-induced LHCII phosphorylations (Fig. 3, lanes 5 and 6).

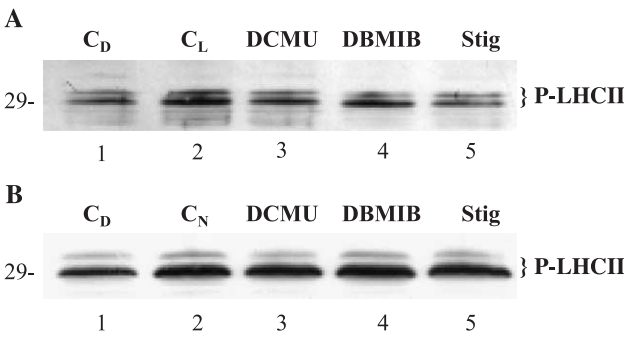

Fig. 2. Effects of DCMU, DBMIB and stigmatellin on (A) light- and (B) NaCl-induced LHCII phosphorylation in *D. salina* thylakoid membranes. Thylakoid membrane proteins from dark-adapted *D. salina* were phosphorylated at 25 °C for 20 min in the light (about 200 μmol photons m^{-2} s^{-1}) (A) or in darkness with the addition of 0.3 M NaCl (B) in the absence or presence of 10 μM DCMU (DCMU, lane 3), 5 μM DBMIB (DBMIB, lane 4) or 5 μM stigmatellin (Stig, lane 5). C_D (A and B, lane 1) indicates the dark control; C_L (A, lane 2) and C_N (B, lane 2) indicate the light- and NaCl-induced LHCII phosphorylation, respectively. The positions of phosphorylated LHCII proteins recognized by the P-Thr antibody are indicated.

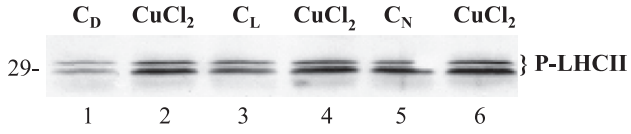

Fig. 3. Effects of $CuCl_2$ on LHCII phosphorylation in *D. salina* thylakoid membranes. Thylakoid membrane proteins from dark-adapted *D. salina* were phosphorylated at 25 °C for 20 min in the absence (lanes 1, 3 and 5) or in the presence (lanes 2, 4, and 6) of 50 μM $CuCl_2$ in dark (lanes 1 and 2) or light (about 200 μmol photons m^{-2} s^{-1}) (lanes 3 and 4) conditions or in darkness with addition of 0.3 M NaCl (lanes 5 and 6). C_D indicates the dark control; C_L and C_N indicate the light- and NaCl-induced LHCII phosphorylation, respectively. The positions of phosphorylated LHCII proteins recognized by the P-Thr antibody are indicated.

3.3. Effects of oxidants on light- or NaCl-induced LHCII phosphorylation in D. salina thylakoid membranes

To further determine the role of the redox state of electron carriers, we tested the effects of oxidizing treatments on NaCl-induced LHCII phosphorylation. Fig. 4 shows that treatment with ferredoxin and $NADP^+$, which oxidizes the cytochrome b_6f complex [16], inhibited light-induced LHCII phosphorylation (Fig. 4A) but not NaCl-induced LHCII phosphorylation (Fig. 4B). However, while 0.1 mM ferricyanide was reported to fully oxidize both cytochrome *f* and the iron–sulfur redox center of the Rieske protein [41], this oxidant induced LHCII phosphorylation in isolated *D. salina* thylakoid membranes even at a concentration of 10 mM (Fig. 5, lanes 1 and 2). Furthermore, it did not abrogate NaCl-

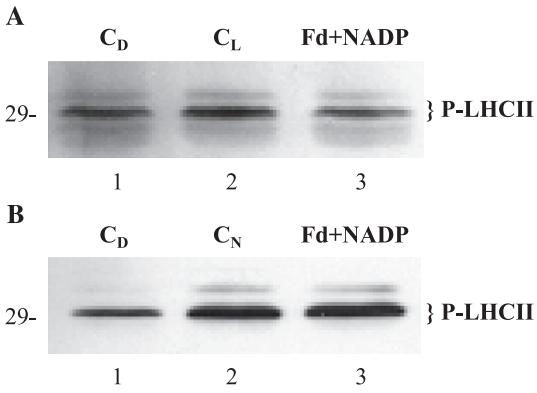

Fig. 4. Effects of ferredoxin and NADP on (A) light- and (B) NaCl-induced LHCII phosphorylation in *D. salina* thylakoid membranes. Thylakoid membrane proteins from dark-adapted *D. salina* were phosphorylated at 25 °C for 20 min in the light (about 200 μmol photons m^{-2} s^{-1}) (A) or in darkness with the addition of 0.3 M NaCl (B) in the absence or presence of 5 μM ferredoxin and 2 mM NADP (Fd+NADP, lane 3). C_D (A and B, lane 1) indicates the dark control; C_L (A, lane 2) and C_N (B, lane 2) indicates the light- and NaCl-induced LHCII phosphorylation, respectively. The positions of phosphorylated LHCII proteins recognized by the P-Thr antibody are indicated.

