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Isolation and characterization of vesicles originating from the chloroplast grana margins

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A membrane fraction composed of right-side-out vesicles and deriving from the periphery of the granal stacked region of spinach thylakoid membranes has been isolated by means of sonication and separation in an aqueous two-phase system, and its biochemical and photochemical properties examined. This vesicle population, referred to as the chloroplast granal margins, is enriched in PSI as compared to the central core of the grana, has a Chl *a*/*b* ratio of between 3.0 and 3.3, and on a per chlorophyll basis contains the least amount of cytochrome *f* as compared to both the central core of the grana and the stroma lamellae domains. Notably, however, the margins represent the region of the thylakoid most enriched in the 64 kDa kinase. P700 and room temperature fluorescence induction kinetics show that the granal margins contain PS I with a larger antenna size than the PS I from the stroma lamellae and that the PS II of the margins is, with respect to the antenna size, more like the PS II from the stroma lamellae (i.e., PS IIβ). The margin vesicles also contain the ATPase complex as do the other stroma exposed areas of the thylakoid.

Introduction

Biochemical fractionation and electron microscopy have made it clear that there exists an extensive lateral segregation of the photosynthetic protein complexes [1–4]. This segregation is currently interpreted in terms of a domain model where discrete regions within the plane of the membrane are delineated according to their relative compositional and hence functional differences of photochemistry. Furthermore, the degree by which individual domains differ from one another, both qualitatively and quantitatively, is not fixed but sensitive to environmental conditions, such as temperature and incident light intensity and spectral qualities [2,4,5].

Ultrastructural examination of the thylakoid reveals the existence of at least three distinguishable regions: (1) the appressed or granal stacks, (2) the non-appressed stroma and end membranes, and (3) the highly curved grana margins which Anderson et al. suggest may comprise between 11 and 30% of the non-appressed area of the thylakoid (Ref. 6, and see Fig. 1a). The mere fact that the granal margin region displays such a unique membrane conformation makes the isolation of vesicles from this area of the thylakoid of interest but as of yet, the only direct biochemical examination of the margin region in an isolated state has been accomplished through the application of the detergent Tween-20, an agent which appears to preferentially solubilize the margins while leaving the rest of the thylakoid more or less intact [7]. Assuming all of the protein components released into the detergent supernatant derived entirely from the disrupted ends of the grana, their results indicated that the ATPase, cytochrome b_6/f, and PS I complexes are all present in the margin area. LHC II, however, was noticeably absent.

In the present study we have used a non-detergent method to isolate and characterize a sub-thylakoid membrane fraction which we suggest derives from the margin region of the grana domain. This project is a follow-up to the earlier studies on sonication of thylakoids which demonstrated that intact granal vesicles could be further fragmented to obtain a membrane fraction more highly enriched in PS II [8–13]. It was surmised that this final membrane fraction (referred to as the 'BS' fraction) represented the central core of the grana and that the membrane material removed from it

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Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; F_m, maximum fluorescence intensity; F_v, variable fluorescence; PS II, Photosystem II; PS I, Photosystem I; LHC II, light harvesting chlorophyll *a*/*b* protein; Y100, stroma lamellae fraction of yeda pressed thylakoids; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

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by the fragmentation-separation process represented the outer annuli of the granal disc [1,9,13,14]. Further study of the sub-domains of the grana revealed qualitative as well as quantitative differences in protein composition amongst the individual fractions, including the existence of a potential third thylakoid domain found to be enriched in PS I and depleted in cytochrome b_6/f with respect to the other granal sub-domains [13]. This fraction was tentatively located at the outer edge of the grana, the margins, as it was the first population to be liberated from whole grana vesicles by a single round of sonication [8]. A more detailed description of this fraction is presented here. The putative margin fraction, studied here in a more purified state than in the previous reports [8,10,11,13], is characterized in terms of the five major trans-membrane protein complexes found in the thylakoid and the results compared to those properties found in the stroma and grana membrane compartments.

Materials and Methods

Chemicals. Dextran 500 was obtained from Pharmacia (Uppsala, Sweden). Poly(ethylene glycol) 4000 (Carbowax PEG 3350) was supplied by Union Carbide (New York, NY).

Phase partitioning. Separation of thylakoid material always took place in a phase system of the following composition: 5.7% (w/w) Dextran 500/5.7% (w/w) poly(ethylene glycol) 3350/10 mM sodium phosphate buffer (pH 7.4)/5 mM NaCl/20 mM sucrose. The temperature was maintained at 4°C. When vesicles were separated by a batch procedure, separation was speeded up by centrifuging the phase in a swing-out type rotor for 3 min at $1000 \times g$. The first partition step consists of mixing the two-phase system, separating under centrifugal force, transferring the top phase to a clean tube, and adding an equal volume of fresh bottom phase to this the top phase vesicle fraction (T1) and an equal volume of fresh top phase to the bottom phase vesicle fraction (B1). Thereafter the partition steps are considered extractions so that T1, for example, is extracted two more times with bottom phase to yield T3.

