Transient Decrease of Light-harvesting Complex II Phosphorylation Level by Hypoosmotic Shock in Dark-adapted Dunaliella salina

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Abstract This study investigated the regulation of major light harvesting chlorophyll a/b protein (LHCII) phosphorylation by hypoosmotic shock in dark-adapted Dunaliella salina cells. When the external NaCl concentration decreased in darkness, D. salina LHCII phosphorylation levels transiently dropped within 20 min and then restored gradually to basal levels. The transient decrease in LHCII phosphorylation levels was insensitive to NaF, a phosphatase inhibitor. Inhibition of intracellular ATP production by addition of an uncoupler or an ATP synthase inhibitor increased LHCII phosphorylation levels in D. salina cells exposed to hypoosmotic shock. Taken together, these results indicate that hypoosmotic shock inhibits the LHCII phosphorylation process. The related mechanism and physiological significance are discussed.

Key words ATP content; Dunaliella salina; hypoosmotic shock; light harvesting chlorophyll a/b protein (LHCII) phosphorylation

Plants have evolved many mechanisms for adapting to changes in environmental conditions. One such mechanism is the reversible phosphorylation of the major light harvesting chlorophyll a/b proteins (LHCII), which represents a system for balancing the excitation energy between photosystem I (PSI) and photosystem II (PSII) under fluctuating light conditions [1−3]. Overexcitation of PSII results in a reduction of the plastoquinone pool and subsequent plastoquinol occupation of the quinol oxidase site in the cytochrome b_{6}f (Cyt b_{6}f) complex, leading to activation of LHCII kinase and phosphorylation of LHCII. Interaction of phosphorylated LHCII with PSI enhances PSI light absorption. Preferential excitation of PSI results in oxidation of the plastoquinone pool and subsequent deactivation of LHCII kinase. The permanently active phosphatase dephosphorylates LHCII, leading to its gradual reassociation with PSI. In this way, plants can regulate excitation energy distribution and optimize light utilization [1−3].

In the unicellular green alga Chlamydomonas, LHCII kinase remains active in darkness due to plastoquinone reduction by chlororespiration, which is enhanced under conditions of ATP depletion [4,5]. In these cells, phosphorylation of LHCII in darkness is accompanied by migration of the Cyt b_{6}f complex from the PSII to PSI membrane domains, thus increasing cyclic electron flow and ATP generation upon illumination [4,5]. Thus, LHCII phosphorylation also enables the alga to adapt to intracellular ATP demands.

The halotolerant green alga Dunaliella salina is distinguished for its adaptation to media ranging in salinity from 50 mM to 5 M NaCl by the accumulation of intracellular glycerol [6,7]. Harrison and Allen [8] reported that dark incubation could induce LHCII phosphorylation in D. salina cells, as is the case in Chlamydomonas. Our group [9] further observed that LHCII phosphorylation in D. salina resembles that of spinach in terms of light-mediated control in isolated thylakoid membranes. However, we observed that spinach and D. salina differed with respect to NaCl sensitivity. Treatment with NaCl induced LHCII phosphorylation in D. salina thylakoid membranes under both light and dark conditions, whereas in spinach thylakoid membranes, NaCl did not induce LHCII phosphorylation. This work was supported by a grant from the Major State Basic Research Development Program of China (No. G1998010100.)

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phosphorylation in the dark, and even inhibited light-induced LHCII phosphorylation. Furthermore, we found that hypertonic shock increased LHCII phosphorylation levels in dark-adapted *D. salina* cells [9].

Here, we investigated the regulation of LHCII phosphorylation by hypoosmotic shock, another stress *D. salina* occasionally has to deal with in extreme osmotic environmental conditions. New insights into this system might improve our overall understanding of the regulation and physiological significance of LHCII phosphorylation in the halotolerant green alga.

**Materials and Methods**

**Plant materials and hypoosmotic shock treatments**

*D. salina* cells were grown in an artificial hypersaline medium containing 1.5 M NaCl at 25 °C in a 12 h light (fluorescent lamp, 70 µmol photons·m⁻²·s⁻¹)/12 h dark cycle, with continuous shaking (100 rpm). When the concentration of chlorophyll (Chl) was approximately 12 µg/ml, as determined by the method of Arnon [10], *D. salina* cells were kept in darkness without shaking for approximately 3 h. Hypoosmotic shock was carried out by incubating dark-adapted cells acclimated to 1.5 M NaCl with an artificial hypersaline medium containing 0.5 M NaCl or other chemical reagents for 20 min (or for the indicated time period) before measurements. Treatments with dicyclohexylcarbodimide (DCCD) (5 µM) or nigericin (5 µM) were performed by incubating *D. salina* cells with either reagent for 20 min.

