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BRIEF COMMUNICATION

Effects of high temperature exposure of spinach intact plants and isolated thylakoids on light-harvesting complex 2 protein phosphorylation

M. SATPATHY and P. MOHANTY*

School of Life Sciences, Jawaharlal Nehru University, New Delhi-110067, India

Abstract

After a 6 min exposure of isolated thylakoids to 43 °C, the extent of phosphorylation of light-harvesting complex of photosystem 2 (LHC2) was higher than in control thylakoids kept at 25 °C. Similarly, the exposure of intact spinach plants to 43 °C in dark for 11 h induced higher extent of thylakoid LHC2 phosphorylation than in control plants kept at 25 °C. The induced ability of LHC2 for enhanced phosphorylation may enable better energy distribution in favour of photosystem 1.

Additional key words: photosystem 2, *Spinacia oleracea*.

In higher plant chloroplasts, radiant energy is predominantly absorbed by both photosystem 1 (PS1) which is mostly present in stroma thylakoids and by PS2 which is located mostly in grana thylakoids. The unequal energy absorption by the two photosystems creates an imbalance in the energy distribution between the photosystems (Whitmarsh and Govindjee 1995). In higher plants, the PS2 which contains much of the chlorophyll (Chl) *b* in its light-harvesting complex (LHC2), acts as an antenna for funneling excitation energy to PS2 reaction centres. Also, the energy absorbed by PS2 antenna is distributed between the two photosystems for optimal rates of electron flow (Ghanotakis and Yocum 1990). This distribution of excitation energy is controlled by redox dependent protein phosphorylation (Allen *et al.* 1981). The reduced state of plastoquinone (PQH_2) activates a kinase which phosphorylates LHC2. Upon phosphorylation, a fraction of LHC2 separates from PS2 core in the appressed membranes and migrates to the non-appressed thylakoid regions (Larsson *et al.* 1983, Staehelin and Arntzen 1983).

Temperature affects the migration of LHC2 (Bukhov and Mohanty 1999). PS2 and a portion of its LHC2 migrate into the stroma thylakoids leaving mainly free LHC2 behind in the appressed membranes (Larsson *et al.* 1987). Besides temperature, other stress factors (*e.g.* herbicide *SAN 9785* in pea) affect the LHC2 migration from appressed to non-appressed region in the chloroplast (Joshi *et al.* 1995).

Most studies on the effect of elevated temperature on thylakoid membranes have been carried out in *in vitro* systems (isolated chloroplasts or PS2 enriched membranes). Therefore we report here the *in vivo* heat induced enhancement in LHC2 protein phosphorylation in comparison with *in vitro* heat treated samples.

Spinach (*Spinacia oleracea* L.) leaves were harvested from 4 to 6-week-old plants grown in experimental garden. Plants were uprooted and washed thoroughly with water. The plants were kept in the Hoagland's half strength nutrient solution and esposed to heat treatment (43 or 48 °C) for 11 h in the dark. Control plants were kept at temperature 25 °C and relative humidity 60 % in the dark for the same time period.

Thylakoids from control and heat treated spinach leaves were isolated according to Nakatani and Barber (1977). The fresh leaves were cut into small pieces and homogenised in ice-chilled isolation buffer (buffer A)

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Abbreviations: Chl - chlorophyll; LHC2 - light harvesting complex of photosystem 2; PS - photosystem; ATP - adenosine triphosphate ; SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis.

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*author for correspondence; fax:(+ 91) 11 616 5886, e-mail: pmohanty@jnuiv.ernet.in

containing 0.4 M sorbitol, 15 mM tricine (pH 7.8), and 5 mM $MgCl_2$. The homogenate was filtered through four layers of miracloth and centrifuged at 3 000 *g* for 5 min. The supernatant was discarded and the pellet was washed in buffer B containing 10 mM tricine (pH 7.8), 10 mM NaCl and 5 mM $MgCl_2$. After a repeated centrifugation at 3 000 *g* for 5 min, the pellet was resuspended in the buffer C containing 0.1 M sorbitol, 10 mM NaCl and 5 mM $MgCl_2$. Heat treatment (43 or 48 °C) was given for 6 min in a *Julabo F-10* water bath (Type *UC/5*, Seelbach, Germany) to isolated thylakoids.

