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Phosphorylation of thylakoids and isolated subthylakoid vesicles derived from different structural domains of the thylakoid membrane from spinach chloroplast

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Abstract

(1) Thylakoids from spinach chloroplasts were phosphorylated, fragmented by sonication, and then fractionated by aqueous two-phase partitioning to yield membrane fragments, deriving from different structural domains of the membrane: grana, grana margins, grana core and stroma lamellae. The photochemical activities of PS Iα and PS IIα, located in the grana, and PS Iβ and PS IIβ, located in the stroma lamellae, were compared for phosphorylated and control thylakoids. The antenna size (reflected by the K_m value) and maximum activity (V_{max}) of PS IIα declined by 19 and 23%, respectively, while for PS IIβ the antenna size and V_{max} decreased by 4 and 12%, respectively. No significant changes in antenna size were detected for either grana PS Iα or stroma lamellae PS Iβ. Counter-current distribution was used for the quantitative separation of grana and stroma lamellae vesicles. Upon phosphorylation, the stroma lamellae fraction increased from 30% to 35% of the total, based on total absorbance at 680 nm. This increase can be explained by partial unstacking of the grana periphery and appressed membranes near the fret regions. Portions of the previously stacked membranes can therefore break and separate with the stroma exposed membrane. In addition, since the grana margins contain PS Iα (with 40% larger antennae than PS Iβ), which is functionally connected to LHC II, it is to be expected that some of these PS Iα units will also enter the stroma lamellae fraction and thus help contribute to a lower chlorophyll a/b ratio and a small increase in the average PS I antenna size of the stroma lamellae fraction from phosphorylated thylakoids. It is concluded that the incidence of partial destacking of the grana, which occurs due to the phosphorylation of LHC II and PS II polypeptides, may promote the exposure of the granal PS Iα centers to the aqueous stroma and increase cyclic electron flow around Photosystem I and thereby ATP production over NADPH production. (2) Subthylakoid vesicles, representing the different structural domains, were also examined for their properties following an incubation in presence of light and ATP. Phosphorylation of membrane proteins including LHC II and PS II associated polypeptides was observed in membrane fractions deriving from the grana lamellae and, to a lesser extent, the grana core. Three unidentified polypeptides of 15, 20 and 22 kDa were the most abundantly labeled polypeptides in the stroma lamellae fraction. No membrane proteins became phospho-labeled in the grana margin fraction.

Keywords: Thylakoid membrane organization; Grana margin; Membrane domain; Protein phosphorylation; Aqueous two-phase partitioning; LHC II protein kinase

1. Introduction

It is now well established that each of the two types of photosystem in spinach chloroplasts is heterogeneous with respect to antenna size. We now recognize the existence of at least two sub-types, designated as α and β, for both PS I and PS II, which can be distinguished by the different amounts of light-harvesting antenna chlorophyll a/b binding protein (LHC II) funneling light energy to the reaction center. The α centers, PS Iα and PS IIα, contain more LHC II and have a larger antenna size as compared to the β type photocenters, PS Iβ and PS IIβ [1–4].

The α and β photosystems are segregated from one another in the thylakoid membrane, a structure which consists of both an appressed domain, the grana partitions, and non-appressed domains, the stroma lamellae, grana

Abbreviations: PS, Photosystem; Chl, chlorophyll; CCD, counter-current distribution; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel; PBQ, phenyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; cyt, cytochrome; MV, Methyl viologen; DMSO, dimethylsulfoxide; P700, reaction center of PS I. ; CP43, PS II (psbC) polypeptide; D1 and D2, PS II reaction center polypeptides; LHC II, Light harvesting complex II.

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end membranes and grana margins. PS IIα and PS Iα are located in the grana core and grana margins, respectively, while PS IIβ and PS Iβ are located in the stroma lamellae [1–5].

It has been suggested that the two photosystems in the grana, PS IIα and PS Iα, perform an oxygenic non-cyclic electron transport, whereas the site of cyclic electron transport is located in the stroma lamellae [5]. Although the PS IIα and PS Iα are segregated in the grana, where PS Iα is located in the peripheral annulus of the grana disc and PS IIα in the core, there is a large contact surface between the two domains which, according to the model, should allow for an efficient electron transport between the two photosystems via the electron carriers; plastoquinone, plastocyanin and the cytochrome *b/f* complex.

Changes in the environmental conditions, such as light intensity, influence the organization of the photosynthetic apparatus within the thylakoid membrane. For example, the over-excitation of PS II (or PS I) is thought to induce a redistribution of the light-harvesting antennae (LHC II) between the two photocenters. Therefore, at low light intensities the excitation energy reaching the two photosystems is in a constant and dynamic equilibrium [6,7]. In addition, redistribution of the cytochrome *b/f* complex between grana and stroma lamellae has been suggested to act as a mechanism for augmenting or attenuating (depending on the light conditions) the ATP/NADPH ratio at different light intensities [8]. The reversible phosphorylation of several thylakoid membrane proteins is assumed to be the key biochemical mechanism for regulation of energy capture, electron transport and production of ATP and NADPH (see [9] for a review).

In this paper we use two approaches to study the effect of protein phosphorylation on the domain organization of the thylakoid membrane. Firstly, intact thylakoids were phosphorylated and then fragmented in order to isolate subthylakoid fragments representing the different structural domains of the thylakoid, the stroma lamellae, the grana core and the grana margins. Secondly, non-phosphorylated thylakoids were fragmented, the sub-thylakoid fragments isolated and examined for their respective abilities to undergo membrane protein phosphorylation. We show here that certain subthylakoid membrane fractions can also become phosphorylated when illuminated in the presence of ATP.

2. Materials and methods

2.1. Chemicals

Dextran 500 was obtained from Pharmacia (Uppsala, Sweden). Poly(ethylene glycol) 4000 (Carbowax PEG 3350) was purchased from Union Carbide (New York, NY, USA). [γ-^{32}P]ATP was prepared as described in Ref. [10]. Ferredoxin from spinach [F3013] and NADPH was supplied from Sigma Chemical Company (St. Louis, MO, USA).

2.2. Chloroplast isolation

Spinach (*Spinacia oleracia L.*) was grown at 20°C with a light period of 12 h and incident light intensity of 300 μE m^{-2} s^{-1}. The spinach leaves were dark-adapted 24 h prior to chloroplast isolation. The spinach chloroplasts were isolated in 50 mM sodium phosphate buffer (pH 7.4), 5 mM $MgCl_2$, 300 mM sucrose and osmotically broken in 5 mM $MgCl_2$ as described in [1]. The thylakoids were washed twice in 10 mM Tricine (pH 7.4), 5 mM $MgCl_2$, 300 mM sucrose, once in 10 mM sodium phosphate buffer (pH 7.4), 5 mM NaCl, 1 mM $MgCl_2$, 100 mM sucrose and resuspended in the same medium to a chlorophyll concentration of 4 mg/ml.

