

Radiation inactivation analysis of thylakoid protein kinase systems in light and in darkness

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Abstract

Chloroplast thylakoid contains several membrane-bound protein kinases that phosphorylate thylakoid polypeptides for the regulation of photosynthesis. Thylakoid protein phosphorylation is activated when the plastoquinone pool is reduced either by light-dependent electron flow through photosystem 2 (PS2) or by adding exogenous reductants such as durohydroquinone in the dark. The major phosphorylated proteins on thylakoid are components of light-harvesting complex 2 (LHC2) and a PS2 associated 9 kDa phosphoprotein. Radiation inactivation technique was employed to determine the functional masses of various kinases for protein phosphorylation in thylakoids. Under the photosynthetically active radiation (PAR), the apparent functional masses of thylakoid protein kinase systems (TPKXs) for catalyzing phosphorylation of LHC2 27 and 25 kDa polypeptides were 540 ± 50 and 454 ± 35 kDa as well as it was 448 ± 23 kDa for PS2 9 kDa protein phosphorylation. Furthermore, the functional sizes of dark-regulated TPKXs for 25 and 9 kDa proteins were 318 ± 25 and 160 ± 8 kDa. The 9 kDa protein phosphorylation was independent of LHC2 polypeptides phosphorylation with regard to its TPKX functional mass. Target size analysis of protein phosphorylation mentioned above indicates that thylakoid contains a group of distinct protein kinase systems. A working model is accordingly proposed to interpret the interaction between these protein kinase systems.

Additional key words: dark-regulated protein phosphorylation; functional mass; light-dependent protein phosphorylation; light-harvesting complex 2; photosystem 2.

Introduction

Protein phosphorylation plays an essential role in cellular signalling, gene expression, and metabolic regulation of living cells. Chloroplast thylakoid membranes presumably contain several distinct membrane-bound protein kinases that phosphorylate thylakoid proteins for the regulation of photosynthesis (Bennett 1977, Allen 1992). The kinases are activated when the plastoquinone (PQ) pool is reduced by light-dependent electron flow or by exogenous reductants in the dark (Aro and Ohad 2003, Zer and Ohad 2003, Allen 2005, Buchanan and Balmer 2005). The most apparently phosphorylated proteins are components of light-harvesting chlorophyll (Chl) complex 2 (LHC2), the 27 and the 25 kDa polypeptides,

and a minor protein of photosystem 2 (PS2) at 9 kDa (Larson *et al.* 1987). The phosphorylation of LHC2 was implicated in redistribution of excitation energy between two photosystems during state transition and in the protection against inhibition of PS2 by excessive excitation by photosynthetically active radiation (PAR) (Haldrup *et al.* 2001, Allen 2003, Vink *et al.* 2004). Phosphorylation of 9 kDa *psbH* gene product was suggested to be involved in an early event of photo-inhibition as its phosphorylation could lead to a stabilization of Q_A at PS2 in the light and cause the decrease in PS2 photochemical activity and electron transferring (Packham 1987, O'Connor *et al.* 1998).

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Abbreviations: DBMIB – 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCCD – dicyclohexylcarbodiimide; DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP – dichlorophenol-indophenol; DMBQ – 2,5'-dimethylbenzoquinone; DQH₂ – durohydroquinone; EDC – 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; FSBA – 5'-*p*-fluorosulfonyl-benzoyl adenosine; G₆PDH – glucose-6-phosphate dehydrogenase; LHC – light-harvesting complex; NEM – N-ethylmaleimide; PAR – photosynthetically active radiation; PC – plastocyanine; PQ – plastoquinone; PQH₂ – plastoquinone; PS – photosystem; Q_A – the primary quinone electron acceptor of PS2; TCA – trichloroacetic acid; TPKX – thylakoid protein kinase system.

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Thylakoid protein phosphorylation exhibits substrate specificity indicating multiple systems of protein kinases on the membranes. Two polypeptides of 38 and 25 kDa were firstly identified as protein kinases by photoaffinity labelling of 8-azido ATP (Lin *et al.* 1982). In accordance with the sensitivity to PAR it was shown that there were a light-dependent protein threonine kinase and a light-independent protein serine kinase on thylakoid membranes (Lucero *et al.* 1987, Vescovi and Lucero 1990). Later, a PS2 core complex associated protein of 58 kDa was suggested to be a kinase to phosphorylate both LHC2 polypeptides and PS2 proteins (Race and Hind 1996). Moreover, three putative thylakoid-associated kinases (TAKs) (molecular mass ~55 kDa) isolated from *Arabidopsis* were proved to interact with cytochrome *b₆* complex and PS2, and could phosphorylate threonine of LHC2 (Snyders and Kohorn 1999). A 60 kDa tyrosine protein kinase catalyzing LHC2 protein phosphorylation was considered to promote the transition to state-2 configuration in response to PAR favouring PS2 (Forsberg and Allen 2001). Currently, a chloroplast thylakoid-associated serine-threonine protein kinase STN7 of 85 kDa from

green alga *Chlamydomonas* was also identified to be involved in state transition (Depège *et al.* 2003, Bellafiore *et al.* 2005). Despite efforts, the exact identities of protein kinases and their control mechanism still remain to be determined (Allen and Race 2002, Aro *et al.* 2004, Allen 2005, Buchanan and Balmer 2005).

