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Specific loss of LHCII phosphorylation in the *Lemna* mutant 1073 lacking the cytochrome b_6/f complex

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The thylakoid protein kinase(s) activity of *Lemna perpusilla* strain 6746 (wild type, WT) and the cytochrome (cyt) b_6/f-less mutant 1073 was compared. Isolated thylakoids of both WT and mutant phosphorylated the polypeptides of 9–15, 29, 32–34 and 40–45 kDa. This kinase(s) activity was light-dependent and could be elicited by addition of duroquinol in the dark. Thylakoids from both WT and mutant phosphorylated histone III-S at comparable rates. However, the redox-controlled phosphorylation of the LHCII polypeptide which could be demonstrated in vitro and in vivo in the WT thylakoids could not be detected under any experimental condition in the cyt b_6/f-less thylakoids. Halogenated quinone analogues known to inhibit reduction of the cyt b_6/f complex inhibited both the electron flow and duroquinol-activated LHCII phosphorylation, but had no effect on the duroquinol-dependent phosphorylation of the other thylakoid polypeptides. These results indicate that the *Lemna* thylakoids contain at least two redox-activated protein kinase(s). A quinone-binding site is involved in the activation of the LHCII kinase system which is rendered inactive in the absence of the cyt b_6/f complex.

Cytochrome b_6/f complex; LHCII; Protein phosphorylation; Thylakoid; *(Lemna)*

1. INTRODUCTION

The phosphorylation of the Chl *a/b*-binding polypeptides forming the light-harvesting antennae complex of PS II plays an important role in the regulation of energy distribution between the two photosystems [1]. The protein kinase responsible for this activity (LHCII kinase) is an intrinsic membrane protein. Its activity is subject to positive redox control exerted by photosynthetic electron flow-dependent reduction of the plastoquinone pool in the light [1,2]. LHCII kinase can be also activated in the dark by addition of various reductants such as dithionite or duroquinol [2,3]. The mechanism whereby the quinones interact with the LHCII kinase system has so far remained obscure. Experimental results obtained in our laboratory with LCHII kinase of thylakoids from various sources and their inhibition by quinone analogues suggested that a specific binding site for reduced quinones might be directly involved in this process (Gal et al., in preparation). The quinone-binding characteristic of this site(s) suggested the possible involvement of the cyt b_6/f complex in the activation of LHCII phosphorylation. Thus, it was of interest to assess the activity of LHCII kinase in a

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Abbreviations: Chl, chlorophyll; cyt b_6/f, cytochrome b_6/f complex; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DIBB, 2,3-diiodo-5-*t*-butyl-1,4-benzoquinone; diuron, 1,3-dichlorophenyldimethylurea; DQ/DQH_2, oxidized/reduced duroquinone; LHCII, light-harvesting chlorophyll *a/b* complex of photosystem II; PQ/PQH_2, oxidized/reduced plastoquinone; PS, photosystem; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; WT, wild type

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mutant specifically defective in the cyt b_6/f complex. The *Lemna perpusilla* 1073 mutant lacks all 4 subunits of this complex [4] but contains active PS II and I complexes [5]. Here, evidence is presented demonstrating that this mutant cannot phosphorylate the LHCII complex whether in vivo or in vitro.

2. MATERIALS AND METHODS

L. perpusilla 6746 (WT) and mutant strain 1073 were grown, and thylakoid membranes were prepared as described by Shahak et al. [5].

Thylakoid protein phosphorylation in vitro was assayed by incubating the membranes (1–2 μg Chl/assay) in a reaction mixture (100 μl), containing 50 mM Tris-HCl (pH 8.00), 10 mM $MgCl_2$, 5 mM NaF and 100 μM [^{32}P]γATP (200–400 cpm/pmol). When indicated, duroquinol (1 mM) [3] was added to the dark-incubated samples. Incubation was continued for 15 min at 25°C, either in the light (100 W/m^2) or in the dark. The incubation mixture was then centrifuged and the membrane pellet was resuspended in electrophoresis sample buffer, and heated for 5 min at 80°C, prior to resolution of the polypeptides by SDS-PAGE as described by Laemmli [6]. Standard M_r markers were purchased from Pharmacia. The gels were stained with Coomassie brilliant blue R, dried and exposed to X-ray film.