Thylakoids were then diluted with buffer C to a Chl concentration of 400 μg cm^{-3}. Reaction mixture included 200 μM cold ATP and 20 mM freshly prepared NaF for the phosphorylation of LHC2. For non-phosphorylated preparation, sample tubes were wrapped completely to prevent any irradiation. Phosphorylation was then induced by transferring sample tubes under 'white light' (400 μmol m^{-2} s^{-1}) at room temperature for 20 min (Larsson *et al.* 1987). The irradiance was attenuated using different neutral density filters (*Balzers*, USA).

To label thylakoid membranes with ^{32}P, they were incubated separately with 0.4 mM [γ-^{32}P] ATP and 20 mM NaF. Reaction of both phosphorylated and non phosphorylated samples was terminated at 60 °C. Then equal volume of sample buffer was added, the sample was boiled for 3 min and then centrifuged in *Eppendorf Minifuge* (*RM12C*, *REMI*, Bombay, India) for 1 min. Sample equivalent to 20 μg of Chl was loaded on 12.5 % SDS-PAGE. Electrophoresis was performed for 12 - 14 h at a constant voltage (70 V for stacking and 100 V for separating gel) on 1 mm thick 12.5 % polyacrylamide gel by the buffer system of Laemmli (1970). After electrophoresis, the gels were stained with Coomassie brilliant blue R-250, destained for the visualisation of actual position of 29 kDa molecular mass protein (LHC2 protein) in our spinach sample. For radiolabelled samples, the gels were dried and amount of radioisotope present in the 29 kDa protein was achieved by using X-ray sensitive film (*Phosphoimager Fuji* film *BAS-1800*). High specific activity [γ-^{32}P] ATP (18.5 × 10^{13} Bq $mmol^{-1}$) was obtained from the *Radiochemical Centre* (*Amersham*, Bucks, UK). Chl concentration was measured in 100 % dimethyl formamide as given by *Porra et al.* (1989).