Radiation inactivation analysis has been used effectively to estimate the functional mass and thus to elucidate the structure-function relationship of many biological molecules and systems including ATP synthase (Wang *et al.* 1988, Ma *et al.* 1993), HIV-1 reverse transcriptase (Sluis-Cremer *et al.* 2003), photosynthetic electron transport chain (Pan *et al.* 1987), progesterone receptor (Wu *et al.* 2004), pyrophosphatase (Jiang *et al.* 2000), and vertebrate hyaluronan synthase (Pummill *et al.* 2001). In this work, we report the functional masses of thylakoid protein kinase system(s) (TPKXs) determined by radiation inactivation technique. From the analysis of functional masses, we demonstrated that the protein phosphorylation on thylakoid membrane represented a group of distinct TPKXs with different regulatory machinery. A working model is proposed accordingly.

Materials and methods

Materials: Fresh spinach (*Spinacia oleracea* L.) was provided by local market. [γ - 32 P]ATP (1.85×10^5 GBq mol $^{-1}$) was obtained from *Amersham Biosciences* (Buckinghamshire, England). Reduced duroquinone (durohydroquinone, DQH $_2$) was prepared according to Izawa and Pan (1977). All other chemicals of the highest reagent grade were purchased from *Sigma* (St. Louis, MO, USA) and *Merck* (Darmstadt, Germany), and used without further purification.

Isolation of thylakoid membranes: Thylakoid membranes were isolated as previously described (Izawa and Pan 1977), and finally re-suspended in the storage medium [25 mM HEPES-NaOH (pH 7.5), 10 mM NaCl, 10 % (v/v) glycerol, 2.5 % (v/v) dimethyl sulfoxide, 2 mM dithiothreitol, 2 kg m $^{-3}$ bovine serum albumin, and 2 kg m $^{-3}$ Chl].

Determination of Chl concentration and enzyme activities: Chl concentration was determined according to Arnon (1949). Enzyme activity of glucose-6-phosphate dehydrogenase (G $_6$ PDH) was measured as the rate of absorption change at 340 nm in the reaction medium [50 mM Tris-HCl (pH 7.8), 3 mM MgCl $_2$, 3 mM NAD $^+$, 3 mM glucose-6-phosphate, and 1 g m $^{-3}$ G $_6$ PDH].

Protein phosphorylation of thylakoid membranes was initiated by adding 0.5 mM [γ - 32 P]ATP (370 kBq) to 170 mm 3 reaction medium [50 mM Tricine-NaOH (pH 7.5), 0.1 M sucrose, 10 mM NaCl, 5 mM MgCl $_2$, and 200 g(Chl) m $^{-3}$ of thylakoid membranes]. The light-

dependent protein phosphorylation was carried out under PAR (1 200 W m $^{-2}$) for 2 min at 25 °C. For dark phosphorylation, 1 mM DQH $_2$ was added to activate the protein kinases at 25 °C for 15 min in the dark. The reaction was terminated by 25 mM NaF and 5 % (v/v) ice-cold trichloroacetic acid (TCA). After centrifugation at 10 000 \times g for 1 min, the pellet was washed twice with 1 cm 3 of 2 % (v/v) ice-cold TCA. The thylakoid proteins were re-suspended in 60 mm 3 sample buffer [62.5 mM Tris-HCl (pH 6.8), 2 % (m/v) SDS, 0.25 % (v/v) 2-mercaptoethanol, 0.0125 % (m/v) bromophenol blue, and 1 % (v/v) glycerol] and ready for SDS-PAGE and autoradiography.

For protein phosphorylation of exogenous substrates by thylakoid membranes, casein and histones II-AS (0.4 kg m $^{-3}$) were incubated in the reaction medium [50 mM Tricine-NaOH (pH 7.5), 0.1 M sucrose, 10 mM NaCl, 5 mM MgCl $_2$, 25 mM NaF, 0.5 mM [γ - 32 P]ATP (185 kBq), and 258 g(Chl) m $^{-3}$ of thylakoid membranes] at 25 °C for 15 min in the dark. The reaction was terminated by 5 % (v/v) ice-cold TCA. Thylakoid membranes were then spun down by micro-centrifuge and the supernatant was subjected to scintillation counting.