When intact thylakoids were phosphorylated, the final wash was carried out in the 'illumination medium' comprised of 10 mM sodium phosphate buffer (pH 7.4), 5 mM NaCl, 5 mM $MgCl_2$, 10 mM NaF and 100 mM sucrose. The thylakoids were then resuspended in the same medium to a chlorophyll concentration of about 1 mg chlorophyll/ml prior to illumination.

2.3. Light-induced protein phosphorylation of intact thylakoids

The thylakoid suspension (1 mg chlorophyll/ml) was incubated for 15 min at 18°C in the illumination buffer with [γ-^{32}P]ATP, at a final concentration of 0.2 mM, 20 μCi/ml. The incubation took place in a shallow, horizontal Plexiglas tray (20 ml of sample in a 3.5 cm wide and 25 cm long tray) with illumination from above (200 μE m^{-2} s^{-1} white light) under continuous mixing. After the illumination, the thylakoids were washed twice in non-radiolabelled illumination buffer and resuspended to 4 mg chlorophyll/ml in the same medium prior to addition to the polymer mixture as described in Section 2.4. The control thylakoids were illuminated in the absence of ATP.

2.4. Membrane fragmentation by sonication followed by two-phase partitioning

2 g of the thylakoid suspension (4 mg chlorophyll/ml) were added to 9.66 g of a polymer mixture to give the final concentrations: 5.7% (w/w) Dextran T500, 5.7% (w/w) PEG 4000, 10 mM sodium phosphate buffer (pH 7.4), 5 mM NaF, 1.6 mM $MgCl_2$, 1 mM NaCl and 20 mM sucrose. This 'sample system' was then incubated on ice for 45 min and sonicated using a Vibra-cell ultrasonic processor Model VC 500 (Sonics and Materials, Danbury, CT, USA) equipped with a 1/2 inch horn. The sample system was sonicated six times for 30 s each with resting intervals of 1 min in a cylindrical aluminium tube im-

mersed in ice/water. The ultrasonic exposure had an intensity output setting of 7, with 20% duty pulses.

After sonication, 6.43 g of pure lower phase and 5 g of pure top phase from an aqueous two-phase system comprised of 5.7% (w/w) Dextran T500, 5.7% (w/w) PEG 4000, 10 mM sodium phosphate buffer (pH 7.4), 5 mM NaF and 20 mM sucrose (henceforth known as 5.7:5.7#1) were then added to the sonicated sample system. The sample system was then mixed at 4°C and centrifuged at 2000 × g to separate the phases. The upper and lower phases, enriched in thylakoid fragments from stroma and grana lamellae respectively, were separated and each washed twice by 10 ml of fresh lower or upper phase respectively. The final upper phase fraction, after being washed twice by fresh lower phase, constituted the stroma lamellae membrane fraction (T3) and the final lower phase fraction, after two washes by fresh upper phase, constituted the grana lamellae membrane fraction (B3) [1]. Similarly and in exactly in the same manner as just described, the grana lamellae fraction (B3) could be sonicated further (12 × 30 s), and the resulting fragments separated by two-phase partitioning to yield new upper and lower phase fractions representing the grana margins and grana core, respectively. The top and lower phases containing the subthylakoid vesicles were diluted three times with 10 mM sodium phosphate buffer (pH 7.4), 5 mM NaCl, 10 mM NaF, 100 mM sucrose and pelleted at 100 000 × g for 90 min. The pellets were resuspended in the dilution medium containing 5% DMSO and stored in liquid nitrogen. Alternatively, the vesicles, representing the different structural domains, were resuspended in illumination buffer and immediately phosphorylated as described in Section 2.6.

2.5. Counter-current distribution

Counter-current distributions of sonicated thylakoids and sonicated grana lamellae vesicles were carried out in a centrifugal counter-current distribution apparatus as described by Åkerlund [11]. For sonicated thylakoids, 6.43 g of the lower phase and 5.0 g of the top phase were added to the sample system after sonication. Following sonication, control and phosphorylated thylakoids were compared directly in the same experiment by loading three cavities on opposite halves of the plate (containing a total of 60 cavities) with 1.8 ml of the respective thylakoid sonicates. The remaining 54 cavities were loaded with 1.8 ml of the phase system 5.7:5.7#1 (0.9 ml of the top phase and 0.9 ml of the lower phase). 25 operative cycles, comprising 30 s mixing, 90 s centrifugation and one transfer each, were carried out. Following the run, each of the cavities was collected into a centrifuge tube and the vesicles were released from the polymers by addition of 'dilution medium' composed of 10 mM sodium phosphate buffer (pH 7.4), 10 mM NaF and 100 mM sucrose. Absorbance at 680 nm was recorded for each cavity and cavities were then selected, pooled and collected by centrifugation at 100 000 × g for 90 min. The pellets were resuspended in a small amount of 'dilution medium' containing 5% DMSO, the chlorophyll content and a/b ratios determined according to Arnon [12] and stored in liquid nitrogen.

Intact grana vesicles (B3) were also phosphorylated (see Section 2.6), sonicated and fractionated by countercurrent distribution and compared to their non-phosphorylated and sonicated counterparts. In this case, an equal volume of fresh top phase (5.7:5.7#1) was added to the grana sonicate, suspended in the lower phase, prior to loading as described above.

2.6. Phosphorylation of isolated vesicles representing different structural domains of the thylakoid membrane

Isolated grana lamellae (B3), grana core, grana margin and stroma lamellae (T3) vesicles were phosphorylated in the illumination buffer, as described for isolated thylakoids in Section 2.3. After phosphorylation the vesicles were diluted in $MgCl_2$ free illumination medium and pelleted by centrifugation at 100 000 × g for 90 min. The pellet was resuspended in 'dilution medium', the chlorophyll content determined and the vesicles either frozen in liquid nitrogen for later analysis, or, in the case of B3, directly sonicated in a two-phase system for analysis by countercurrent distribution as described in Section 2.5.

2.7. Analysis

Chlorophyll was determined according to Arnon [12]. In the countercurrent distribution experiments (Figs. 1 and 4) the amount of membrane was estimated by absorbance at 680 nm which gives an approximate value of the relative content of chlorophyll in the grana and stroma lamellae fractions. By comparison with the acetone method of Arnon, we found that the grana lamellae fraction (chlorophyll $a/b = 2.4$) is underestimated by 3% compared to the stroma vesicles (chlorophyll $a/b = 5.0$).

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed according to the method of Laemmli [13] with linear gradients of 12–22.5% acrylamide and in the presence of 4 M urea. Gels were stained with Coomassie brilliant blue and the relative content of LHC II (25 and 27 kDa polypeptides) in the different fractions estimated from the peak area (optical absorbance, A) after scanning the gel by a laser densitometer (Personal Densitometer SI, Molecular Dynamics, Sunnyvale, CA, USA). Phosphorimages of dried SDS gels were obtained by a phosphorimager (PhosphorImager SI, Molecular Dynamics) and the ^{32}P incorporation was quantified from peak areas after lane profile analysis of the phosphorimage (Table 2).