Vesicle isolation. Spinach (*Spinacia oleracia L.*) was grown at 20°C with a light period of 12 h and incident light intensity of 400 $\mu E/m^2$ per s. Chloroplast thylakoids were isolated as in Ref. 15. The thylakoids were subjected to sonication followed by aqueous two-phase partitioning by a batch procedure according to Ref. 15. This yielded the B3 and T3 fractions in the bottom and top phases, respectively. The vesicle phase suspensions were diluted with the buffer: 10 mM sodium phosphate buffer (pH 7.4)/5 mM NaCl/100 mM sucrose by at least 3-fold and pelleted at 100 000 $\times g$ for 90 min, after which they were resuspended in a small amount of the same buffer + 5% DMSO, the chlorophyll concentration read according to Arnon [16], and the sample stored in liquid nitrogen.

An additional phase system could be run along side the system described above so that a second B3 fraction could be used for isolating the margin fraction which was accomplished in the following manner. Fresh top phase (volume equalling approximately one-tenth the volume of the bottom phase) was added to the B3 bottom phase fraction and the contents were sonicated 6×30 s with 1-min resting intervals (180s) using the same ultrasonic exposure settings as for whole thylakoids, with a 1/2″ horn, under continuous cooling, all as described in Ref. 15. The sonicated B3 was either loaded onto a counter-current distribution plate for fractionation or separated according to the batch procedure below.

Batch procedure isolation of margins. Fresh top phase (equal volumes of top and bottom phases) was added to the sonicated B3 vesicles and one partition step was carried out. The resulting top and bottom phases constituted the 180s and B3-180s fractions respectively. The 180s fraction (which we denote as margins) could be cleaned of contaminating inside-out vesicles by means of three additional extraction steps to obtain a purified margin fraction defined here as 180sT3 (enriched margins). The corresponding bottom phase fraction deriving from 180s was referred to as 180sB3 (Fig. 1b).

Counter-current distribution. An apparatus where phase separation is speeded up by centrifugation was used [17]. The experiment was run exactly as is described in detail in Ref. 15.

Analysis. SDS-polyacrylamide gel electrophoresis was performed in the buffer system of Laemmli [18] in the presence of 4 M urea with an acrylamide gradient from 12 to 22.5%.

Proteins were dissociated by incubation in a water bath at 75°C for 5 min in the presence of 5% 2-mercaptoethanol (Carl Roth). The electrophoresis was run at 4°C.

Two-dimensional gel electrophoresis was carried out essentially as described by O'Farrell [19] with modifications which will be described in detail in a forthcoming publication (Yu and Albertsson, unpublished data).

The concentration of P700 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 $mM^{-1} cm^{-1}$ [20]. The reaction buffer contained 0.02% (w/w) SDS, 2 mM methylviologen, 2 mM sodium ascorbate, 15 mM Tricine (pH 7.8), 5 mM $MgCl_2$, 10 mM NaCl, 400 mM sucrose, and thylakoid vesicles for a final chlorophyll concentration of between 20–30 μM.

The rate of photooxidation of P700 was measured according to Melis [21] using an Aminco DW-2 spectrophotometer working in the dual wavelength mode. The measuring wavelength was set at 700 nm while the reference beam was set at 730 nm. The optical path length of the cuvette for the measuring beam was 10 mm and for the actinic beam 4 mm. In front of the photomultiplier was a RG 665 Schott filter, while in front of the actinic light source was placed a 566.9 nm bandpass filter, a cut-off 600 nm filter, and a neutral filter so that the light intensity was 30 $\mu E/m^2$ per s. Signal averaging was performed with a Nicolet instrument corporation model 527 instrument. Samples were first dark-adapted for 30 min and then incubated in a buffer containing: potassium cyanide (150 mM)/Tricine-KOH (pH 7.8, 90 mM)/$MgCl_2$ (1 mM)/sucrose (100 mM) at a chlorophyll concentration of 300 μM for 2 h on ice. After incubation the samples were diluted 10-times with the same buffer except without KCN and the kinetics measured in the presence of 50 μM DCMU and 200 μM methylviologen. The time course of absorbance decrease was evaluated by hand by drawing the best fit curve through the trace and the data plotted semilogarithmically as befits a first order reaction.

Cytochrome *f* was determined by an immunological method using antiserum raised in rabbits against cytochrome *f* from spinach (Sigma). The specificity of the serum was determined by Western blotting [22]. Rocket immunoelectrophoresis was run as described in Ref. 15. The concentration of the cytochrome *f* standard from Sigma was determined spectroscopically.

Fluorescence emission kinetics at 685 nm were measured at a right angle to the excitation light. The excitation light was filtered by two longpass filters in combination with a cut-off 600 nm filter to give green light spanning the 380–580 nm wavelength region. In addition, neutral filters were used to obtain a light intensity of 30 $\mu E/m^2$ per s. An interference filter (690 nm HBW 70 nm) was placed in front of the photomultiplier to block extraneous light. The fluorescence induction was performed at room temperature in the presence of DCMU (20 μM) using an Aminco DW-2 spectrophotometer. The signal was amplified by a Tetronix TM 503 differential amplifier and further processed by a nicolet signal averager model 527. After recording the trace, the half-rise time ($t_{1/2}$) of the area growth over the induction curve was determined. F_m was established by a second excitation sweep of longer duration (10 s). The reaction buffer contained: sucrose (400 mM)/NaCl (10 mM)/$MgCl_2$ (5 mM)/Tricine (pH 8.0, 50 mM) and membrane fraction (5 μg Chl/ml). Samples were dark-adapted for 5 min prior to excitation.