**ATP content measurement**

*D. salina* cells were suspended in 50 mM Tricine-NaOH (pH 8.0) and then killed with boiling water. ATP contents were measured with a luciferin-luciferase assay, as previously described [11].

**Isolation of thylakoid membranes**

Thylakoid membranes were isolated according to the method of Kim *et al.* [12]. Briefly, dark-adapted *D. salina* cells were suspended in sonication buffer (100 mM Tris-HCl, pH 6.8, 5 mM MgCl₂, 0.2% polyvinyl pyrrolidone K30, 3 mM aminocaproic acid, 1 mM aminobenzamidine and 0.2 mM phenylmethylsulfonyl 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. Samples were then centrifuged at 40,000 g for 20 min at 4 °C, and the pellets were re-suspended in sonication buffer at 1 mg Chl/ml.

**Thylakoid membrane protein analysis and immunoblotting**

Thylakoid membranes were solubilized in 0.5 M Tris-HCl (pH 6.8), 7% sodium dodecyl sulfate (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 [12]. Thylakoid membrane proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15% acrylamide, 0.5% bisacrylamide and 4 M urea) using 0.75 cm×6 cm×8 cm slabs on a Miniprotein III cell system (Bio-Rad, Hercules, USA), with each sample containing 5 µg Chl. The separated polypeptides were stained with 0.1% Coomassie brilliant blue R or electrophoretically transferred to Hybond ECL nitrocellulose membranes with a semi-dry transfer cell for immunoblot analysis. Phosphorylated thylakoid membrane proteins were detected with rabbit polyclonal anti-phosphothreonine (P-Thr) antibody (Zymed) [9,11,13,14]. The relative amount of phosphorylated LHCII was quantified using a software Gel-Pro analyzer 3.0.

**Results**

In thylakoid membranes, phosphorylated proteins mainly belong to PSII core proteins and LHCII. Different from phosphorylation of PSII core proteins, LHCII phosphorylation is known to require not only reduction of the plastoquinone pool but also reduction of the Cyt *b,f* complex [4]. Fig. 1(A) shows that two peptides with an apparent molecular mass of about 29 kDa were recognized by the anti-phosphothreonine antibody. We have observed that the light-induced phosphorylation of both peptides was sensitive to Cyt *b,f* complex reduction inhibitors [13], and thus they were identified as LHCII proteins.

Previous evidence indicated that shock induced LHCII phosphorylation in dark-adapted *D. salina* [9]. Here, we observed that 20 min of hypoosmotic shock induced the opposite effects: LHCII phosphorylation levels declined as the external NaCl concentration decreased from 1.5 to 1.0, 0.5 or 0.3 M (Fig. 1). Interestingly, resuspension of *D. salina* cells adapted to 1.5 M NaCl in hypoosmotic media containing sucrose, glycerol or mannitol (at osmotic concentrations equivalent to 0.5 M NaCl, 0.93 Os/kg) decreased LHCII phosphorylation levels to an even larger extent (Fig. 2), indicating that the transient LHCII dephosphorylation is affected not only by hypoosmotic conditions but also by salt ions.

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As shown in Fig. 3, transfer of *D. salina* cells to hypoosmotic medium induced a transient decrease in LHCII phosphorylation levels (20 min), followed by a gradual restoration to near basal levels within 4 h. This result indicates that the observed decrease in LHCII phosphorylation levels is a response to transient decreases in external salt concentration, instead of steady hypoosmotic conditions.

The phosphorylation level of LHCII results from the combined effects of LHCII phosphorylation by LHCII kinase and LHCII dephosphorylation by phosphatase. To investigate the individual contribution, we tested the effects of NaF, a phosphatase inhibitor, *in vivo* and *in vitro* [15–17] on hypoosmotic shock-induced decreases in LHCII phosphorylation. In the presence of 20 mM NaF, an increase (Fig. 4, C<sub>NaF</sub>) in the LHCII phosphorylation level was detected, suggesting that the chemical agent actually entered the cells and inhibited phosphatase activity. However, hypoosmotic shock could still decrease LHCII phosphorylation levels (Fig. 4, H<sub>0</sub>) even in the presence of 20 mM NaF (Fig. 4, H<sub>NaF</sub>), indicating that the decrease in the LHCII phosphorylation level is likely due to transient inhibition of LHCII phosphorylation by kinase, but not...
due to stimulation of dephosphorylation of phosphorylated LHCII by phosphatase.