Two-dimensional gel electrophoresis was carried out according to O'Farrell [14] with modifications as described by Yu et al. [15].

The concentration of P700 (reaction center of PS I) was

obtained with an Aminco DW -2 spectrophotometer operated in a split beam mode. The amplitude of the light-minus-dark absorbance change at 700 nm was measured using an extinction coefficient of 64 mM^{-1} cm^{-1} [16]. The reaction mixture contained 15 mM Tricine (pH 7.8), 5 mM $MgCl_2$, 10 mM NaCl, 400 mM sucrose, 2 mM sodium ascorbate, 2 mM methyl viologen 0.02% (w/w) SDS and thylakoid vesicles for a final chlorophyll concentration of 20 μM.

The kinetics of P700 photooxidation was measured according to Melis [17]. Thylakoid vesicles were dark adapted on ice for 30 min prior to 2 h incubation in 90 mM Tricine-KOH (pH 7.8), 150 mM KCN, 1 mM $MgCl_2$, 5 mM NaF, 100 mM sucrose and 300 μM chlorophyll in the dark [18]. After incubation the samples were diluted ten times in KCN-free incubation medium and the kinetics were measured in the presence of 50 μM DCMU and 200 μM methyl viologen. A DW-2 Aminco spectrophotometer working in a dual-wavelength mode was used to measure the absorbance change at 700 nm with 730 nm as a reference. In front of the photomultiplier was a RG 665 Schott filter and the actinic beam was transmitted by a 380–600 nm broad band filter, a 566.9 nm interference filter, and attenuated by a neutral filter to give an uniform light between 500 and 600 nm and an intensity of 25 μE m^{-2} s^{-1}. Signal averaging was performed with a Nicolet instrument corporation model 527 instrument. The best-fit curve through the trace was drawn by hand and the data were plotted semilogarithmically as befits a first-order reaction. The slope was taken as equal to the rate constant K_{P700} s^{-1}. In each experiment, two measurements composed of 12 overlaid traces were carried out for grana vesicles (B3) while 5 overlaid traces were carried out for the stroma lamellae vesicles (T3).

PS II electron transport from H_2O to phenyl-*p*-benzoquinone (PBQ) was measured by the oxygen electrode in 30 mM sodium phosphate buffer (pH 6.5), 3 mM NaCl, 5 mM NaF, 0.2 mM PBQ, 60 mM sucrose and thylakoid membranes to a chlorophyll concentration of 25 μM. The excitation light was transmitted by a 630 nm cut-off filter (RG 630) and attenuated by a transformer to the right photon flux density measured with a LI-COR (LI-70) quantum meter.

Cytochrome *f* was determined spectrophotometrically from the reduced minus oxidized absorbance change at 554 nm [19] using an absorption coefficient of 17.2 cm^2 $mmol^{-1}$. The reaction buffer contained 8 mM sodium phosphate buffer (pH 7.4), 4 mM NaCl, 80 mM sucrose, 1% Triton X-100 and a thylakoid membrane fraction (150 μM chlorophyll). After registration of the baseline (540–570 nm), 25 μl of 0.1 M hydroquinone were added to the sample cuvette. An equal volume of 0.1 M potassium ferricyanide was added to the reference cuvette and the absorbance difference recorded.

3. Results

3.1. Phosphorylation of intact thylakoids followed by fractionation

Membrane dynamics – sub-thylakoid domains

Counter-current distributions of sonicated control and phosphorylated thylakoids are depicted in Fig. 1. The peaks to the left represent the vesicles derived from the grana and the peaks to the right represent the vesicles derived from the stroma lamellae.

In the control experiment the grana peak accounts for 70% of the total absorbance at 680 nm. Upon light-induced protein phosphorylation, this value declined to 65%, while the content of the peak to the right, representing stroma lamellae, increased from 30% to 35% based on the absorbance at 680 nm. At the same time, the chlorophyll *a/b* ratio of the stroma lamellae is reduced from 4.9 to 4.7, while the *a/b* ratio of the grana vesicles remains fairly constant (Fig. 1).

Effect of protein phosphorylation on PS II and PS I

The chlorophyll *a/b* ratios, PS I and cyt *b/f* contents and PS II and PS I light-harvesting activities of sub-thylakoid membrane fragments isolated from phosphorylated and non-phosphorylated thylakoids using the batch proce-

Table 1
Properties of sub-thylakoid membrane fractions derived from phosphorylated (P) and control (C) thylakoids

Membrane fraction	Yield % Chl	Chl *a/b* (mol/mol)	K_{P700} (s^{-1})	P700/Chl (mmol/mol)	PS II V_{max}	Activity K_m	Cyt *f*/Chl (mmol/mol)
Grana lamellae C (B3)	23	2.3	4.3 (2.7%)	1.2 (2.1%)	146	315	1.7 (7.4%)
Grana lamellae P (B3)	19	2.3	4.2 (2.6%)	1.2 (2.8%)	112	387	1.6 (9.5%)
Stroma lamellae C (T3)	17	4.4	3.2 (8.6%)	4.3 (4.0%)	71	602	1.2 (6.3%)
Stroma lamellae P (T3)	20	4.1	3.4 (2.6%)	4.0 (3.2%)	63	626	1.2 (2.4%)
Thylakoids C	100	3.0		2.7 (4.3%)	97	467	1.4 (3.1%)
Thylakoids P	100	3.0		2.7 (2.7%)	83	574	1.3 (2.7%)

Phosphorylated and control thylakoids were fragmented and the vesicles derived from grana and stroma lamellae were separated by aqueous two phase partitioning as described in Section 2.4. The experimental values of the kinetic rate constant, K_{P700}, of photooxidation is the average of four separate experiments. The PS II activity was measured from H_2O to PBQ. V_{max} is expressed as O_2/mol chlorophyll per h. The K_m and V_{max} values were determined from the inverse plot (Fig. 2). The values given within parentheses are relative standard deviation of four separate experiments.

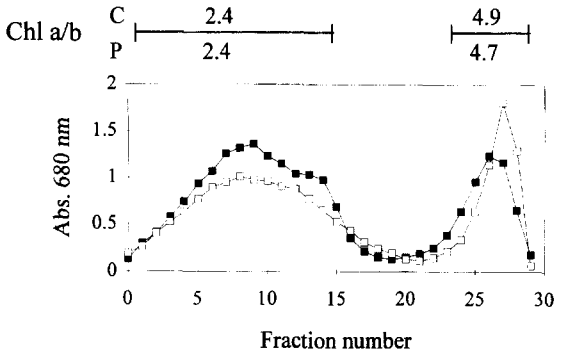

Fig. 1. Counter-current distribution diagram of sonicated control (filled squares) and phosphorylated (open squares) thylakoids. The peaks to the left represent the appressed membranes and the peaks to the right the unappressed membranes. Note the decrease in the chlorophyll a/b ratio (top of figure) for the unappressed membranes derived from phosphorylated thylakoids.

dure of partitioning (see Section 2.4) are presented in Table 1. Note as well the decrease and corresponding increase in yields of the grana and stroma lamellae fractions, respectively, following the phoshorylation of whole thylakoids (Table 1).