Proton translocation studies were carried out essentially according to Andersson et al. [23]. Sample frac-

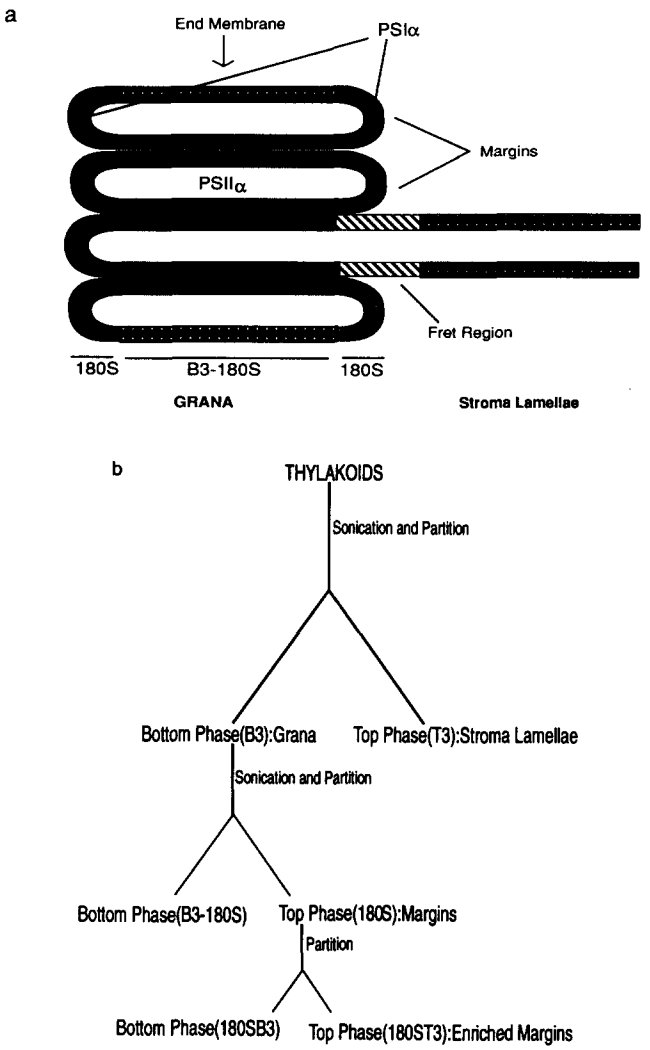

Fig. 1. (a) A schematic diagram illustrating the different domains of the thylakoid membrane plus the proposed origin of two sub-thylakoid fractions isolated by aqueous two-phase partitioning of sonicated B3 vesicles. The 180s population is heterogeneous yielding the 180sB3 and 180sT3 vesicle fractions, the latter of which is proposed to derive from the stroma exposed periphery of the grana; (b) a flow chart describing the batch preparation of the various sub-thylakoid membrane fractions. The (B3-180s) fraction represents the 'grana depleted of margins' (partitions).

TABLE I

The yields and chlorophyll *a*/*b* values for the membrane fractions described in Fig. 1b as obtained using the batch procedure of vesicle isolation

Fraction	Chlorophyll *a*/*b*	% of thylakoid	% of Grana (B3)
T3	4.4	22	
B3	2.4	41	100
B3-180s	2.3	24	60
180s	2.6	8	19
180sB3	2.4	3	8
180sT3	3.3	1	3

tions were washed twice using KCl (40 mM)/sucrose (400 mM) pelleting the samples each time at 100 000 × *g* for 1 h at 4°C. The samples were then resuspended in the same solution at 1.5 μg Chl/μl. A temperature-controlled light permeable reaction vessel was used for the measurements. The reaction medium contained in addition to the washing solution: phenyl-*p*-benzoquinone (PpBQ) (0.37 mM) and membrane fraction to 150 μg Chl/ml. The starting pH was 6.0 and the final volume was 1 ml. The pH electrode was immersed in the reaction medium and the medium illuminated using a slide projector fitted with broad band filters to provide red light between 600 and 700 nm. The maximum intensity was 500 $\mu E/m^2$ per s. A recorder was connected to the pH meter and the change in pH was traced with time.

Results

Preparation of margins

Partitioning in dextran-PEG aqueous two-phase systems is a technique which can be used to separate biomolecules and cellular particles based on their surface associated properties [24–26]. When thylakoids are fragmented by sonication and partitioned three times a bottom phase preparation of inside-out vesicles deriving from the grana region and denoted as 'B3' is obtained [15]. Thin sections of B3 vesicles range in diameter from 50 to 600 nm with an average length of 300 nm [9]. The vesicles therefore display the same average dimensions as for the original granal stacks [3]. Further sonication of the B3 population, in an aqueous two-phase system, results in a new vesicle population (which we refer to as 180s, Fig. 1b) which partitions to the top phase and has an average diameter 2–3-times smaller than the original B3 parent population (compare B3 with S3 in Fig. 3 of Ref. 9). The 180s (margins) population can be cleaned of contaminating inside-out vesicles by subjecting them to three additional extraction steps with fresh bottom phase so that a final top phase fraction is isolated, which is enriched 2.5-fold in PS I as compared to the B3 fraction, comprises 1–2% of the chlorophyll in the thylakoid and represents what we believe to be the outermost edges of the grana