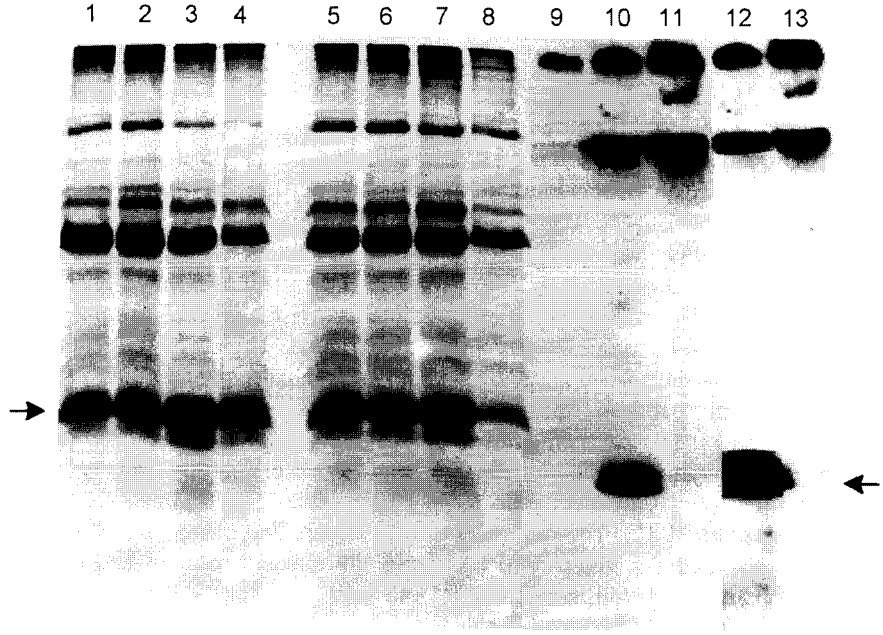

Fig. 1. Polypeptide profiles (SDS PAGE) for spinach samples exposed *in vivo* (*lanes 1 - 4, 9 - 13*) or *in vitro* (*lanes 5 - 8*) to temperature of 25 (*lanes 1, 2, 5, 6, 9 - 11*), 43 (*lanes 3, 7, 12*) or 48 (*lanes 4, 8, 13*) °C and then kept in dark (*lanes 1, 5, 11, 13*) or light (*lanes 2 - 4, 6 - 8, 12*) for 20 min (*lanes 1 - 8, 10* - 13). 200 μM ATP and 20 mM NaF was added to samples *1* to *8*. Some samples (lanes *9* to *13*) were phosphorylated in the presence of 0.4 mM [γ-^{32}P]ATP and 20 mM NaF.

The SDS-PAGE profiles for samples exposed for 11 h to elevated temperature *in vivo* (Fig. 1, lanes 1 to 4) showed that the presence of ATP did not change the band intensity in light or dark but a small increase in the band intensity at 29 kDa protein was seen in the sample heated at 43 °C (lane 3). In a parallel experiment with isolated thylakoids exposed to heat treatment for 6 min *in vitro* (Fig. 1, lanes 5 to 8) ATP did not change the band intensity but an increase in the band intensity was evident at 29 kDa protein in the sample heated at 43 °C (lane 7). The results suggest that the increase in band intensity may be associated with phosphorylation.

In the presence of 0.4 mM [γ-^{32}P]ATP and 20 mM NaF, isolated spinach thylakoids exposed in light

(400 μmol m^{-2} s^{-1}) for 20 min incorporated radioisotope into several membrane polypeptides. Single polypeptide of LHC2 (29 kDa) was the most heavily labelled phosphoprotein in both control and 43 °C heated samples (Fig. 1, *lanes 10* and *12*, respectively). However, isolated thylakoids of control samples and after 20 min in dark showed no phosphorylation (*lanes 9* and *11*). *Lane 13* represent the isolated thylakoids from the leaf of 43 °C heated sample incubated for 20 min in dark but showed significant enhancement of LHC2 phosphorylation in light for 20 min (*lane 12*) as compared with that of control sample (*lane* 9). Thus the incorporation of [γ-^{32}P]ATP was temperature dependent.

Further we checked the kinetics of both *in vivo* and *in vitro* heat-treated samples at 43 °C because significant enhancement of LHC2 was observed at this particular temperature. Both the samples showed rapid phosphorylation during first 20 min of sample irradiation but thereafter the amount of LHC2 declined (Table 1).

The decline in the amount of LHC2 after 20 min could have been due to the inactivation of kinase, reduction in the number of sites remaining to be phosphorylated, or concomitant dephosphorylation by the phosphoprotein phosphatase. At high temperature above 45 °C, the normal thylakoid structure is usually lost. The membranes become highly convoluted and appear to break down to form a mass of small fuse vesicles (Gounaris *et al.* 1984).

The *in vitro* phosphorylation of LHC2 in spinach has already been reported (Larsson *et al.* 1987) but the study of effect of heat exposed to intact plants is of physiological importance. Our results showed that elevated temperature enhanced LHC2 phosphorylation either because of activation of kinase or by supression of phosphatase activity. Our earlier findings showed that exposure to high temperature enhances the LHC2 migration from grana to stroma lamellar region (Joshi *et al.* 1995). The present results demonstrate that also the *in vivo* heat treatment causes enhanced phosphorylation of LHC2 and its consequent migration to the stroma lamellae.

Table 1. The kinetics of [γ-^{32}P] incorporation to LHC2 in the thylakoids isolated from 43 °C *in vivo* or *in vitro* heat-treated samples.

Time [min]	*In vivo* heat treated [count s^{-1}]	*In vitro* heat treated [count s^{-1}]
10	334	340
20	347	348
30	239	300
40	88	185

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