Photochemical activities and antennae sizes were measured for both PS I and PS II of the grana (B3) and stroma lamellae (T3) vesicles. In order to study the electron transport properties of PS II, light saturation curves of oxygen evolution, with PBQ as an electron acceptor, were constructed for both B3 and T3 membrane preparations from phosphorylated and non-phosphorylated thylakoids. The data were then plotted in the straight-line format, V/I vs. V (Fig. 2), in order to determine the maximum velocities (V_{max}) of oxygen evolution and the relative antenna sizes (K_m) for each membrane sample (Table 1). The latter is obtained from the negative slope (equal to $-1/K_m$) of the V/I vs. V plot with the assumption that the turnover rates are constant.

The negative slope and V_{max} of the grana vesicles decreased upon protein phosphorylation by 19% and 23%, respectively (Table 1), indicating a corresponding decrease in the functional antenna size and maximum activity. By contrast, the V_{max} for PS II in the stroma lamellae vesicles from phosphorylated thylakoids is reduced much less (12%) and the negative slope is almost unaffected (4% decrease). The corresponding changes for the thylakoid (14% reduction in V_{max} and 18% increase in K_m) lie between the values obtained for the grana and stroma lamellae vesicles as one would expect from a weighted contribution of the grana and stroma lamellae domains (85% of the PS II is in the grana and 15% in the stroma vesicles [1]). Thus, the functional antenna size of PS IIα in the grana is reduced upon phosphorylation, while the antenna size of PS IIβ in the stroma lamellae is virtually unaffected.

The antenna sizes of the PS I populations located in the grana and stroma lamellae membrane domains were examined by measuring the kinetics of P700 photooxidation. Weak green light oxidizes P700 and the rate of the concomitant absorption change is proportional to the antenna size [17]. As seen in Table 1. the first-order rate constant (K_{P700}) for the grana PS Iα is 4.3 s^{-1} compared to 3.2 s^{-1} for the PS Iβ of the stroma lamellae. This means that the antenna size of PS Iα is 34% larger than PS Iβ, which is in agreement with previous results [1,3]. Neither the antenna size of PS Iα of the grana vesicles nor the PS Iβ antenna size of the stroma lamellae vesicles was affected significantly by phosphorylation of the thylakoid membrane proteins (Table 1).

Distribution of the cytochrome b/f complex

It has been reported that the cytochrome b/f complex changes its distribution between the grana and stroma lamellae during state transitions [8]. It was therefore of interest to study the content of cytochrome f in the grana and the stroma lamellae vesicles derived from control and phosphorylated thylakoids. The concentration of cytochrome f/chlorophyll in our grana and stroma lamellae vesicles remained the same (Table 1). We found, therefore, no increase in the cyt f content per chlorophyll in the stroma lamellae vesicles after phosphorylation. However, since the amount of stroma lamellae fraction is greater following phosphorylation (+16%, see Fig. 1), the *total* amount of cyt b/f in the stroma lamellae fraction has increased.

^{32}P-labeling of LHC II in the different membrane domains derived from phosphorylated thylakoids

Thylakoids phosphorylated in the presence of [γ-^{32}P] ATP were fractionated and the vesicles analyzed by gel electrophoresis and phosphorimaging. The data in Table 2

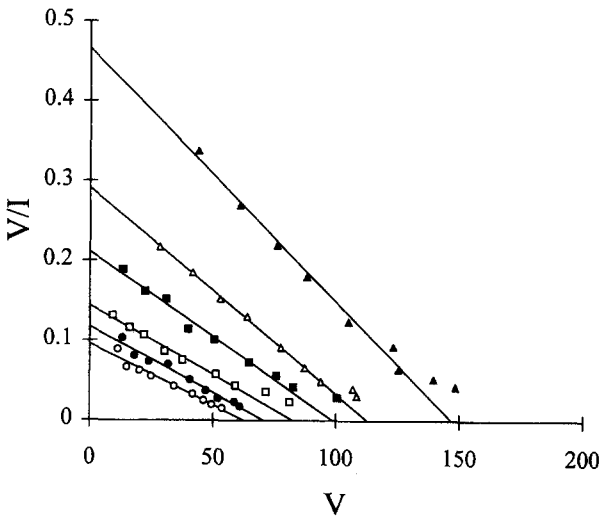

Fig. 2. V/I vs. V plot for PS-II-mediated electron transport for thylakoids (squares), grana (B3, triangles) and stroma lamellae (T3, circles) vesicles from control (closed symbols) and phosphorylated (open symbols) thylakoids. V is the PS II activity in mol O_2/mol Chl per h and I is the light intensity in μE m^{-2} s^{-1}. The slope is equivalent to $-1/K_m$. A smaller K_m reflects a relatively larger antenna size.

Table 2
^{32}P-labeled LHC II polypeptides in fractions derived from phosphorylated thylakoids

Membrane fraction	Chl a/b (mol/mol)	^{32}P-LHCII/LHCII (counts/A)
Thylakoid	3.0	470
Stroma lamellae (T3)	4.5	796±31
Grana lamellae (B3)	2.4	359±20
Grana margins	2.7	409±24
Grana core	2.2	322±27

Two separate measurements were normalized with respect to thylakoids and the relative counts for LHC II in SDS-gels is given per LHC II protein unit based on optical absorbance (A) from Coomassie-stained gels (see Material and methods).

show that the specific labeling of LHC II is highest in the stroma lamellae vesicles and lowest in the grana core vesicles which is in agreement with Andersson et al. [20]. The phosphorylated LHC II is rather evenly distributed on a chlorophyll basis in our fractions, whereas the PS II core complex polypeptides CP43, D1, D2 and the 9 kDa and an unidentified 12 kDa polypeptide are found predominantly labeled in the grana-derived fractions [21].

Two-dimensional electrophoretic protein patterns for vesicles originating from stroma lamellae (Fig. 3a) and grana lamellae (Fig. 3b) show that LHC II polypeptides (25 and 27 kDa) are found predominantly in the grana fraction, whereas the α and β subunits of the ATPase, 59 and 56 kDa, respectively, are found predominantly in the stroma fraction. A comparison of the phosphorimages of the stroma lamellae fraction (Fig. 3c) and the grana fraction (Fig. 3d) show that two unidentified polypeptides of molecular masses 16–22 kDa (arrows) and pI values lower than 5.0 are found weakly labeled in the stroma lamellae fraction but are not found in the grana fraction.