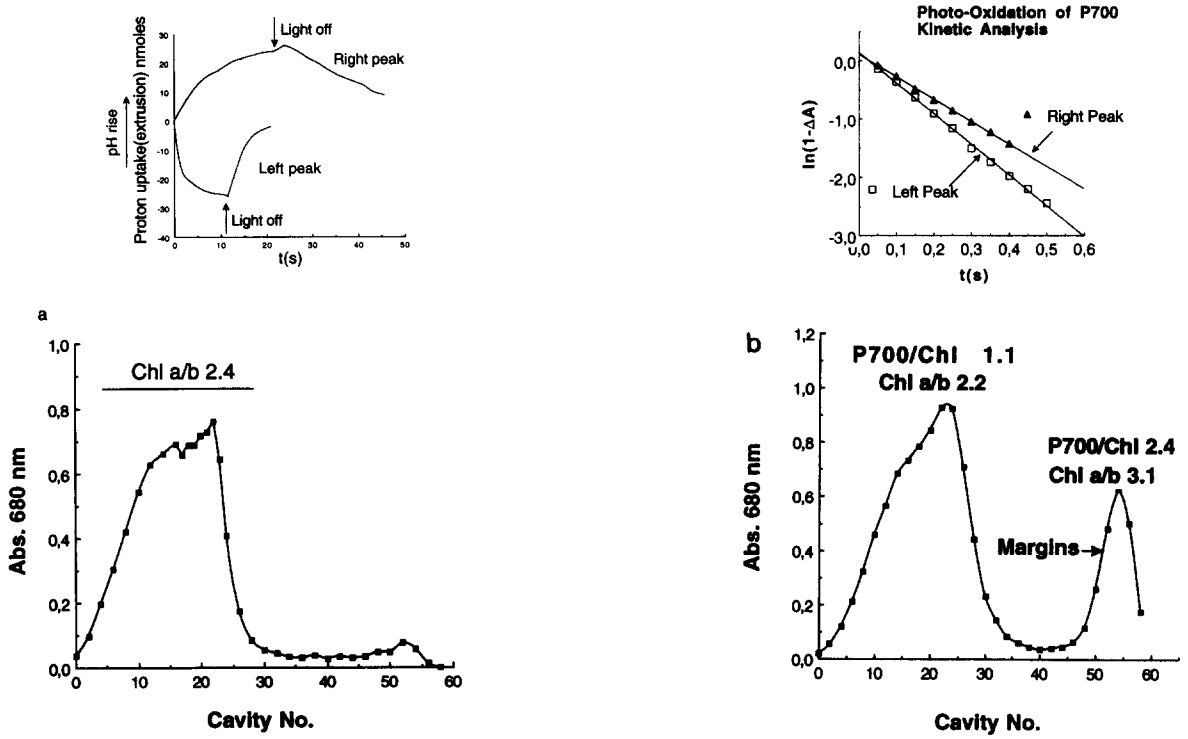

Fig. 2. (a) The counter-current distribution pattern of B3 vesicles (grana vesicles). The CCD apparatus was programmed for 53 transfers. The cavities comprising the bulk of the peak were pooled and the chlorophyll *a*/*b* ratio determined. (b) the CCD pattern obtained for sonicated B3 vesicles. Note the appearance of a new peak to the right (margins). The Chl *a*/*b* and P700/Chl (mM/M) properties for each of the vesicle populations are indicated above each peak. The peak to the left represents the remainder of the grana ('grana depleted of margins' (partitions)). (Inset upper left) Proton translocation measurments of vesicles pooled from each of the peaks. The light was turned on at time zero. Vesicles deriving from the right hand peak took up protons upon illumination, a behavior also observed for native intact thylakoids, thereby gaining the designation 'right-side-out'. Vesicles comprising the left-hand peak are 'inside-out'. (Inset upper right) Kinetic traces for time course of P700 photooxidation. The steeper slope observed for the vesicles of the left-hand peak reflects a faster rate of oxidation and hence larger antenna size for PS I.

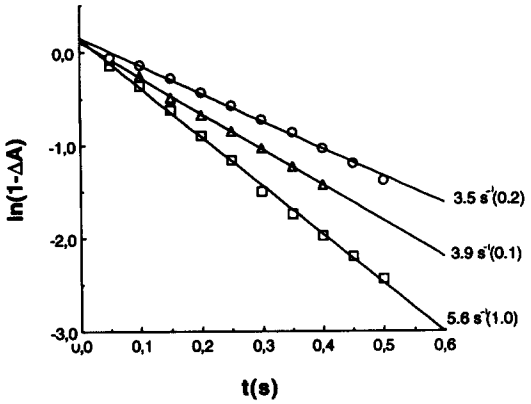

Fig. 3. The first order kinetic analysis of P700 photooxidation absorbance data for the grana depleted of margins (partitions) (squares), stroma lamellae (circles), and enriched margins (triangles) membrane fractions. Six different preparations were measured. The above values represent the average over all experiments and the standard deviations are given in the parentheses.

stacks. This membrane fraction is referred to as 180sT3 (enriched margins) (Fig. 1a and b). Table I presents the properties of these membrane fractions including the yields and chlorophyll a/b values.