For quantitative measurements of LHCII phosphorylation the stained LHCII polypeptide bands, as identified by immunoblotting with anti-*Lemna* LHCII antibodies, were excised from the dried gels, and Cerenkov radioactivity was counted.

Phosphorylation of histone III-S in vitro was carried out as in [7]. The reaction mixture (100 μl) contained 15 μg thylakoid protein, 50 mM Na-Tricine, pH 8.0, 10 mM $MgCl_2$, 5 mM NaF, 5 mM DTT and when indicated, 25 μg histone III-S (Sigma). Incubation was carried out at 25°C for 20 min in room light intensity.

For in vivo phosphorylation of the thylakoid polypeptides plants were incubated in growth medium containing 20 μCi [^{32}P]orthophosphate/ml (1 μCi/nmol) for the desired time and illumination conditions, after which thylakoid membranes were prepared as described above. All experiments were carried out with freshly prepared thylakoids. Chlorophyll was measured according to Arnon [8], and protein according to Peterson [9].

3. RESULTS AND DISCUSSION

3.1. *In vitro phosphorylation of WT and mutant thylakoid polypeptides*

The absence of cyt b_6, cyt f, Rieske protein and subunit IV in the mutant membranes, as demonstrated before by immunoblotting [4] was confirmed (not shown). However, no differences could be detected between the LHCII polypeptides of the mutant as compared to those of the WT with respect to their electrophoretic mobility either in the absence (fig.1) or presence of urea (not shown). Moreover, the LHCII fragments generated by trypsin and chymotrypsin treatment had the same electrophoretic mobility in both WT and mutant (not shown). However, while the LHCII bands of the WT thylakoids were heavily phosphorylated no

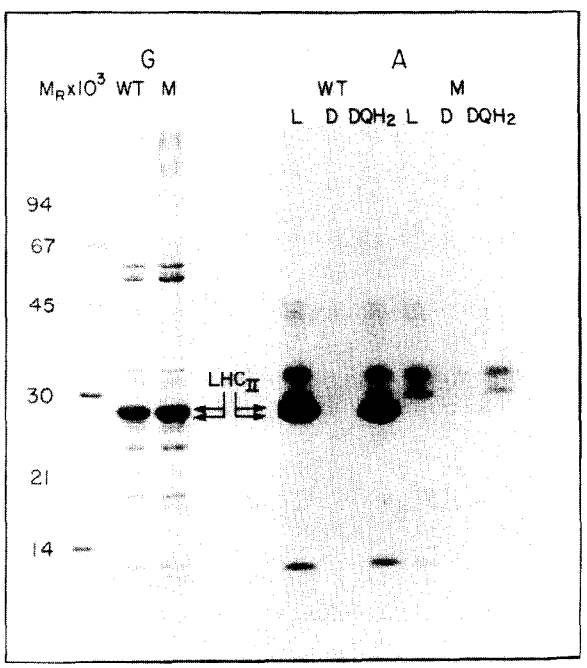

Fig.1. In vitro phosphorylation pattern of WT and mutant thylakoid polypeptides. Thylakoids were incubated with [^{32}P]γATP, using the kinase assay conditions as described in section 2. G, stained gel; A, autoradiogram; L, D, DQH_2, samples incubated in the light, dark or dark with addition of 1 mM DQH_2; M, mutant; WT, wild type; values for standard M_r markers are indicated.

^{32}P incorporation could be detected in the LHCII polypeptides of the mutant (fig.1). In contradistinction to LHCII, several thylakoid polypeptides of 9, 29, 32–34 and 42–45 kDa were phosphorylated in the mutant as well as in the WT (fig.1) as also observed in thylakoids of other higher plants and algae [10,11]. The kinase(s) involved in this process in both types of thylakoids was (were) active in the light, inactive in the dark and could be activated by addition of duroquinol in the dark (fig.1).