The 64 kDa phosphoprotein

The phosphorylated polypeptides in the range 60–65 kDa probably represent an autophosphorylated protein ki-

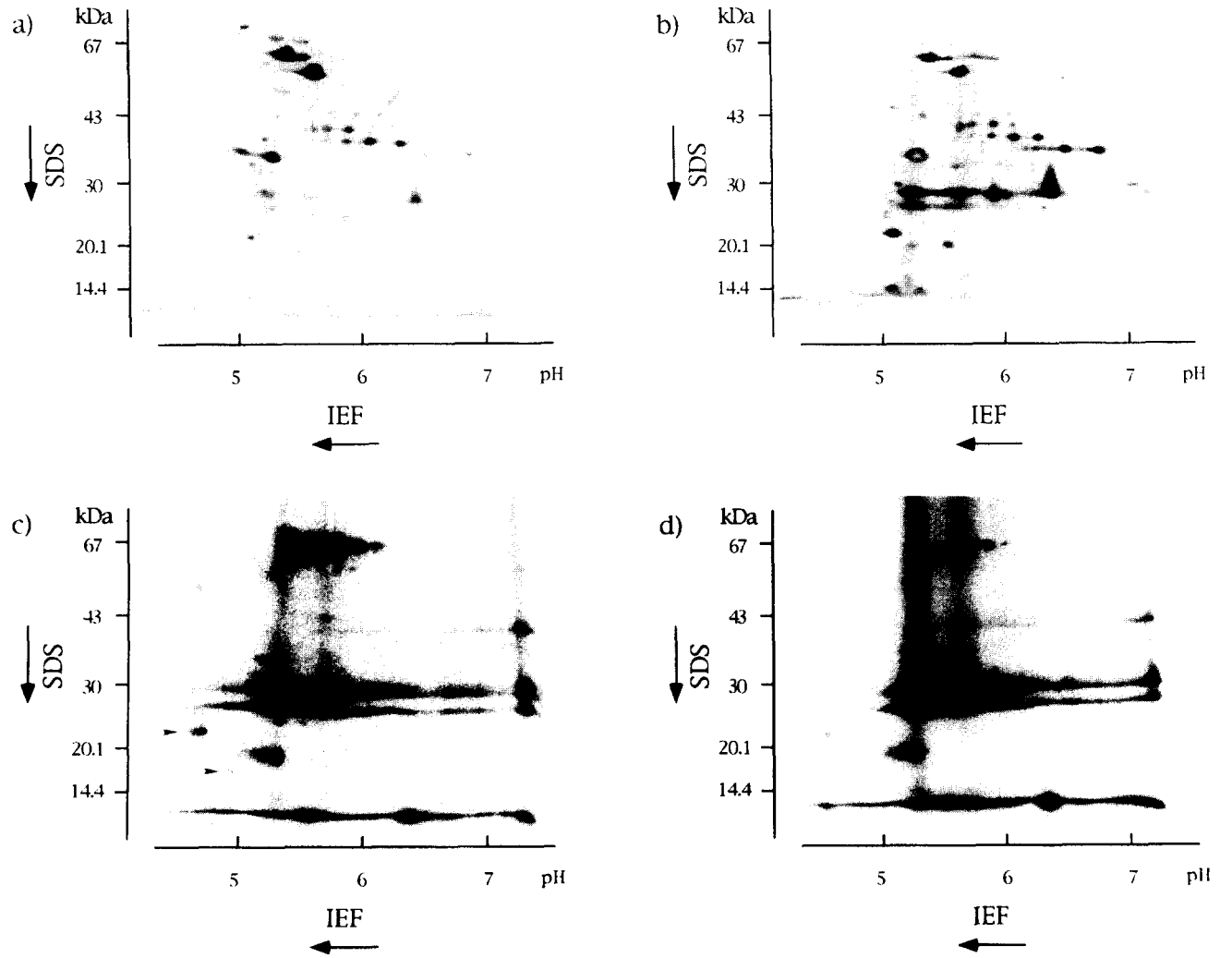

Fig. 3. Two-dimensional gel electrophoretic fractionation of stroma lamellae (a) and (c); and grana lamellae (b) and (d) from phosphorylated thylakoids. Gels in (a) and (b) were silver-stained and the subsequent phosphorimages are shown in (c) and (d). Arrows in (c) designate two unidentified phosphoproteins located in the stroma lamellae fraction but not in the grana.

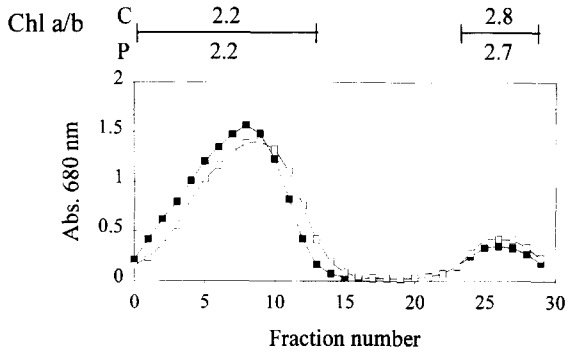

Fig. 4. Counter-current distribution of sonicated, control (filled squares) and phosphorylated (open squares), grana lamellae vesicles. The peaks to the left are derived from the core of grana and the peaks to the right are enriched in the grana margins. Note the decrease in the chlorophyll *a/b* ratio (top of figure) and increase in yield for the grana margins vesicles derived from phosphorylated grana vesicles.

nase of 64 kDa (claimed to be the LHC II protein kinase) [22]. Antibodies to this 64 kDa protein react against two polypeptides having similar molecular masses but different p*I* values, which, in non-phosphorylated thylakoids, are both found to be enriched in the grana margins but virtually absent in the stroma lamellae [2,15]. However, in phosphorylated thylakoids we find the 64 kDa phosphoprotein(s) in both the stroma lamellae and the grana lamellae fractions (Fig. 3c and d), indicating that some of the margin membrane is found in the stroma lamellae fraction after phosphorylation.

3.2. Phosphorylation of isolated subthylakoid vesicles

Membrane dynamics – sub-grana domains

Intact grana lamellae vesicles were incubated in the light in the presence of ATP (Section 2.6), washed and added to an aqueous two-phase system, fragmented by sonication, and, finally, fractionated by way of counter-current distribution (Fig. 4). The grana-core-enriched vesicle population partitions with the lower phase (peak to the left in Fig. 4) and the grana-margin-enriched vesicle population partitions with the upper phase (peak to the right in Fig. 4) [2]. Slightly more material, 16% vs. 13% based on total absorbance at 680 nm, and a lower chlorophyll *a/b* ratio was found for the grana margin peak obtained from phosphorylated grana vesicles (Fig. 4).