Fig. 2a and b display the fractionation patterns obtained upon counter-current distribution (CCD) of the B3 starting material before and after sonication. This method allows one to analyze the surface heterogeneity of a vesicle population [24–26] and indicates in this case that our starting material, unsonicated B3, consists of essentially one major vesicle population. However, if the B3 membrane fraction is sonicated and then loaded onto the CCD apparatus, an additional peak appears to the right consisting of right-side-out vesicles as judged by proton translocation studies (see inset, upper left Fig. 2b). This new population (comprising approximately 20% of the chlorophyll in the grana) released by ultrasound then represents our candidate for the margin region. The fact that these two vesicle populations are so well separated makes it possible to devise a batch procedure for preparing the margin fraction, a procedure exactly analogous to the procedure used for separating sonicated thylakoids in the very first step (Fig. 1b and Materials and Methods).

P700 kinetics

It was of interest to know if the PS I contained in this new peak appearing after sonication of B3 vesicles was of the α sub-type [14,15]. This was determined by measuring the rate of photooxidation of the reaction center chlorophyll of PS I, P700. These data along with the relative concentrations of P700/Chl are given in Fig. 2b. The left peak, representing a 'grana depleted of margins' (grana partitions) fraction, contained a lower PS I/Chl as compared to the right peak, but the antenna size of the PS I population of the left peak was considerably larger than for the PS I partitioning with the putative margin fraction. This conclusion is reached by a first order kinetic analysis of the P700 photooxidation trace where the slope of the line is directly proportional to the antenna size. The difference is on the order of 30% (inset, upper right in Fig. 2b). However, when we compared all three of the primary regions of the thylakoid with respect to the PS I antenna size we found a modest (10–15%) but consistently faster rate of P700 photooxidation for the margins as compared to the stroma lamellae (Fig. 3). The average difference in antenna size between the PS I complexes located in the stroma lamellae and the 'grana depleted of margins' (partitions) membrane fractions was 38%.

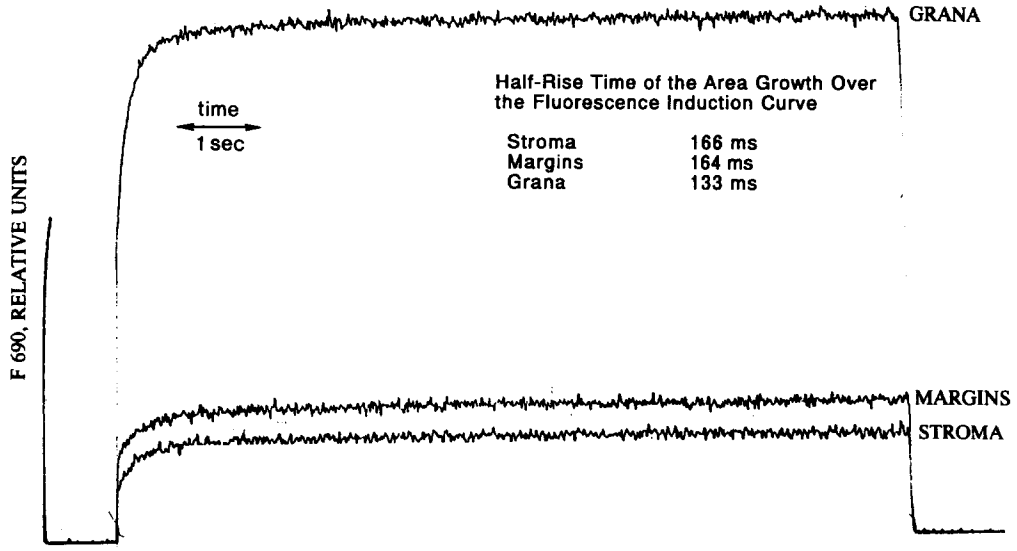

Fig. 4. The room temperature fluorescence induction curves for the grana partitions (B3-180s), stroma lamellae (T3), and enriched margin (180sT3) regions. The measured $t_{1/2}$ values are given in milliseconds.

PS II properties

We also examined the PS II properties of the enriched margin fraction and compared them to those of the stroma lamellae and grana membranes. Since the yield of the margins is so low (1–2% of the chlorophyll in the thylakoid using the batch procedure), we chose to use fluorescence induction as an economical means to measure both the antenna size as well as the relative concentrations of PS II present in each of the fractions [27–29]. Fig. 4 shows the three induction curves, measured at room temperature in the presence of DCMU, obtained for the three primary domains of the thylakoid, the 'grana depleted of margins' (partitions), the stroma lamellae, and the margins (enriched). Each trace can be directly compared since all were obtained at the same concentration of chlorophyll. The significantly greater maximum fluorescence intensity observed for the grana partitions compared to the stroma lamellae and margin fractions indicates not only the greater number of PS II centers per total chlorophyll in the grana partitions but also the larger antenna size of these centers. The margins emit only slightly more fluorescence than do the stroma lamellae vesicles. To estimate the antennae sizes of the three populations, we measured the fluorescence kinetics between 0 and 0.5 s (traces not shown). The half-rise time of the variable fluorescence portion of the induction curve (F_v), whose value is inversely proportional to the antenna size of PS II [11], was calculated (inset, Fig. 4) and indicates that the antennae sizes of the PS II complexes located in the grana margins and stroma lamellae are about the same.