Thylakoid kinase(s) phosphorylate(s) soluble exogenous substrates such as casein or histone either when still integrated in the membrane [12] or after purification. In the latter case redox control toward LHCII polypeptides was lost [7]. To determine whether there is a substantial loss of overall kinase activity in the mutant, we have compared the capacity to phosphorylate such substrates in WT and mutant thylakoids. As demonstrated in table 1, the total activity of the thylakoid kinase(s), using histone as a substrate, was slightly higher in the mutant as compared to that of the WT thylakoids.

These results demonstrate that despite the similarities of LHCII polypeptides of both WT and mutant thylakoids, and the equal kinase activity of both thylakoids toward endogenous and added substrates, LHCII was not phosphorylated in the mutant. The LHCII kinase system of the mutant could not be activated in vitro either by light-dependent electron flow or by reduction with DQH_2 which could activate the phosphorylation of other thylakoid polypeptides.

3.2. *In vivo phosphorylation of thylakoid polypeptides of WT and mutant* Lemna

Lack of LHCII kinase activity in the mutant as assayed in vitro could be due to the loss or reduction of LHCII phosphatase activity which is required for the turnover of the phosphate ester bonds and regeneration of open phosphorylation sites [1,2]. If this was the case no free sites for phosphorylation of LHCII could be available in the isolated thylakoids, since those would be already phosphorylated in vivo. To test this possibility *Lemna* plants were incubated for 3 h in growth medium containing [^{32}P]orthophosphate in white light, followed by exposure to far-red (710 nm) and then red (650 nm) light to induce state transition and related changes in the phosphorylation or dephosphorylation of LHCII [1,2,13]. The results of such an experiment (fig.2A) show an expected increase in phosphorylation of LHCII in the WT thylakoids following transfer from far-red to red light (state transition) as compared to those of plants kept in far-red light only. As opposed to WT plants, no phosphorylation of LHCII could be detected in the thylakoids of the *Lemna* mutant (fig.2A).

To ensure that even a residual LHCII kinase activity would be detected in the mutant, plants were grown for 48 h in the presence of [^{32}P]orthophosphate. However, no LHCII phosphorylation could be detected in the mutant thylakoids in this experiment (fig.2B).

In addition, we have measured the state 1–state 2 transition by the effect of continuous red and far-red illumination on chlorophyll fluorescence

Table 1

Phosphorylation of histone III-S by *Lemna* WT and mutant 1073 thylakoids

Thylakoid source	Phosphorylation (pmol ^{32}P incorporated · mg^{-1} thylakoid protein · min^{-1})		
	Endogenous (− histone)	Total (+ histone)	Net histone phosphorylation
Lemna WT	8.4	120.6	112.2
Lemna mutant	6.2	130.0	123.8
Chlamydomonas reinhardii	49.8	216.5	166.7

Histone S-III phosphorylation was carried out as described in section 2. For comparison, the activity of *Chlamydomonas* thylakoids [20] under similar conditions was also measured. The phosphorylation of histone III-S was not markedly changed when carried out in the light, dark, or dark in the presence of DQH_2