Protein phosphorylation in isolated sub-thylakoid fractions representing the different structural domains of the thylakoid membrane

Subthylakoid vesicles representing the different structural domains were prepared from dark-adapted spinach by

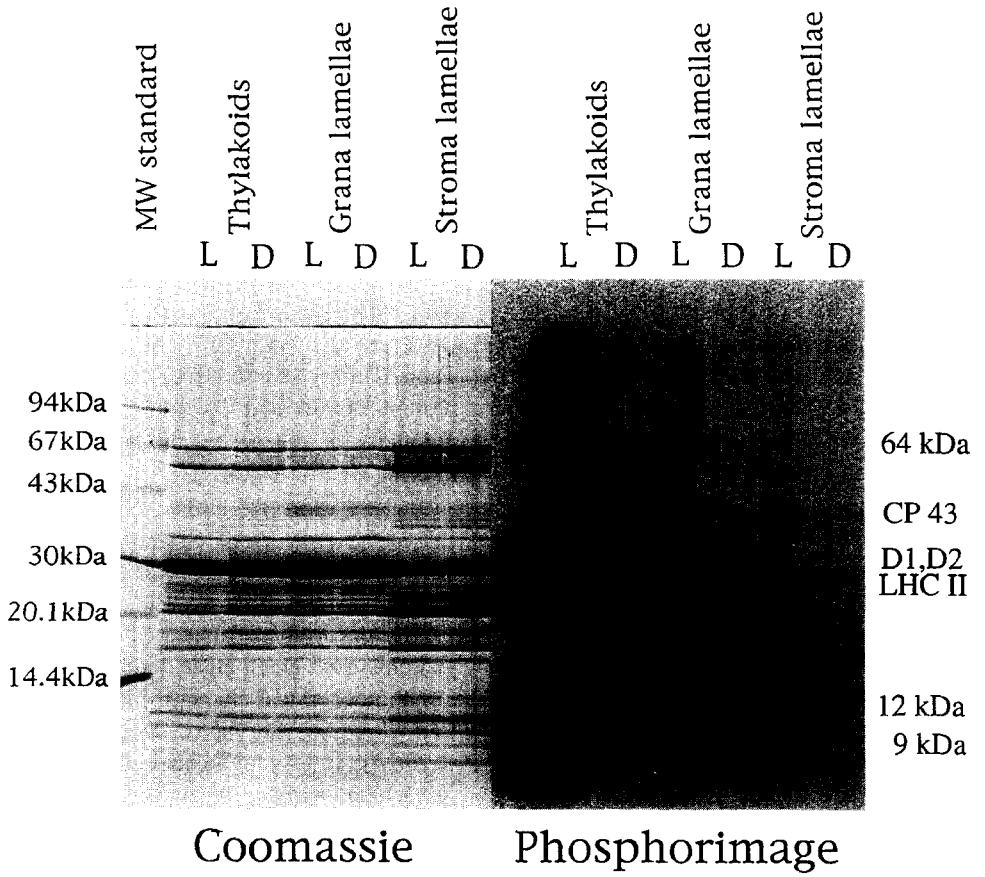

Fig. 5. SDS-PAGE and subsequent phosphorimage of thylakoids, grana and stroma lamellae vesicles. The subthylakoid vesicles were phosphorylated after fragmentation of the thylakoid. Incubation in the presence of [γ-^{32}P]ATP took place either in the light (L) or in the dark (D).

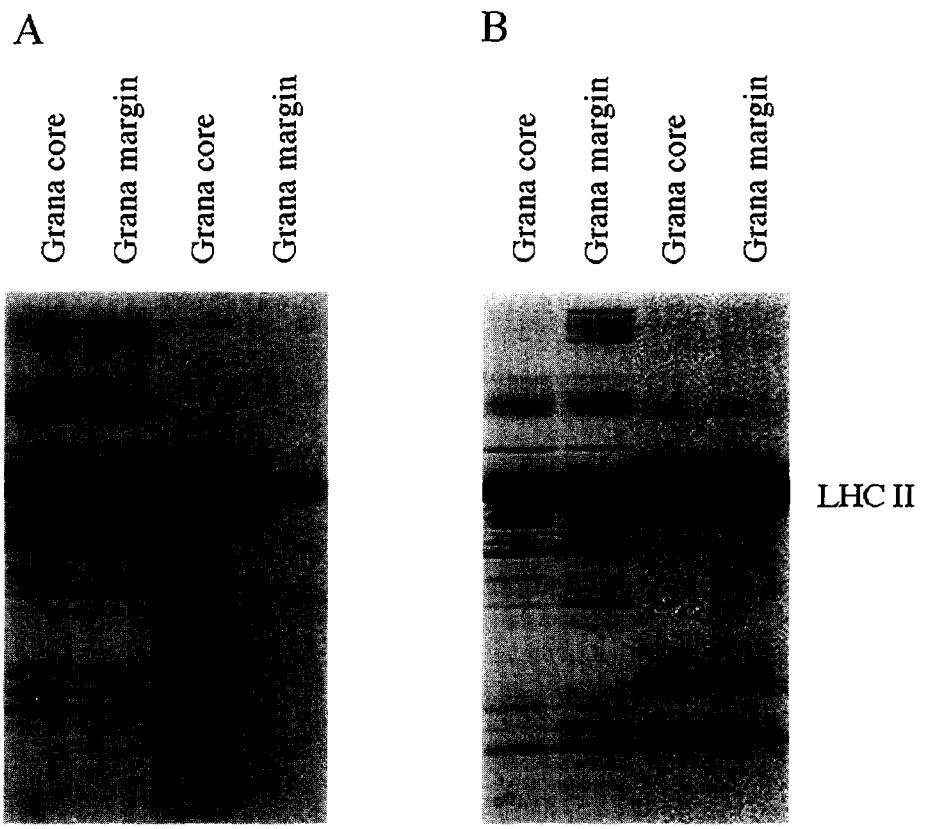

Fig. 6. SDS-PAGE and subsequent phosphorimages of the sub-grana fractions, grana core and grana margin. In (A) The grana core and grana margin vesicles were phosphorylated after fragmentation of the grana whereas in (B) the grana lamellae vesicles (B3) were first phosphorylated and then fractionated further followed by separation of vesicles derived from grana core and grana margin. The first two lanes in A and B are stained with Coomassie whereas the other two are the subsequent phosphorimages.

the batch procedure as described in Section 2.4 and then incubated separately in light in the presence of [γ-^{32}P]ATP (Section 2.6). SDS-PAGE was carried out for each of the membrane samples and the protein visualized by Coomassie staining and phosphorimaging (Fig. 5). In general, less radioactivity is incorporated into membrane protein when subthylakoid fractions are used instead of intact thylakoids. The LHC II apoproteins and the PS II core complex polypeptides D1, D2 (30–34 kDa) and CP 43, and other polypeptides of molecular weights 9, 12 and 62–65 kDa became labeled in the grana vesicles (B3) but not in the stroma lamellae vesicles (T3) (Fig. 5). Interestingly, polypeptides in the molecular mass range 15–22 kDa became labeled in the stroma lamellae fraction (T3) under plastoquinone reducing conditions, the same conditions that induce the phosphorylation of the polypeptides in the grana vesicles (B3) (Fig. 5). This may reflect the existence of a possible heterogeneity and segregation of protein kinase specificity in the thylakoid. When the sub-grana vesicles, grana core and grana margins, were incubated in the light and in the presence of [γ-^{32}P]ATP, phosphorylated polypeptides were found in only the grana core fraction (Fig. 6a). If, however, grana lamellae vesicles (B3) were phosphorylated and then fractionated into the sub-grana domains, grana margins and grana core (see Section 2.4 and Fig. 4), both vesicle populations contained phosphorylated LHC II and PS II associated polypeptides (Fig. 6b). Furthermore, we found that the specific labeling of LHC II was highest in the grana margin as compared to the grana core (Fig. 6b). The same result is obtained when whole thylakoids are phosphorylated and then fragmented (compare the values for the grana core and margins in Table 2). The results favor the idea that both the LHC II protein kinase and its substrate(s) are enriched in the periphery of the grana, the margins, where we find the highest specific labelling.