For inhibition studies, 0.5 mM dicyclohexylcarbodiimide (DCCD), 0.5 mM 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC), 0.6 mM 5'-*p*-fluoro-sulfonyl-benzoyladenine (FSBA), and 0.75 mM N-ethylmaleimide (NEM) were added into the above reaction medium to determine the activities of thylakoid protein kinases under the same conditions.

SDS-PAGE and autoradiography: Electrophoretic separation of thylakoid membrane proteins was performed on 15 % (m/v) polyacrylamide slab gel. The gels were stained with Coomassie blue or with silver stain and then subjected to autoradiography at -70°C . Quantification of protein phosphorylation was carried out using an *Ultrosan XL*-Enhanced Laser Densitometer with an analytical software (*GelScan XL, Pharmacia LKB*).

Irradiation was performed with a ^{60}Co irradiator (approximately 1.11×10^6 GBq) and the dose rate was 7.00×10^{-3} Mrad s^{-1} determined as previously described (Pan *et al.* 1987, Wang *et al.* 1988, Ma *et al.* 1993, Tzeng *et al.* 1996, Jiang *et al.* 2000, Wu *et al.* 2004). Internal

Results

PAR-dependent protein phosphorylation of thylakoid membrane: Under PAR exposure, thylakoid membrane proteins were phosphorylated in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The phosphoproteins of thylakoid membrane could be visualized by autoradiography (Fig. 1). The major phosphoproteins of LHC2 27 kDa (P27), LHC2 25 kDa (P25), and the PS2 9 kDa (P9) proteins were radioactively labelled beside two unidentified polypeptides of 18 kDa (P18) and 16 kDa (P16). Exposure of the thylakoid membranes to γ -rays resulted in inhibition of protein phosphorylation (Fig. 1, *first lane from left*). The apparent degree of inactivation of thylakoid kinase activities followed a simple exponential decrease with increasing dosages (Fig. 2). Semi-logarithmic plots of relative activities *versus* irradiation doses gave the D_{37} values of 1.36 ± 0.13 , 1.61 ± 0.12 , and 1.69 ± 0.08 Mrad for γ -radiation inactivation of thylakoid protein kinase system(s) (TPKXs) catalyzing the 27, 25, and 9 kDa polypeptides phosphorylation, respectively. Consequently, the functional masses of the light-dependent TPKX₂₇ and TPKX₂₅ were calculated as 540 ± 50 kDa and 454 ± 35 kDa as well as that of TPKX₉ was 448 ± 23 kDa (Table 1). These values were further subtracted the molecular mass of endogenous protein substrates which were also irradiated. Obviously, the functional masses of the TPKXs to phosphorylate 27, 25, and 9 kDa proteins were different, indicating that these polypeptides were possibly phosphorylated by distinct protein kinase systems.

Dark-regulated protein phosphorylation of thylakoid membrane: The major phosphoproteins resulting from dark-regulated phosphorylation were 25 and 9 kDa proteins (Fig. 3) after thylakoid membranes were activated by reductant DQH₂ in the dark. Upon irradiation by γ -ray, the level of protein phosphorylation was also decreased with increasing dosages. The D_{37} values for radiation inactivation of dark-regulated TPKX₂₅ and TPKX₉ were 2.25 ± 0.18 and 4.56 ± 0.24 Mrad, respectively (Fig. 4). Likewise, the functional masses of dark-

standardization of radiation exposure was carried out with G₆PDH. Both chloroplasts [$2 \text{ kg}(\text{Chl}) \text{ m}^{-3}$] and standard enzyme (200 g m^{-3}) were irradiated at $-18 \pm 2^{\circ}\text{C}$.

Functional size was calculated according to the equation of Beauregard and Potier (1985) as

$$\log m = 5.89 - \log D_{37,t} - 0.0028 t$$

where m is the molecular mass [Da], $D_{37,t}$ is the dose of radiation [Mrad] required to reduce the total activity to 37 % of that found in unexposed control at temperature t [$^{\circ}\text{C}$]. The molecular masses obtained were then corrected with that of G₆PDH as standard enzyme (McIntyre and Churchill 1985).