In *Chlamydomonas*, LHCII phosphorylation is modulated by ATP demand. Depression of respiratory ATP synthesis in darkness results in the activation of LHCII kinase and LHCII phosphorylation, while ATP restoration by way of photophosphorylation under illumination decreases LHCII phosphorylation levels [18]. Thus, we examined the possible role of ATP content in the regulation of LHCII phosphorylation by hypoosmotic shock in *D. salina* cells. It was shown that intracellular ATP content was increased by 22% when dark-adapted *D. salina* cells were subjected to hypoosmotic shock, which may be related to the stimulation of respiration [19]. The ATP content increase in hypoosmotically shocked *D. salina* cells was blocked by the addition of 5 µM DCCD, an ATP synthase inhibitor, or 5 µM nigericin, an uncoupler (Fig. 5). Consistent with these observations, addition of DCCD and nigericin, which block ATP synthesis, also increased LHCII phosphorylation levels in stressed *D. salina* [Fig. 5(B,C), H and H_{Nig}].

**Discussion**

Previous studies with isolated thylakoid membranes have demonstrated that LHCII kinase is activated when the intersystem electron carriers are reduced. Thus, LHCII phosphorylation is traditionally induced by exposing the thylakoids to white light in the presence of ATP [14,20–23], while the inactivation of LHCII kinase is usually obtained by oxidizing plastoquinone with a light-dark transition [24]. However, dark-adapted green algae (*Chlamydomonas* and *Dunaliella*) still exhibit LHCII phosphorylation activity, probably due to chlororespiration-dependent reduction of the plastoquinone pool [8,25,26]. Nevertheless, the inactivation process in dark-adapted green algae is not well understood. Here, our results reveal that an
decrease in external osmotic pressure leads to a transient decrease in LHCII phosphorylation levels in dark-adapted *D. salina*, providing a useful system for future *in vivo* studies of “dark” down-regulation of LHCII phosphorylation in green algae.

Rokka *et al.* [24] reported that abrupt transfer of isolated spinach thylakoids to heat-shock induced a rapid decrease in the phosphorylation levels of the LHCII and PSII core proteins, and attributed this effect to activation of phosphatase. Here, our results show that hypoosmotic shock-induced decrease of phosphorylated LHCII is insensitive to phosphatase inhibitor in dark-adapted *D. salina* cells. Thus, the decrease in LHCII phosphorylation levels induced by hypoosmotic shock in dark-adapted *D. salina* cells is probably due to inhibition of LHCII phosphorylation, but not stimulation of LHCII dephosphorylation.

Bulté *et al.* [18] have investigated ATP control on LHCII phosphorylation in dark-adapted *Chlamydomonas* and found that a decrease in ATP content activates LHCII kinase while the inactivation process needs ATP synthesis. Here we observed that the changes in ATP content were correlated with LHCII phosphorylation levels in hypoosmotically shocked *D. salina* cells in the presence or absence of DCCD or nigericin, suggesting that increases in ATP content might be related to the inactivation of LHCII kinase in *D. salina* cells following hypoosmotic shock. Conversely, we have reported salt-induced activation of LHCII kinase in *D. salina* [9,27], and the present results reveal that hypoosmotical treatment of *D. salina* cells with non-ionic medium decreases LHCII phosphorylation levels more effectively than treatment with ionic medium (Fig. 2). Thus, transient decreases in intracellular and extracellular ion concentration upon hypoosmotic shock may also contribute to the inactivation of LHCII kinase.

Notably, treating hypoosmotically shocked cells with DCCD led to a large decrease in ATP content without a further increase in LHCII phosphorylation levels compared with that in un-shocked cells. This observation was different from the results obtained with *Chlamydomonas* [18] and un-shocked *D. salina* cells [27] where a decrease in ATP content caused LHCII phosphorylation. These results suggest that other factors might also be involved in hypoosmotic shock-induced decreases in LHCII phosphorylation. Zer *et al.* [28,29] reported that LHCII phosphorylation was regulated not only by kinase activity but also by the exposure and access of the LHCII phosphorylation site to protein kinase. Maeda and Thompson [30] reported that chloroplast envelope expanded in *Dunaliella* in the case of hypoosmotic shock. Taken together, the conformational changes in chloroplast structure may be unfavorable to the access of the LHCII phosphorylation site to LHCII kinase. However, further experiments will be required to confirm these points.

We have reported that salt shock decreases intracellular ATP content and induces state II transition associated with LHCII phosphorylation [9]. On the contrary, the present results show that hypoosmotic shock increases intracellular ATP content and induces a transient decrease in LHCII phosphorylation. The dephosphorylated LHCII is suggested to interact with PSII. Such reorganization of photosynthetic apparatus will optimize relative efficiency of PSII activity and favor linear electron flow upon re-illumination [11], and thus decrease ATP synthesis to balance the ATP supply in the case of hypoosmotic shock. In this way, state transitions enable *D. salina* to adjust the NADPH/ATP ratio according to physiological conditions when it is subjected to osmotic shocks.

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