4. Discussion

The occurrences of phosphoproteins in chloroplast thylakoids from green algae and higher plants and the attending photochemical changes observed for the photosystems following phosphorylation have been extensively reviewed [9,23,24]. It is widely held that, at least in the case of spinach thylakoids, the protein kinase activity responsible for catalyzing the covalent modifications of PS II and LHC II polypeptides is enriched in the grana domain [2,15,25,26] and that the activity and substrate specificity of the kinase is regulated by the redox status of the plastoquinone pool

[27] and its interaction with the cyt b/f complex, respectively [25].

It has been suggested that one of the functions of the phosphorylation of LHC II is to regulate the relative turnover rates of PS I and PS II by modifying the relative antennae sizes of the two photosystems. This can be accomplished either by dissociation of phosphorylated LHC II from PS II in the grana followed by lateral movement of a portion of the phosphorylated LHC II to the stroma lamellae and association with PS I, or by only dissociation of LHC II from PS II. In both cases there is a reduction of the PS II antennae size relative to the antenna size of PS I. Since we have proposed a three-domain model for the thylakoid membrane where the PS IIα and PS Iα photosystems located in the grana core and margins respectively carry out linear electron transport and the PS Iβ photosystems in the stroma lamellae participate in mainly cyclic electron transport [5,28], and since the compositions of the thylakoid domains are critical to the photochemistry taking place in and across these regions, it was of interest to study the effects that phosphorylation has on the two types of granal photosystem (PS IIα and PS Iα) as well as how the protein kinase activity influences the domain organization of the grana core, the grana margins, and the stroma lamellae.

In this regard, the sonication and aqueous two phase partitioning technique [29] is well suited for studying the post-phosphorylation events occurring within and between the grana and stroma lamellae compartments. Recently, the non-detergent isolation method has been shown to be an effective tool for separation of the core and margins of the grana of spinach thylakoids [2,30] and for the demonstration of the heterogeneous nature of the stroma lamellae [21,29].

4.1. Phosphorylation and sonication of whole thylakoids

Counter-current distribution of sonicated thylakoids resolves two populations of vesicles representing the grana and stroma lamellae domains (Fig. 1) [1]. The increase in yield of the stroma lamellae and the corresponding loss of grana during those times when LHC II protein kinase activity is high (Table 1 and Fig. 1) could be due to partial destacking of the grana. Such destacking has been observed by electron microscopy of thin sections of thylakoids [31,32], linear dichroism [33] and sub-fractionation studies [34]. Since the stacked region of the thylakoid is the origin of the inside-out grana vesicles (left peak in Fig. 1) [35], unstacking will decrease the yield of the inside-out vesicles and increase the yield of the right-side-out vesicles (the right hand peak in Fig. 1). It is reasonable to assume that this destacking occurs mainly in the peripheral region of grana and in the fret region. It is likely that the initial phosphorylation of LHC II occurs here in the periphery of the grana where the 64 kDa polypeptide claimed to be the kinase is preferentially located in non-phosphorylated thylakoids. When either whole thylakoids or isolated inside-out vesicles are phosphorylated, the highest specific labelling of LHC II in the grana is found in the margins (Table 2 and Fig. 6B, and see [21]).

The peripheral region, the grana margin, contains relatively less LHC II compared to the center of the granum. If LHC II is responsible for the stacking forces of the grana, and they decline by protein phosphorylation, then the grana margin material will be more easily broken off the grana core by sonication. We therefore suggest that phosphorylation causes unstacking of the appressed membranes at the grana margins and near the fret regions. The newly exposed areas are therefore broken off the grana and are co-isolated with the stroma exposed membranes contributing to the increase in yield of the right hand peak of phosphorylated thylakoids in Fig. 1. The chlorophyll a/b ratio of the grana margin vesicles is about 2.7 [2] and will hence lower the cholorophyll a/b ratio of the stroma lamellae (from 4.9 to 4.7). No subsequent change in the chlorophyll a/b ratios is observed for the grana fractions from control and phosphorylated thylakoids (the left hand peaks in Fig. 1). This can be explained, since the chlorophyll a/b ratio of the margins (2.7) is closer to the chlorophyll a/b ratio of the grana vesicles (2.4) than the chlorophyll a/b ratio of the stroma lamellae vesicles (4.9). Furthermore, the loss of membrane material from the left peak (7%) representing the grana is relatively less than the concomitant gain of membrane material in the right-hand peak (16%) representing stroma lamellae (Fig. 1).

The grana margins have been characterized elsewhere [2]. They contain PS I of the α type which is functionally connected to a 'special' pool of LHC II and which has a 30–40% larger antenna as compared to the PS Iβ in the stroma lamellae [4]. The PS II present in margins is of the β type, i.e., with a small antenna size as PS IIβ in the stroma lamellae. However, unlike PS IIβ of stroma lamellae the PS IIβ of grana margins are Q_B-reducing with respect to acceptor side properties [30].

When the thylakoids are phosphorylated with [γ-^{32}P]ATP we find that the specific LHC II phosphorylation, i.e., ^{32}P per LHC II protein unit, is highest in the stroma lamellae fraction. This is in agreement with other fractionation studies on phosphorylated thylakoids [20]. These results have been interpreted as a detachment of peripheral LHC II from PS II in the grana and a lateral movement of this LHC II out to the stroma lamellae. Since there was no concomitant increase of the PS II core polypeptides in the stroma lamellae fraction (Y100), this was taken as an evidence for selective movements of the peripheral LHC II to the stroma lamellae and not movement of the entire PS II complex [36,37]. However, we have previously suggested an alternative explanation for the increase in LHC II content in the stroma lamellae fraction after phosphorylation [21]. According to this, most of the LHC II of the grana margin is attached as peripheral LHC II to PS Iα and not to PS II [2,4,30]. Partial destacking after protein

phosphorylation results in more grana margin material and material near the fret regions being sonicated off the grana. Small vesicles derived from the grana margins, enriched in PS Iα, with its attached LHC II, will then partition with the stroma lamellae fraction and increase its LHC II content and lower the chlorophyll a/b ratio. We have previously shown that our stroma fraction is a heterogeneous population containing vesicles having different chlorophyll a/b ratios [21,29]. This explanation is supported by the present investigation, however, whether the LHC II attached to PS Iα becomes phosphorylated requires further investigation. It is of interest that recent studies based on French press fractionation, followed by sucrose density gradient experiments, show that the phosphorylated stroma lamellae fraction is a heterogeneous vesicle population containing vesicles of different sizes and having different chlorophyll a/b ratios [38].