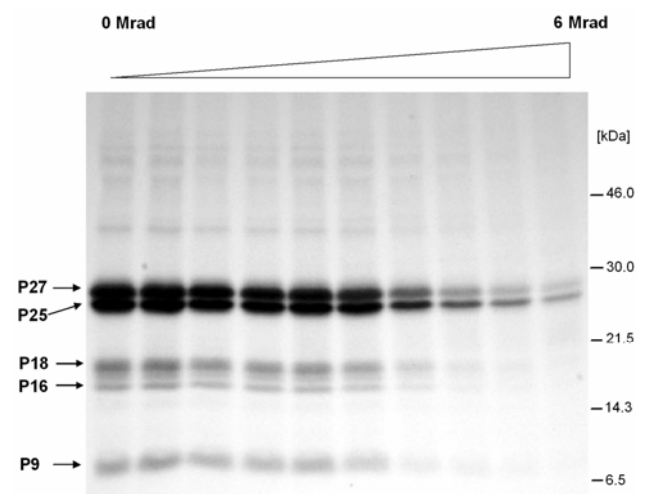


Fig. 1. PAR-dependent protein phosphorylation of thylakoid membranes after the exposure to various dosages of ^{60}Co . Thylakoid membranes were ^{60}Co affected at various dosages and sequentially PAR affected (1200 W m^{-2}) for 2 min at 25°C in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The samples were then subjected to SDS-PAGE and autoradiography as described under Materials and methods. It shows that the level of protein phosphorylation decreased with increasing irradiation doses.

regulated TPKX₂₅ and TPKX₉ were calculated as 318 ± 25 and 160 ± 8 kDa, respectively. The estimated functional masses were ascribed to those of protein kinases without the involvement of PAR-induced electron transfer apparatus. The functional masses of TPKX₂₅ and TPKX₉ were not identical in the light and in the dark.

Functional sizes of protein phosphorylation using exogenous substrates: Evidence showed that thylakoid membranes could phosphorylate several exogenous protein substrates such as casein and histones (Lucero *et al.* 1982, 1987).

Table 1. Summary of functional mass analysis of thylakoid protein kinase systems. Functional mass of thylakoid protein kinase system (TPKX) catalyzing protein phosphorylation was determined by radiation inactivation technique as described in Materials and methods.

TPKX/protein substrates	D ₃₇ [Mrad]	Functional size including protein substrate [kDa]	Functional size excluding protein substrate [kDa]
A. Light-dependent proteins			
TPKX ₂₇ /27 kDa	1.36±0.13	567±50	540±50
TPKX ₂₅ /25 kDa	1.61±0.12	479±35	454±35
TPKX ₉ /9 kDa	1.69±0.08	457±23	448±23
B. Dark-regulated proteins			
TPKX ₂₅ /25 kDa	2.25±0.18	343±25	318±25
TPKX ₉ /9 kDa	4.56±0.24	169±8	160±8
C. Exogenous substrates in darkness			
TPKX _{casein} /casein	4.20±0.18	—	210±9
TPKX _{histone} /histone II-AS	5.53±0.28	—	157±8

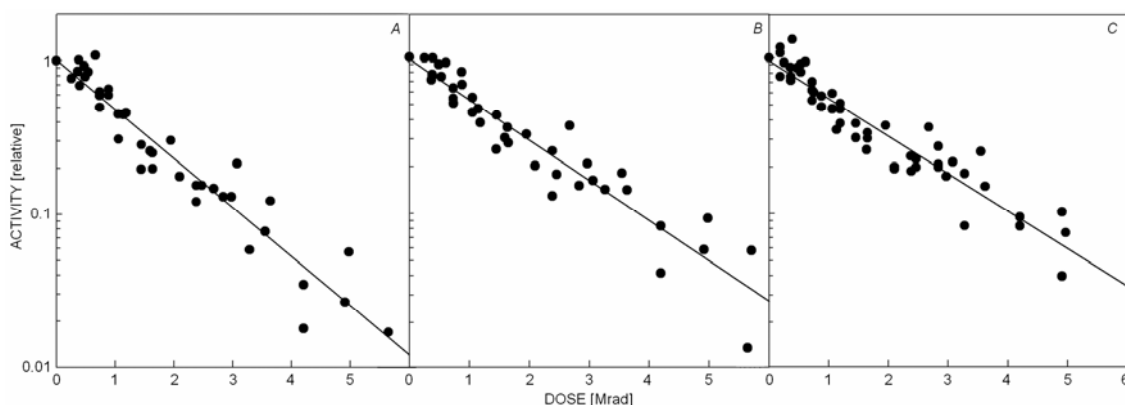


Fig. 2. Radiation inactivation of irradiation-dependent protein phosphorylation of thylakoid membranes. Relative phosphorylation of thylakoid 27 (A), 25 (B), and 9 (C) kDa proteins was plotted against various irradiation doses. Intersects at 37 % of control activities give D₃₇ values of 1.36±0.13, 1.61±0.12, and 1.69±0.08 Mrad, yielding functional masses of 540±50, 454±35, and 448±23 kDa for TPKX₂₇ (A), TPKX₂₅ (B), and TPKX₉ (C) systems, respectively.