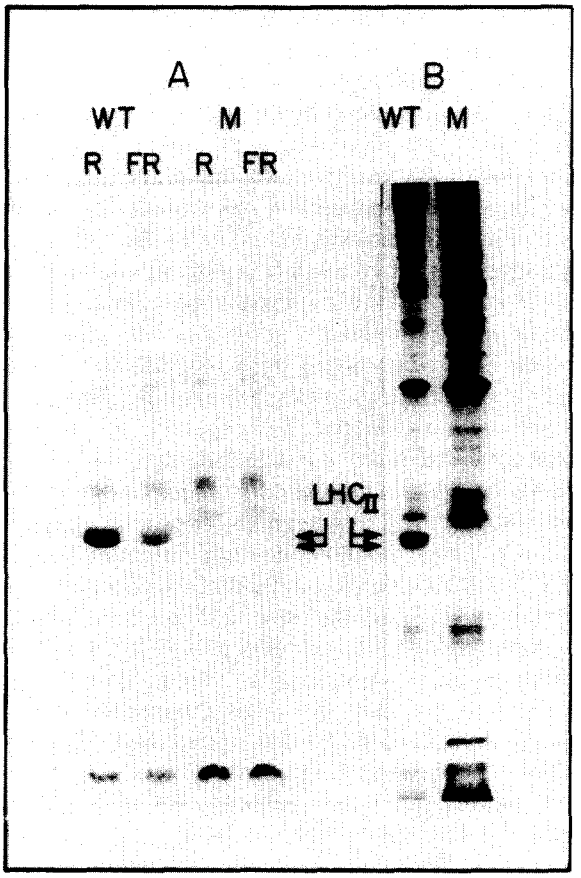

Fig.2. In vivo phosphorylation of WT and mutant thylakoid polypeptides. WT and mutant *Lemna* were incubated with [^{32}P]orthophosphate at 25°C, as described in section 2. A, plants were incubated for 3 h in white light (7 W/m^2) and then subjected to 60 min illumination with 720 nm light (FR), or 30 min illumination with 710 nm light followed by 30 min illumination with 650 nm light (R), (20 W/m^2 each). B, plants were grown for 48 h in white light in the presence of [^{32}P]orthophosphate. WT, wild type; M, mutant. Arrows indicate the location of LHCII polypeptides.

yield under modulated measuring illumination of whole *Lemna* plants [14]. The results clearly indicate normal behaviour of WT, while no state transition could be detected in the mutant plant (not shown). As in the in vitro experiments, in both WT and mutant thylakoids the polypeptides of 32–34 and 45–50 kDa were phosphorylated. In the mutant a 29 kDa polypeptide which is not an LHCII component was heavily phosphorylated as well (fig.2A).

Table 2

Inhibition of LHCII phosphorylation in *Lemna* WT thylakoids by various quinone analogs

	Experimental conditions	
Additions	Light	Dark + DQH_2
None	100	100
Diuron (10μM)	0	170
Atrazine (10 μM)	13	131
DBMID (50 μM)	0	32
DIBB (10 μM)	0	0

LHCII kinase activity was measured and analyzed as described in section 2 and is expressed as % of control (450 and 350 cpm/LHCII polypeptide bands in the light- or dark-incubated sample respectively)

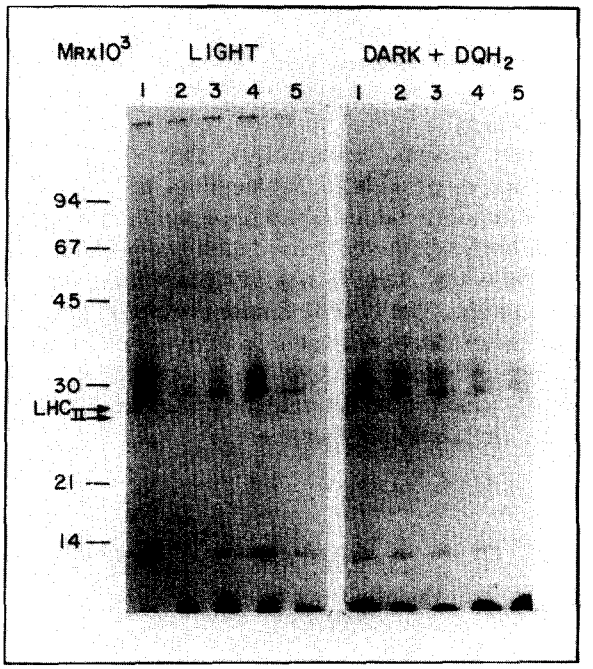

Fig.3. Effect of quinone analogs on the in vitro phosphorylation of mutant thylakoid polypeptides. Experimental conditions as described in section 2. Only the autoradiogram of the SDS-PAGE-resolved polypeptides is shown; lanes: 1, control (no additions); 2, diuron, 10 μM; 3, atrazine, 10 μM; 4, DBMIB, 50 μM; 5, DIBB, 10 μM. M_r($\times 10^3$), M_r markers indicated. Arrows indicate the location of the LHCII polypeptides which are not phosphorylated.

