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Evidence for light-dependent and light-independent protein dephosphorylation in chloroplasts

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Abstract A number of photosystem II (PSII) associated proteins, including core proteins D1, D2 and CP43, and several proteins of the LHCII complex, are phosphorylated by a thylakoid-bound, redox-regulated kinase(s). We demonstrate here that the compound propyl gallate is an effective inhibitor of LHCII phosphorylation in vivo while having little effect on PSII core protein phosphorylation. Using this inhibitor, we demonstrate that LHCII dephosphorylation is insensitive to light in vivo. Taken together with our previous conclusion (Elich et al., EMBO J. 12 (1993) 4857–4862) that PSII core protein dephosphorylation is light-stimulated, our data suggest the presence of multiple phosphatases responsible for thylakoid protein dephosphorylation in vivo.

Key words: Chloroplast phosphatase; Kinase inhibitor; LHCII kinase; Photoregulation; Photosynthesis; Duckweed; *Spirodela oligorrhiza*

1. Introduction

Regulated protein phosphorylation and dephosphorylation is ubiquitous in signal transduction pathways regulating diverse cellular functions in animals [1]. Plants also use protein phosphorylation as a mechanism to regulate different metabolic processes in response to both internal and environmental cues [2]. The first plant proteins shown to be phosphorylated were the light harvesting chlorophyll a/b apoproteins (LHCII) of PSII [3]. Subsequently, a number of PSII core proteins were also shown to be phosphorylated, viz., D1, D2, CP43 and the psbH product [4]. Phosphorylation of PSII-associated proteins has been widely studied due to the fundamental importance of photosynthesis.

PSII associated proteins are phosphorylated by thylakoid-associated, redox-regulated kinase activity [4]. The demonstration that phosphorylation of LHCII can be distinguished from that of PSII core proteins by various inhibitors and mutants suggests the presence of multiple kinases or modes of regulation. Phosphorylation of LHCII is generally thought to regulate energy distribution between the two photosystems [4–6]. The role of PSII core protein phosphorylation is less clear; however, it has been implicated in regulating D1 metabolism [7].

In vitro studies have demonstrated that PSII phosphoproteins can be dephosphorylated by thylakoid-bound phosphatase activity that is insensitive to light or redox control [8,9]. In contrast, in vivo studies have indicated light-stimulated dephosphorylation of D1 [7,10] as well as D2 and CP43 [10]. Previously, to specifically examine the regulation of dephosphorylation, we found it necessary to inhibit endogenous protein kinase activity [10]. In the case of PSII core proteins, it was demonstrated that they underwent little phosphorylation in the dark, and that their light-dependent phosphorylation could effectively be inhibited with the PSII inhibitor, 3-(3,4-dichlorophenyl-1,1-dimethylurea) (DCMU) [10–13]. In the case of LHCII, however, substantial dark phosphorylation was observed precluding us from ascertaining potential light regulated phosphatase activity.

In the present study we have identified propyl gallate (PG) as a specific inhibitor of LHCII phosphorylation in vivo. This compound effectively inhibits LHCII phosphorylation both in the light and in darkness, allowing us to specifically examine potential regulation of dephosphorylation in the absence of interfering kinase activity. We show that LHCII dephosphorylation in vivo is not significantly affected by light, consistent with in vitro studies on isolated thylakoids.

2. Materials and methods

Axenic cultures of *Spirodela oligorrhiza* were grown as previously described [13]. Procedures for in vivo phosphorylation, thylakoid isolation, SDS-PAGE, and autoradiography were as previously described [10,13,14].

3. Results

To determine whether LHCII dephosphorylation is light-dependent, it was first necessary to inhibit dark phosphorylation of these proteins [10]. Fortuitously, we examined the effect of propyl gallate (PG), a free radical scavenger previously shown to inhibit D1 degradation [15], on thylakoid protein phosphorylation in vivo. As previously found [10], incubation of *Spirodela* plants for 3 h in the light in the presence of [^{32}P]orthophosphate resulted in the phosphorylation of a number of PSII proteins including D1, D2, CP43, LHCII and the psbH gene product (Fig. 1, lane 1). In the presence of 1 mM PG, however, a dramatic and specific inhibition of LHCII phosphorylation was observed (Fig. 1, lane 2, LHCII band).