Therefore, we investigated the functional mass of thylakoid protein kinases using these two substrates. To make the system simple, we only observed the dose responses of thylakoid protein kinase activity for casein and histones' phosphorylation at dark. Casein and histone II-AS were incubated with thylakoid in a medium containing [γ -³²P]ATP at dark and recovered in supernatant after centrifugation. Then the incorporated ³²P as protein kinase activities was determined by scintillation counter. As a result, the protein kinase activities for casein and histone II-AS phosphorylation decreased with increasing radiation dose (Fig. 5). The thylakoid protein kinases for casein and histone II-AS phosphorylation showed single exponential function of radiation dose. The lines were determined from least square fits of average from 10 assays ($r > 0.98$). From analysis of data using linear regression, the D₃₇ values of 4.20±0.27 and 5.53±0.28 Mrad were obtained for thylakoid protein kinases for casein and histone II-AS

phosphorylation, yielding functional masses of 210±11 (TPKX_{casein}) and 157±8 (TPKX_{histone}) kDa, respectively. The functional mass included the protein kinase core complex and their possible regulatory moiety, if any. However, no activator machinery was involved since the reaction was carried out at dark in the absence of reductant. The functional masses of these two protein kinases are similar to those using endogenous protein substrates.

Effect of inhibitors on light-dependent and dark-regulated protein phosphorylation using endogenous and exogenous substrates: An attempt was made to distinguish the induction of thylakoid protein phosphorylation reflecting the respective kinase systems in light and in darkness. We therefore measured the inhibition of FSBA, DCCD, EDC, and NEM on light-dependent and dark-regulated TPKXs using endogenous and exogenous substrates, as shown in Table 2.

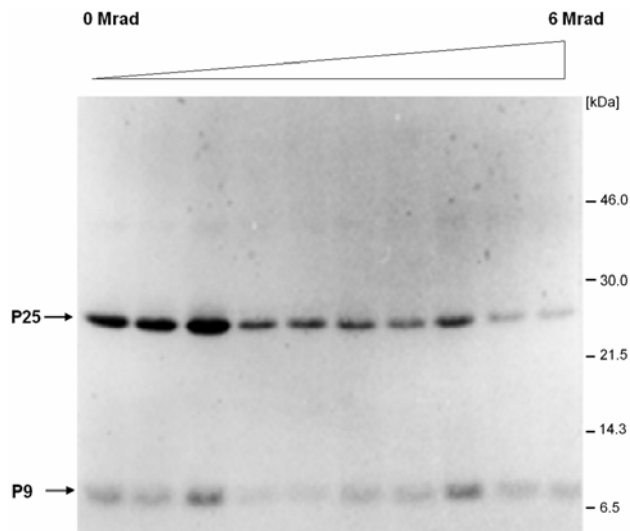


Fig. 3. PAR-dependent protein phosphorylation of thylakoid membranes activated by durohydroquinone in the dark. Experiments were carried out as indicated in Fig. 1 except the protein phosphorylation was performed in the presence of 1 mM durohydroquinone in the dark.

With addition of FSBA, the phosphorylation of LHC2 under PAR was almost completely abolished by 95 % while that for the 9 kDa phosphoprotein was inhibited by 35 %. However, the phosphorylation of histone II-AS was decreased by 45 % with addition of FSBA but that of casein was not significantly affected. The difference in

Discussion

Protein phosphorylation of thylakoid membrane in light and in darkness: Protein phosphorylation on thylakoid membrane has been investigated for years. Several membrane proteins of chloroplast thylakoids were phosphorylated under the control of the plastoquinone or thiol redox states in light or in darkness (Allen 1992, Jennings *et al.* 1996, Aro and Ohad 2003, Allen 2005, Buchanan and Balmer 2005). The most apparently phosphorylated proteins were components of LHC2, the 27 and 25 kDa polypeptides, and a minor protein of PS2 at 9 kDa (Rintamäki *et al.* 2000). The major effects of the phosphorylation on these components resulted in PS2 heterogeneity and then the control of excitation energy distribution between different types of reaction centres (Haldrup *et al.* 2001, Allen 2003, 2005).