Gel electrophoresis

The protein compositional differences amongst the three domains was studied with denaturing gel electrophoresis (Fig. 5). Five separate fractions are shown whose derivation is explained in Fig. 1b. The SDS-PAGE method is quite effective at revealing the polypeptide characteristics which distinguish the stroma lamellae (T3) and grana (B3) membrane regions from one another. The ATPase complex, found exclusively in the stroma-exposed areas of the thylakoid membrane [30,31], shows up as two intensely stained high-molecular-weight bands (subunits from the F1 portion) in the T3 fraction. In contrast, the B3 fraction is characterized by the abundance of LHC II due to the presence of the large antenna systems coupled to PS IIα and PS Iα in the grana domain [14,15,32]. Noteworthy as well are the PS II and PS I core-associated banding in the B3 and T3 fractions respectively. The enriched margin fraction (180sT3) seems to resemble both the appressed and non-appressed areas of the thylakoid depending upon which proteins one cares to examine. The dark banding of the ATPase complex suggests this fraction derives from a stroma-exposed

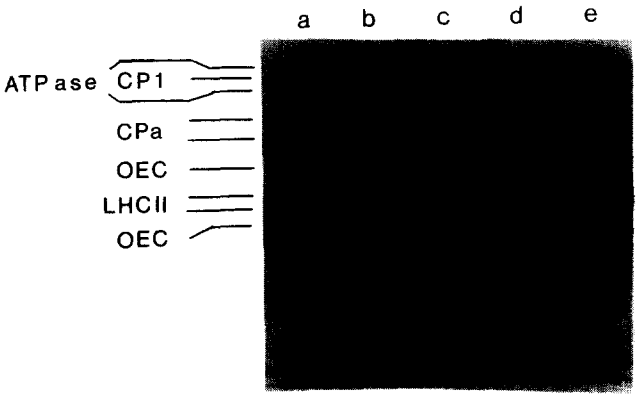

Fig. 5. SDS-PAGE fractionation of the (a) T3, (b) B3, (c) 180s, (d) B3-180s, and (e) 180sT3 membrane fractions (see flow chart in Fig. 1b).

region, yet the prominent PS II core banding resembles the grana regions. The LHC II proteins also stain darker in our margin fraction than in the stroma lamellae, further supporting its proximity to the appressed domain.

Higher resolution of the protein compositions for the three domains was obtained by two-dimensional gel electrophoresis. It has been shown that antibodies against the 64 kDa membrane bound kinase react against two protein spots focusing in the pH range 5.8–6.4, and that these spots stain darkest for the margin fraction of fragmented thylakoids (180s) as compared to the stroma lamellae (Y100) or central core of the appressed region (BS) [33]. These gels are also presented here for purposes of clarity, and as additional evidence in support of the domain character of the margin fraction which appears to be enriched in the LHC II protein kinase (Fig. 6). Note, however, that in this series of gels the T3 membrane fraction (Fig. 6D) represents the stroma lamellae and not Y100 as published previously.

Cytochrome f

A quantitative immunoassay was used to measure the amounts of cytochrome *f* present in each of the membrane fractions and the results plotted against the corresponding amounts of PS I in each fraction (Fig. 7). This type of approach to the study of domain organization in the thylakoid membrane, referred to as 'nearest neighbor analysis', was used previously by Albertsson et al. to show that when, with cytochrome *f* and PS I as domain markers, the stroma lamellae and sub-domains from the grana compartment, as well as unfractionated thylakoids, were compared, a triangular relationship was observed which was used to support the model that the thylakoid membrane consists of at least three domains, two of which are not nearest neighbors [13]. An identical analysis has been carried out here comparing our margin fraction to the stroma

lamellae and grana fractions (Fig. 7). It is clear that amongst these three membrane samples the enriched margins (180sT3) represent the most cytochrome *f*-depleted area of the thylakoid, a characteristic which distinguishes from either the grana core or stroma lamellae. We find here, just as was shown before by Andreasson et al. and Albertsson et al. [13,15], that the grana-derived fractions (B3, and B3-180s) are slightly