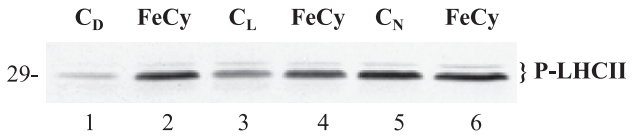

Fig. 5. Effects of ferricyanide on LHCII phosphorylation in *D. salina* thylakoid membranes. Thylakoid membrane proteins from dark-adapted *D. salina* were phosphorylated at 25 °C for 20 min in the absence (lanes 1, 3 and 5) or in the presence (lanes 2, 4 and 6) of 10 mM ferricyanide in dark (lanes 1 and 2) or light (about 200 μmol photons m^{-2} s^{-1}) (lanes 3 and 4) conditions or in darkness with addition of 0.3 M NaCl (lanes 5 and 6). C_D indicates the dark control; C_L and C_N indicate the light- and NaCl-induced LHCII phosphorylation, respectively. The positions of phosphorylated LHCII proteins recognized by the P-Thr antibody are indicated.

induced LHCII phosphorylation and even stimulated light-induced LHCII phosphorylation in *D. salina* thylakoid membranes (Fig. 5, lanes 3–6). These results contrast sharply with the previously reported inhibitory effects of ferricyanide on light- and low pH-induced LHCII phosphorylation in isolated thylakoid membranes from other species, and LHCII phosphorylation in *Acetabularia mediterranea* thylakoid membranes [8,14–17].

4. Discussion

Since the discovery of thylakoid membrane protein phosphorylation, LHCII phosphorylation in vitro has traditionally been induced by light [8–11,13]. Electron flow inhibitor studies have indicated that light activates the protein kinase via reduction of electron carriers, not through direct kinase excitation [8–11,13]. As observed in other species [8,9,13,14,16], here we observed that treatments with oxidants (ferredoxin and NADP) or photosynthetic electron flow inhibitors (DCMU, DBMIB or stigmatellin) inhibited light-induced LHCII phosphorylation in *D. salina* thylakoid membranes, indicating that plastoquinone and the cytochrome b_6f complex are involved in light-induced activation of LHCII kinase; this is consistent with the most widely accepted redox-controlled regulation mechanism.

In contrast, LHCII phosphorylations have been reported in some dark-adapted organisms in vitro. In *A. mediterranea*, thylakoid membrane LHCII phosphorylation activity persists in darkness for hours [14,15], and a low pH-induced LHCII phosphorylation was reported to occur in darkness in spinach thylakoid membranes [16,17]. Both of these dark LHCII phosphorylations are insensitive to PSII-dependent plastoquinone reduction inhibitors, but are sensitive to oxidants and inhibitors of cytochrome b_6f complex reduction [14–17]. These results lead to the suggestion that the kinase is activated and maintained in its active state as long as plastoquinol is bound (but not oxidized) at the Qo site of the cytochrome b_6f complex. Unlike the case in *A. mediterranea* thylakoid membranes, the present study showed that *D. salina* thylakoid membranes had no LHCII phosphorylation activity in darkness. DCMU treatment did not inhibit NaCl-induced LHCII phosphorylation in dark-adapted *D. salina* thylakoid membranes, indicating that the PSII-dependent induction of plastoquinone is not related to the phosphorylation process. Furthermore, neither oxidants (NADP and ferredoxin) nor cytochrome b_6f complex reduction inhibitors (DBMIB, stigmatellin) inhibited NaCl-induced LHCII phosphorylation in darkness, indicating that this process differs from light- and low pH-induced LHCII phosphorylation, and from LHCII phosphorylation in *A. mediterranea* thylakoid membranes. Contrary to the widely accepted theory of redox-controlled regulation mechanisms, our results indicate that the redox state of the cytochrome b_6f complex is likely not involved in NaCl-induced LHCII phosphorylation.

We have previously reported that LiCl, NaCl, KCl, $NaNO_3$, $CaCl_2$, $MgCl_2$, but not glyclerol, could induce LHCII phosphorylation in dark-adapted *D. salina* thylakoid membranes, which led to the suggestion that it is ions that mediate NaCl-induced LHCII phosphorylation. Notably, here we observed that neither addition of $CuCl_2$, an inhibitor of cytochrome b_6f complex reduction, nor oxidizing treatment with ferricyanide inhibited light- or NaCl-induced LHCII phosphorylation, and both salts even induced LHCII phosphorylation in dark-adapted *D. salina* thylakoid membranes as other salts did. All these results indicate that salt affects on LHCII phosphorylation in a more direct way than light does, and the redox state of the cytochrome b_6f complex is irrelevant to the process. One possible explanation is that salt might interact directly with LHCII kinase in *D. salina* resulting in a conformational change from the inactive to active state. However, further experiments will be necessary to assess these points.

We have previously reported that salt induces LHCII phosphorylation in *D. salina* in light or dark conditions but not in spinach thylakoid membranes [25]. The present study further shows that salt affects LHCII phosphorylation through a redox-independent way, which is different from the well-characterized light-induced redox-dependent effect. Salt-induced LHCII phosphorylation is expected to act as an adaptation mechanism to hypersaline environmental conditions.

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