There are many species of protein kinases and different control systems of protein phosphorylation on thylakoid membranes (Vener *et al.* 1998, Carlberg *et al.* 1999, Allen and Race 2002, Hou *et al.* 2003, Zer and Ohad 2003). However, the regulation of these multiple substrates and multiple protein kinases of protein phosphorylation in thylakoid membrane still awaits further understanding. Therefore, we are here to elucidate the identities of protein kinases and their control mechanism

the sensitivity of protein phosphorylation to FSBA indicated the existence of various TPKXs for the phosphorylation of the exogenous and endogenous substrates (Packham 1987). However, we cannot exclude the possibility that all the phosphorylation used the identical site with the involvement of structural and/or moiety regulatory.

The carboxyl group modifiers DCCD (Walters *et al.* 1994) and EDC (Armbrust *et al.* 1994) were also employed to distinguish whether these TPKXs are distinct. We found that the sensitivities of phosphorylation of LHC2 polypeptides and the 9 kDa phosphoprotein to DCCD and EDC were different from those of casein and histone II-AS (Table 2). Moreover, treatment of the thylakoid membrane with NEM (Rintamäki *et al.* 2000), which cross-links thiol groups of protein substrates, turned out a less extent of inhibition in protein phosphorylation. The irradiation-induced phosphorylation of LHC2 was inhibited by about 60 % by NEM but that of 9 kDa phosphoprotein was not influenced. The extent of histone II-AS phosphorylation was decreased by approximate 30 % by NEM, but that of casein phosphorylation was not changed (Table 2). This phenomenon was similar to the observation in the FSBA-treated thylakoids. The sensitivities to DCCD, EDC, and NEM of casein and histone II-AS phosphorylation differed from those of LHC2 and 9-kDa phosphorylation, indicating that the TPKXs phosphorylating endogenous and exogenous substrates were distinct.

using radiation inactivation analysis. The functional masses of light-dependent TPKX₂₇, TPKX₂₅, and TPKX₉ were 540±50, 454±35, and 448±23 kDa, respectively (Table 1). These values are larger than the molecular masses of purified protein kinases (25–85 kDa) identified by other workers (Lin *et al.* 1982, Race and Hind 1996, Snyders and Kohorn 1999, Depège *et al.* 2003). Thus, the functional unit of TPKX probably includes the masses for thylakoid protein kinase (TPK) *per se*, and the activation machinery (X) that contains activators, associated regulators, and structural proteins. The various functional masses could be explained as the involvement of sequential protein phosphorylation or different activation pathways. The protein kinases for LHC2 polypeptides and PS2 9 kDa protein were verified to be discrepant by the selective inhibitors such as FSBA and NEM (Table 2). These protein kinase systems might share the same electron transport chain but possess different activation machinery.

The protein phosphorylation could be activated by exogenous reductants in the dark, excluding the involvement of the light-dependent electron flow through PS2. The dark phosphorylation of 25 and 9 kDa proteins is probably processed to the reorganization of LHC2 in

the membranes during state transition, or the down-regulated activation of other thylakoid kinases after the irradiation exposure of the complex (Zer *et al.* 2003). The functional mass analysis of kinase systems under light-independent conditions gave the different target sizes of TPKX₂₅ and TPKX₉ (Table 1). It was suggested that the TPKX₂₅ and TPKX₉ were activated through electron flow *via* PS2 either dependent or independent pathways. In contrast, the phosphorylation of 27 kDa polypeptide was

not observed in the dark, suggesting either TPKX₂₇ was merely activated by the electron flow *via* PS2 dependent pathways by other control system. Jennings *et al.* (1986) and Aro and Ohad (2003) reported that the 27 kDa polypeptide can be phosphorylated in the dark by ferredoxin-NADPH system. We therefore propose that the activation of TPKX₂₇ does not necessarily directly require the complete reduction of PQ pool but may be regulated by thiol redox control system.

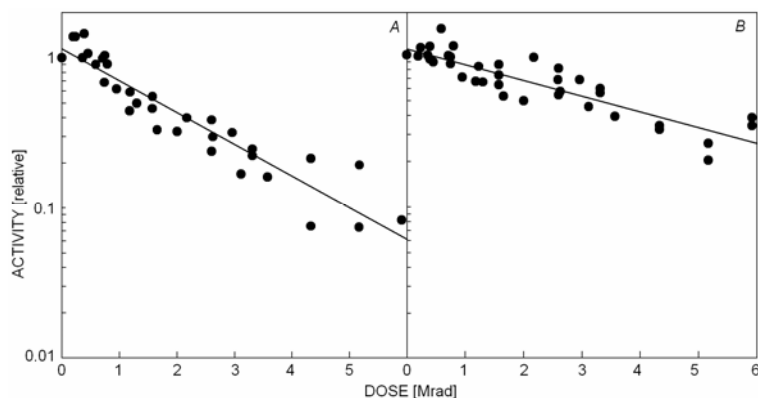


Fig. 4. Radiation inactivation of light-independent protein phosphorylation of thylakoid membranes. Relative phosphorylation of thylakoid 25 (A) and 9 (B) kDa proteins was plotted against various irradiation doses. Intersects at 37 % of control activities give D_{37} values of 2.25 ± 0.25 and 4.56 ± 0.24 Mrad, yielding functional masses of 318 ± 25 and 160 ± 8 kDa for TPKX₂₅ (A) and TPKX₉ (B) systems, respectively.