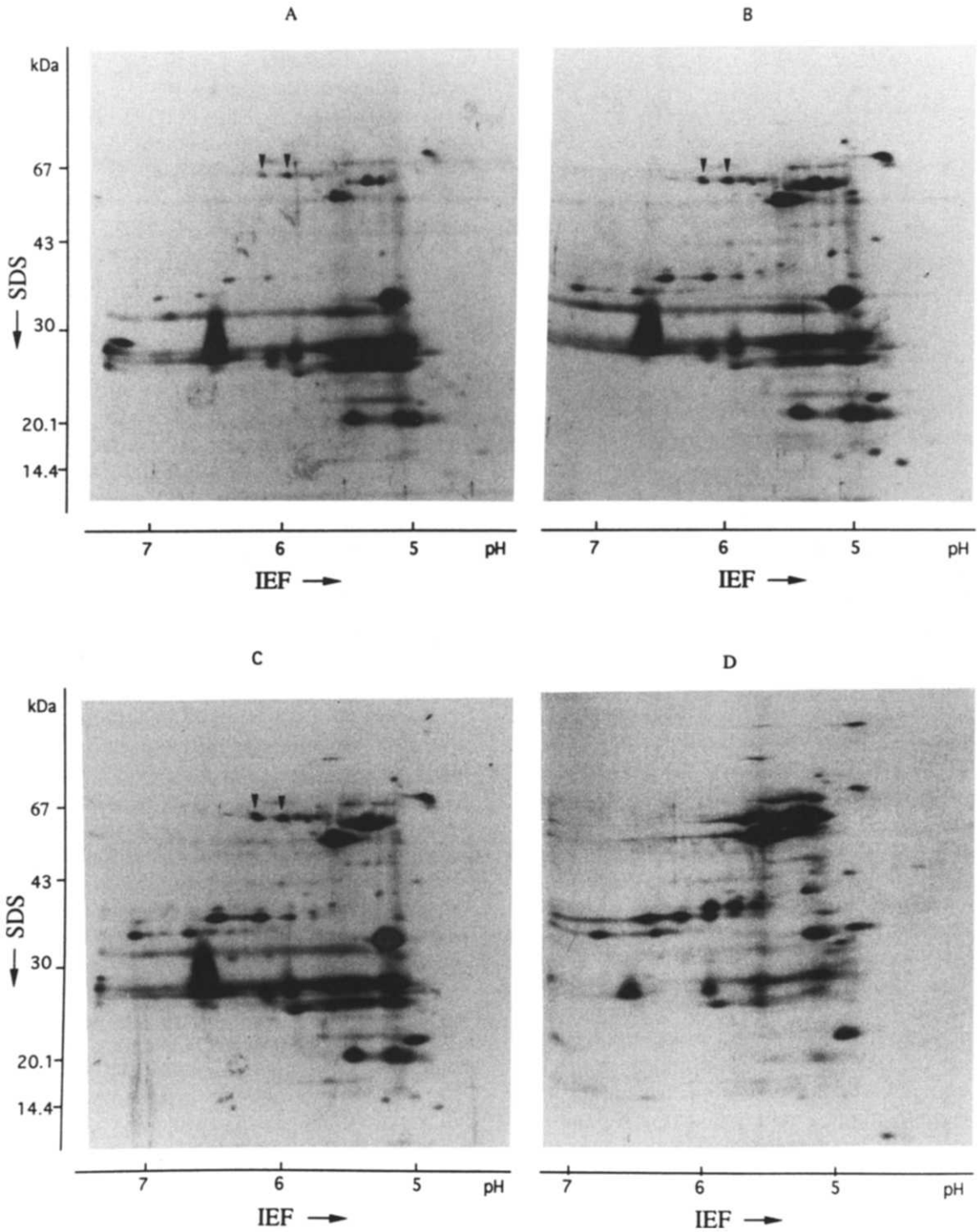

Fig. 6. Two-dimensional gel patterns of: (A) the central core of the grana (BS), (B) the region of the grana between the central core and the stroma (18×30S), (C) the margins (180s), and (D) the stroma lamellae (T3). The arrowheads indicate a group of polypeptides having apparent molecular mass of 64 kDa and focusing in the pH range 5.8–6.4 which tested positive against antibodies of the 64 -kDa kinase of spinach. The isolation and properties of the membrane fraction 'BS' are defined in Refs. 8 and 45. '18×30S' is obtained by sonicating the grana (B3) for the same period of time followed by a partitioning step (Chl *a* / *b* 2.3). Silver staining was used to visualize the proteins.

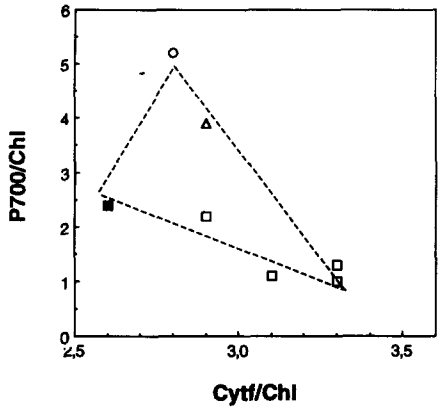

Fig. 7. A comparison of the P700/Chl (mM/M) and cytochrome f/Chl (mM/M) values of selected granal (squares), Y100 (circle), T3 (triangle), and 180sT3 (filled square) fractions. The triangular relationship is indicated where the stroma lamellae, enriched margins, and the central core of the grana represent the three corners (domains). The granal fractions include: B3, B3-180s, 180s, and 180sB3.

more enriched in cytochrome f as compared to the stroma lamellae (Y100, T3).

Discussion

The purpose of this study was to isolate a sub-thylakoid membrane fraction which was representative of the margin region, using the fragmentation-separation approach developed by this laboratory [8,13,15]. The properties of one potential candidate (the 180sT3 sub-thylakoid membrane fraction) are described here.

Our findings show that the 180sT3 vesicle population, deriving from the grana (B3) membrane fraction of sonicated thylakoids, is enriched in PS I, contains less PS II per chlorophyll than for the center of the grana but slightly more than for the stroma lamellae, and has the lowest concentration of cytochrome f per chlorophyll of all the sub-fractions examined. We suggest the 180sT3 membrane fraction represents a stroma exposed region of the granal stacks, possibly the outer edges of the margin area. This reasoning is based on the following evidence.