3.3. *Effect of various quinone analogs on the activity of protein kinase(s) of WT and mutant thylakoids*

Based on the activation of LHCII kinase by PQH_2 and DQH_2 [3] it was generally assumed that a quinone-binding site(s) might participate in the mechanism of LHCII kinase activation. Whether this site(s) is part of the LHCII kinase enzyme itself as recently suggested by Bennett et al. [15] or shared with another thylakoid membrane complex possessing a reduced quinone-binding site(s) such as cyt b_6/f remains to be established. The fact that light-activated LHCII kinase is inhibited by DBMIB [15] as well as by other quinone analogs (Gal et al., in preparation), taken together with the present results, could indicate that the PQH_2-binding site of cyt b_6/f might be essential for the activation of LHCII kinase. Thus, it was of interest to test the effect of quinone analog inhibitors of electron flow on the activity of LHCII kinase.

The results of such experiments are given in table 2 and fig.3. Like diuron and atrazine, DBMIB and DIBB inhibited the activation of LHCII kinase of WT thylakoids by light (table 2) at concentrations at which electron flow to PQ via PS II is inhibited by these quinone analogs [16]. However, unlike diuron and atrazine, DBMIB and DIBB strongly inhibited the LHCII kinase activity elicited by DQH_2 in the dark, indicating that they compete with DQH_2 at a site different from that of diuron binding. Nanba and Katoh [17] have recently reported that DBMIB competes with DQH_2 for the quinone-binding site of the cyt b_6/f complex of *Synechococcus* sp. According to these authors DQH_2 does not reduce PQ but interacts directly with the cyt b_6/f complex at the PQ-binding site [17]. In addition, the *Lemna* mutant does not lack the PQ pool reducible by PS II (cf. [5]), as also detected by measurements of variable fluorescence kinetics (not shown).

In contradistinction to LHCII phosphorylation the light-activated phosphorylation of the 29 and 32–34 kDa polypeptides in the mutant which was inhibited by diuron and DIBB was not significantly inhibited by DBMIB or atrazine. The quinone analogs tested did not inhibit the phosphorylation of these polypeptides when elicited by DQH_2 in the dark (fig.3).

The above results are in agreement with previous reports indicating that the thylakoids contain several kinase activities [15,18], which in *Lemna* are redox-controlled. Furthermore, our data demonstrate that the mechanism of redox-controlled activation of LHCII kinase differs from that of the other thylakoid polypeptides' phosphorylation activated by light or addition of DQH_2 in the dark. It is noteworthy that although being redox-controlled in vitro, the phosphorylation of these other polypeptides in vivo was not correlated with state transitions in the WT (fig.2A).

These results strongly suggest that the cyt b_6/f complex might be involved in the activation of the LHCII kinase. Recently, an alteration of LHCII kinase activity was also reported for a *Chlamydomonas* mutant lacking this complex [19].

Other possible explanations for the loss of LHCII kinase system in the mutant should be considered such as pleiotropic or assembly defects due to loss of a major membrane intrinsic complex. Correlation between physiologically induced structural changes and loss of LHCII kinase activity has been reported before in *Chlamydomonas* [20]. However, sonication or freeze-thawing which recovered the LHCII kinase activity in this case [20] failed to activate the *Lemna* mutant LHCII phosphorylation (not shown). Examination of thin sections by electron microscopy demonstrated the presence of normal arrays of grana stacks and interconnecting stroma lamellae in the chloroplasts of both WT and mutant (not shown).

While these observations do not exclude the above alternative explanation or the possibility that the *Lemna* 1073 which harbors a nuclear, not yet well-defined mutation might have lost LHCII kinase protein(s) as well, the possibility that cyt b_6/f complex plays a specific role in the regulation of the LHCII kinase system remains an attractive working hypothesis to be pursued by further experimental work.

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