Table 2. Effect of inhibitors on the light-dependent and dark-regulated TPKXs in thylakoid membranes. Activities of TPKXs catalyzing endogenous and exogenous protein substrates phosphorylation were determined as described in Materials and methods.

Treatment	Light		Dark	
	Endogenous substrates LHC2	9 kDa	Exogenous substrates Casein	Histone II-AS
Activity [mmol(P) _i kg ⁻¹ (Chl)]	0.28	0.10	0.36	0.60
	Inhibition [%]			
+ FSBA	95	35	0	45
+ DCCD	100	100	45	49
+ EDC	100	35	24	25
+ NEM	60	0	0	30

The difference in functional masses between the light-dependent and dark-regulated TPKX₂₅ and TPKX₉ were calculated as about 136 ± 60 and 288 ± 31 kDa, respectively. This difference represents the sum in molecular mass of electron transport chain through PS2 and protein components around redox regulation site, if any. However, the functional mass of electron transfer chain from H₂O to 2,5'-dimethylbenzoquinone (DMBQ) [*i.e.* H₂O → PS2 → PQ] was measured previously as 174 ± 11 kDa (Pan *et al.* 1987). The difference in molecular size mentioned above is either larger than or close to this value. Thus the activation machinery and/or structural component for protein kinases that phosphorylate LHC2 polypeptides and the 9 kDa protein may not be the same. An extra moiety (approximately in molecular

mass of 106 kDa) to link electron transport through PS2 and regulatory site for protein phosphorylation of LHC2 may be required. The role and identification of this extra moiety still remains to be determined.

Nevertheless, an increasing amount of recent evidence suggests that the moieties of TPKX_{LHC2} and TPKX₉ are distinct and both protein kinase systems operate independently. For instance, several reagents such as DBMIB (PQ antagonist at the Q_o site of cytochrome *b_f* complex) (Zito *et al.* 1999, Yang *et al.* 2001), DCMU (an inhibitor of PS2) (O'Connor *et al.* 1998), FSBA (adenosine analogue) (Farchaus *et al.* 1985), NaF (a phosphatase inhibitor) (Ebbert and Godde 1994), NEM (a reagent reactive to thiol group of protein substrates) (Rintamäki *et al.* 2000), as well as EDC (carboxylation

modifier for protein substrates) (in this study) exhibited preferential inhibition of phosphorylation of LHC2 over the 9 kDa phosphoprotein. In order to clarify whether the functional mass was sensitive to inhibitors, we also estimated the functional masses of $\text{TPKX}_{\text{LHC2}}$ and TPKX_9 under irradiation with addition of FSBA which preferentially inhibits $\text{TPKX}_{\text{LHC2}}$ but does not significantly affect TPKX_9 . We found that the apparent functional mass of $\text{TPKX}_{\text{LHC2}}$ under irradiation was about 618 ± 8 kDa and that of TPKX_9 was 456 ± 22 kDa, irrespective of the presence or absence of FSBA (data from one experiment). That is, the presence of FSBA has no significant effect on the measurement of TPKX functional mass, indicating that the utilization of the radiation inactivation technique is feasible.

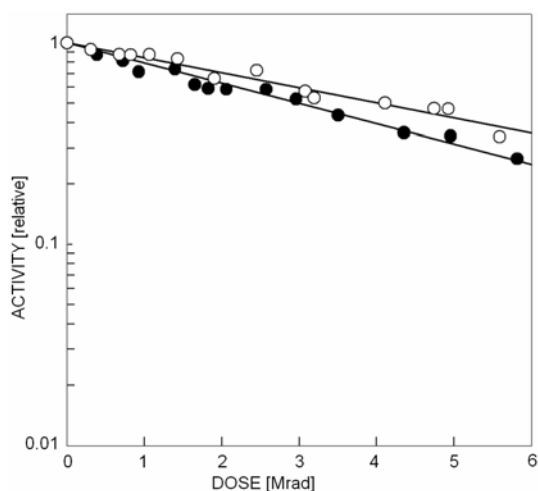


Fig. 5. Radiation inactivation of protein phosphorylation of thylakoid membranes using exogenous substrates. Relative phosphorylation of casein (●) and histone-II-AS (○) was plotted against various irradiation doses. Intersects at 37 % of control activities give D37 values of 4.2 ± 0.24 and 5.53 ± 0.25 Mrad, yielding functional masses of 210 ± 8 kDa ($\text{TPKX}_{\text{casein}}$) and 157 ± 25 kDa ($\text{TPKX}_{\text{histone}}$), respectively.