We next determined whether PG would also inhibit LHCII phosphorylation in the dark under the conditions of our previous study [10]. Plants were incubated with [^{32}P]orthophosphate for 3 h in the light in the presence of both DCMU and PG, washed, and further incubated in the dark in the presence of the inhibitors. Little or no phospho-

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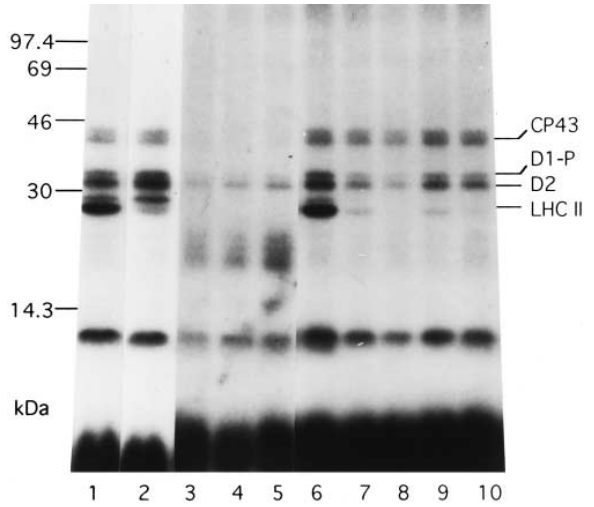

Fig. 1. Propyl gallate inhibition of LHCII dark phosphorylation, and light-insensitivity of LHCII dephosphorylation. One set of plants was labeled with [^{32}P]orthophosphate for 3 h, in the presence (lane 2) or absence (lane 1) of 1 mM propyl gallate. A second set was similarly labeled as the first but in the presence of 1 mM propyl gallate plus 10 μM DCMU (lanes 3–5); plants were then washed and either harvested immediately (lane 3) or further incubated with the inhibitors in the dark for 2 (lane 4) or 4 (lane 5) h. A third set of plants was phosphorylated in the absence of inhibitors (lane 6), then washed and further incubated in the presence of propyl gallate plus DCMU, in the light (lanes 7 and 8) or dark (lanes 9 and 10), for 2 h (lanes 7 and 9) or 4 h (lanes 8 and 10). Thylakoids were isolated and analyzed by SDS-PAGE and autoradiography.

rylation of LHCII was observed up to 4 h of dark incubation (Fig. 1, compare lanes 2 and 3). In contrast, when identical experiments were performed in the absence of PG, substantial phosphorylation of LHCII had been observed previously (Fig. 1, lane 6; see also in Ref. [10], Fig. 3, lane 3).

Using PG to inhibit dark phosphorylation, we examined the question of whether LHCII dephosphorylation is regulated by light. After radiolabeling with [^{32}P]orthophosphate and washing, plants were further incubated in the light or darkness in the presence of DCMU and PG. Similar to our previous study [10], the phosphorylated forms of D1, D2, and CP43 were relatively stable in the dark but rapidly dephosphorylated in the light (Fig. 1, compare lanes 7 and 8 with lanes 9 and 10). In contrast, LHCII was dephosphorylated to approximately the same extent irrespective of light irradiation or dark incubation (Fig. 1, compare lanes 7 and 8 with lanes 9 and 10). We conclude that LHCII dephosphorylation is not significantly regulated by light, and that the apparent light dependence of this process observed previously (see Fig. 1 in Ref. [10]) was due to additional LHCII phosphorylation that occurs in darkness but not in the light in the presence of DCMU.

4. Discussion

We demonstrate here that PG is an effective and specific inhibitor of LHCII phosphorylation in vivo. The differential effect of PG on LHCII versus PSII core protein phosphorylation is consistent with previous evidence for the presence of multiple kinases or regulatory mechanisms; however, in this specific case a substrate-dependent inhibition can not be ruled out. The mode of action of PG in this regard is unknown and beyond the scope of this study. We note, however, that PG was previously shown to inhibit D1 degradation without affecting PSII electron transport [15]. Furthermore, this compound is known to act both as a radical scavenger and a metal chelator [15]. It is also lipophilic and may perturb membranes nonspecifically. In any case, the efficacy and specificity PG exhibits as an inhibitor of LHCII phosphorylation in vivo should make it useful for further studies of the physiological role of this post-translational modification.

The ability of PG to inhibit LHCII phosphorylation allowed us to demonstrate that LHCII dephosphorylation is independent of light in vivo. This result, together with our previous conclusion that PSII core protein dephosphorylation is light-stimulated in vivo [10], suggests the presence of multiple phosphatases responsible for thylakoid protein dephosphorylation in vivo. Our previous demonstration [10] that DBMIB inhibits dephosphorylation of PSII core proteins, but not that of LHCII, is also consistent with this hypothesis. In this regard, we note the identification of two distinct plastid phosphatases, one thylakoid-bound and the other stromal, that can dephosphorylate thylakoid phosphoproteins [16,17]. The existence of a soluble, stromal phosphatase may explain why light regulated dephosphorylation has not been observed in isolated thylakoids ([[18]; Elich and Mattoo, Unpublished], but has been observed in vivo [7,10]. If this is the case, our results would then suggest that the thylakoid bound phosphatase is the sole mediator of LHCII dephosphorylation in vivo.

An interesting component of our studies is the demonstration that LHCII is phosphorylated in vivo to a similar extent in the dark as in the light, at least in *Spirodela* [10]. Furthermore, we have now shown that LHCII dephosphorylation is constitutive and rapid indicating that a constant turnover of ATP is required to maintain the phosphorylated state of LHCII in the dark. An obvious question for the future is why a plant would expend energy in the dark on a modification thought to regulate light distribution between the two photosystems.

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