4.2. The effect of phosphorylation on PS II and PS I

In the present study we find that the main effect of thylakoid protein phosphorylation on the photosystems is the reduction of the functional antenna size of PS IIα in the grana. This decrease in antenna size has been observed in previous studies, see review by Allen [9]. There is virtually no effect on the antenna size of PS IIβ in the stroma lamellae (Table 1 and Fig. 2). We do, however, observe a decrease in the V_{max} for all the fractions, which may be due to photoinhibition (Table 1).

We have proposed elsewhere that the grana lamellae are responsible for linear electron transport while the stroma lamellae carry out cyclic electron transport [3,5,39]. The decrease in antenna size of PS IIα may be related to its function in linear electron transport where it has to cooperate with PS Iα in the grana. It should be noted that PS IIα has a larger antenna than PS IIβ because it has LHC II attached as a peripheral antenna. Since the peripheral LHC II becomes phosphorylated [31,37], this may lead to an uncoupling of the antenna system of PS IIα but not of PS IIβ, which lacks the peripheral LHC II [37].

Neither an increase nor a decrease of the antenna sizes of the PS I in the grana (PS Iα) or of the PS I in the stroma lamellae (PS Iβ) could be detected after phosphorylation (Table 1). Although a small increase (4–5%) in the rate constant for the photo-oxidation of the PS I in the stroma lamellae fraction would be expected as a consequence of destacking and concomitant transfer of grana margin material to the stroma lamellae, these changes are masked by the standard error of the P700 photo-oxidation kinetics measurements (Table 1) ($\pm$10%).

Conflicting results have been reported concerning the functional antenna size of PS I, demonstrating either an increase [40–42] or no increase [43–45] in the antenna size after thylakoid protein phosphorylation. Since phosphorylation of the thylakoid proteins increases the amount of unappressed membrane, the increase in antenna size of PS I in the stroma lamellae, reported in the literature [41,42] can partly be explained by partial unstacking induced by protein phosphorylation. Some grana margin material containing PS Iα (having 30–40% larger antenna than PS Iβ in the stroma lamellae) is thereby broken off the grana during fragmentation and recovered in the stroma lamellae fraction. This leads to an apparent small increase in the antenna size of PS I in the stroma lamellae fraction and also to the appearance of LHC II in this fraction, but it would not lead to an overall increase in the PS I antenna size of the whole thylakoid.

4.3. Phosphorylation of sub-thylakoid vesicles

We found that protein phosphorylation occurs for both right-side out and inside-out sub-thylakoid vesicles but that the protein kinase activities in the grana and stroma lamellae differed with respect to their substrate specificities (Figs. 5 and 6). While the vast majority of the LHC II and PS II protein kinase activity resides in the grana domain, a minor but potentially significant phosphorylation of three low-molecular-mass proteins of 15–22 kDa occurs in the stroma lamellae domain (Fig. 5). These may be the same phosphoproteins which become labelled in whole thylakoids (Fig. 5) and which, following fractionation of phosphorylated thylakoids, are found to be enriched in the stroma lamellae (Fig. 3). These stroma lamellae phosphoproteins have not been previously identified.

In contrast to both of the former membrane fractions, hardly any protein phosphorylation was observed in the grana margin vesicles (Fig. 6a). Even when the plastoquinone pool was reduced in the dark by the addition of ferredoxin and NADPH (data not shown) LHC II and PS II polypeptides failed to become phosphorylated in the grana margin vesicles, whereas the same proteins did become phosphorylated in thylakoids, grana vesicles and grana core vesicles under these reducing conditions. It is remarkable that the right-side-out grana margin vesicles were not able to phosphorylate LHC II, since they are enriched in the 64 kDa protein which is claimed to be the LHC II protein kinase. The LHC II protein kinase is under redox control of the plastoquinone pool [27] and is activated via the cytochrome b/f complex [25].

The ability or inability of the individual membrane fraction to carry out protein phosphorylation may have to do with the actual morphology of the vesicles. Interestingly, it is only the sheet-like vesicles, grana and grana core, which are capable of phosphorylating LHC II and the PS II polypeptides. The LHC II protein kinase might only recognize LHC II as dimers which are transversally connected as might be the case in the inside-out sheet like vesicles but not in the spherical vesicles, the grana margins and stroma lamellae. Electron microscopy of thin sections shows that the inside-out fractions, the grana lamellae and the grana core, are morphologically similar exhibiting a flattened sheet-like character, whereas the right-side-out

fractions, the stroma lamellae and grana margins are more alike, appearing as small spherical vesicles [1,30]. It should be noted that, when grana vesicles (B3) are first phosphorylated and then fragmented, both of the sub-grana populations, grana core and grana margins, contain phosphorylated LHC II (Fig. 6B). This shows that the grana margins were indeed functionally connected to the grana core in our grana preparations (B3).

Our results support the model in which protein phosphorylation reduces the functional antenna size of PS IIα and also conform to the idea that the ratio of grana/stroma lamellae decreases as a consequence of the covalent modifications occuring in and around the grana periphery. Even if none of the phosphorylated and mobile pool of LHC II becomes subsequently attached to PS I, the relative excess of chlorophyll which is functionally associated with PS I compared to PS II will still be greater after phosphorylation than before; one of the basic tenets of the model for state transitions.

4.4. Protein phosphorylation and unstacking related to cyclic and linear electron transport

We have suggested elsewhere a model for the function of the thylakoid membrane according to which the grana, with PS IIα and PS Iα, carry out linear electron transport, while the stroma lamellae, containing mainly PS Iβ, carry out cyclic electron transport [3,5,39]. This model is supported by the following observations and considerations. (1) There is an excess of chlorophyll associated with PS I compared to PS II [5,39] and it is reasonable to assume that this excess of chlorophyll is used to support cyclic electron transport around PS I. (2) There is enough PS Iα in the grana to support linear electron transport with PS IIα [5]. (3) Diffusion of the cytochrome b/f complex and plastoquinone [46–48] is too slow to allow long-range electron transport between PS I in the stroma lamellae and PS II in the appressed grana domain. In addition, it is doubtful that plastocyanin, due to its molecular size and the molecular crowding in the thylakoid lumen where the two inner sides of the thylakoid are in contact [49], can act as a long-range electron shuttler [50].

Partial destacking of the grana after protein phosphorylation may promote the exposure of the granal PS Iα centers to the chloroplast stroma and thereby enhance their participation in cyclic electron transport activity. It has been reported that unstacking of thylakoids increases the rate of cyclic ATP formation [51]. It has also been proposed that state transitions may serve to regulate the proportion of linear and cyclic electron flow and and thereby to balance the NADPH/ATP ratio (see review by Fork and Herbert [52] for references). We therefore suggest that one of the main physiological effects of phosphorylation is to increase the capacity of the thylakoid for cyclic electron transport relative to the capacity for linear electron transport. Vallon et al. have arrived at a similar conclusion, though based on another mechanism involving the selective redistribution of cyt b/f between the grana and stroma lamellae [8].

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