Though the LHC2 protein kinase is specific for threonine residues in endogenous substrate (Michel *et al.* 1988), several thylakoid serine protein kinases could be purified by extraction with detergents and phosphorylate a number of exogenous substrates such as casein and histone II-AS in light and in darkness (Cortez *et al.* 1987). From our results, the functional masses for dark phosphorylation of casein and histone II-AS exogenous substrates were determined as 210 ± 9 kDa ($\text{TPKX}_{\text{casein}}$) and 157 ± 8 kDa ($\text{TPKX}_{\text{histone}}$) (Table 1). We therefore believe that protein kinases for endogenous and exogenous substrates should be different species of kinase complexes on thylakoids.

Other phosphoproteins: There are two unidentified light-dependent phosphoproteins of about 18 and 16 kDa as shown in Fig. 1. There is little information to verify 18 and 16 kDa phosphoproteins but there are a few possible candidates. They might be low-molecular-mass LHC proteins as several phosphopolypeptides of LHC P11, LHC P13, and LHC P17 observed in green alga *C. reinhardtii* in a state-2 configuration during state transition (Ebbert and Godde 1994). Or they might be the subunit V (about 15 kDa) of cytochrome *b₆f* which was able to undergo reversible phosphorylation upon state transition (Hamel *et al.* 2000). Another possible candidate might be a newly identified light-induced phosphorylated *PsaD* protein (18 kDa) in PS1 (Hansson and Vener 2003).

Model for activation of protein kinase systems: Taken together, we propose a working model concerning the activation pathway of various protein kinase systems of thylakoid membrane (Fig. 6). Irradiation causes electron flow through PS2 and progressively results in the reduction of PQ. The PQ could also be reduced by exogenous DQH_2 . The PQH_2 directly or indirectly activates membrane-bound proteins *in situ*, and sequentially phosphorylates protein substrates through some different unknown pathways. The proposed apparatus components

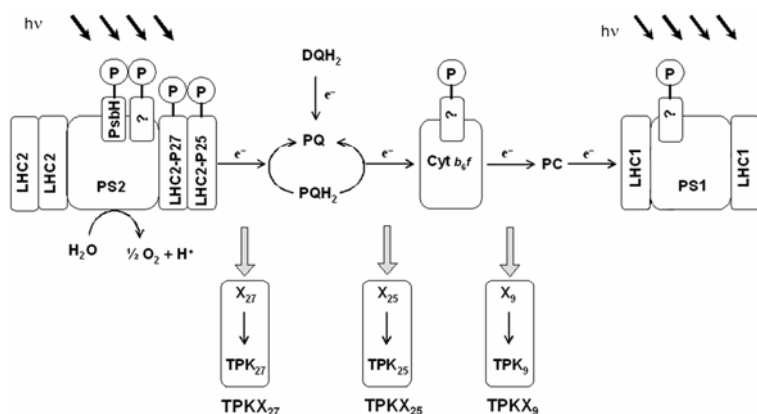


Fig. 6. Proposed model for the protein kinase systems in thylakoid membranes. PQ: plastoquinone; PQH_2 : hydroplastoquinone; DQH_2 : durohydroquinone. TPKX_{27} , TPKX_{25} , and TPKX_9 : proposed protein kinase systems for 27, 25, and 9 kDa proteins of thylakoid membranes, respectively. X_{27} , X_{25} , and X_9 : proposed activation machinery components involved in the protein phosphorylation of 27, 25, and 9 kDa proteins of thylakoid membranes, respectively.

TPKX₂₇, TPKX₂₅, and TPKX₉ are specific for their distinct target substrates of the 27, 25, and 9 kDa polypeptides, respectively. X₂₇, the activation machinery that contains activators, regulators, and structural proteins for catalyzing the LHC2 27 kDa polypeptide phosphorylation, locates before PQ pool. X₂₅, the activation

machinery for catalyzing the LHC2 25 kDa polypeptide phosphorylation, locates after PQ pool. X₉, the activation machinery for catalyzing the PS2 9 kDa polypeptide phosphorylation, locates after Cyt *b₆f*. The exact structure of the proposed apparatus and mechanism of protein phosphorylation remain to be determined.

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