First, the margin population is only obtained by fragmenting (here sonicating) grana vesicles (B3) which implies that the margin vesicles originate from the grana continuum and are not just free stroma lamellae vesicles contaminating the B3 fraction (Fig. 2a and b). Furthermore, according to our model of the mechanics of membrane disruption by the sonication method, sonication of grana vesicles (B3) most likely removes the extreme edges first [9]. Since the average size of our grana vesicles (B3) equal that of the original grana stacks' [15], it is reasonable to assume that the outer edges of B3 vesicles include a major portion of the margin area.

Second, the margin fraction has a unique composition as shown by Figs. 6 and 7. It is relatively depleted in cytochrome f but enriched in the 64 -kDa kinase as compared to both the PS II rich vesicles (B3-180s or BS) originating from the appressed regions and the stroma lamellae vesicles (T3) originating from the non-appressed regions. The enrichment of the LHC II kinase in the margins has been shown by others who used immuno-electron microscopy to localize the enzyme on whole thylakoids [34]. These results together with ours suggest that the LHC II kinase can be used as a marker for the grana margins. This unique protein composition of the 180sT3 membrane fraction is evidence that the margins represent a distinct domain in the thylakoid.

Third, the enriched margin fraction derives from a PS I rich sub-domain of the grana disc comprising at least 20% of the chlorophyll in the grana (Fig. 2b). Since the grana and stroma lamellae domains comprise 60% and 40% of the chlorophyll in the thylakoid, respectively [15], it means that the margin fraction comprises 12% of the total thylakoid or 23% of the non-appressed membrane domain (stroma lamellae + margins). These values agree favorably with earlier estimates of Anderson et al. [6] and Murphy [35] who suggested that the granal margins may comprise between 11 and 30% of the non-appressed domain.

Other reports concerning the domain organization of the thylakoid have proposed a site of cytochrome b_6/f enrichment occurring along the fret region [36–38]. If this is the case then the fraction we have isolated and characterized here is certainly not the vestiges of the frets retained by the inside-out vesicles after their initial fragmentation from the stroma lamellae.

Although the chloroplast grana margins are slightly depleted in the cytochrome b_6/f complex in relation to the grana core and stroma lamellae, it should be stressed that the depletion is only relative; a significant amount of the complex still exists in the margin area (2.6 mM/M Chl by the immunological method), and at this time there is no reason to believe that this deficiency is sufficient to prevent its apparent role as an activator of the 64 kDa LHC II kinase [34].

It has been known for some time that the grana contain a considerable amount of PS I [15,39–41]. For example, using mechanical fragmentation, Sane et al. [40] reported in 1970 that while PS I could be isolated essentially free from PS II, the PS II containing heavy or grana fraction always contained some PS I which, in whole spinach chloroplasts, '... is located in grana regions, [and] exists in much closer association with PS II than the second type of Photosystem I, which is located in the stroma lamellae.' An immune-labelling study of PS-II-rich BBY particles showed that there may exist an outer PS I-rich area surrounding the PS II core of

the grana when it was demonstrated that antibodies formed against the PS I reaction center bound to the periphery of the PS II particles [42]. Atta-Asafo-Adejei et al. showed that the addition of exogenous plastocyanin to inside-out (B3) vesicles stimulated whole chain electron transport by over 400% [41]. It was concluded by the authors that these PS I centers participating in the latent electron transport lay close enough to the appressed region of the grana to partition with the granal PS II centers after fragmentation by yeda press.

In recent publications from this laboratory we have shown that about 36% of the PS I centers are located in the grana [1,15]. Furthermore, the PS I from the grana (PS Iα) is distinct from that of the stroma lamellae (PS Iβ) in that PS Iα has a larger antenna size with more Chl *b* than PS Iβ [14,32]. About 40% of the grana chlorophyll is associated with PS Iα [1,43]. It was suggested that PS IIα and PS Iα of the grana carry out oxygenic non-cyclic electron transport while PS Iβ of the stroma lamellae catalyzes cyclic electron transport [1,43].

We find that the PS I population contained in the enriched margin fraction (180sT3) has a slightly larger antenna size (10–15%) as compared to the PS I in the stroma lamellae, but that its antenna size does not approach that observed for the PS I remaining behind in the grana (30% larger than for PS I in margins) (Fig. 3). Using values of moles Chl/P700 obtained from measurements of stroma lamellae vesicles [15,43] and correcting for the presence of PS IIβ in these vesicles, one can estimate that roughly 200 chlorophyll molecules are associated with the smallest native PS I unit (PS Iβ) in the thylakoid membrane [1,43]. The addition of one LHC II trimer containing 42 molecules of chlorophyll (14 chlorophylls per monomer; Kühlbrandt, personal communication) would increase the antenna size by about 21%. An additional LHC II trimer would increase the antenna size by 42% compared to PS Iβ. It is conceivable that we are detecting a PS I center of intermediate antenna size (240 Chl) as compared to the large PS Iα (280 chl, 'grana depleted of margins' (partitions), Fig. 1b) and small PS Iβ (200 Chl, stroma lamellae).

In addition, the kinetics of room temperature variable fluorescence indicates that the PS II centers in the margin membrane are, with respect to the antenna size, like the smaller β subtype since the half-rise times of the variable fluorescence of both the stroma lamellae, known to contain primarily PS IIβ [44], and margin fractions were quite similar (Fig. 4).

Fractionation of the polypeptide components of the membrane populations by SDS-PAGE revealed that our margin vesicles contain a significant amount of LHC II in contrast to the Tween-20 fraction of Webber et al. [7] (Fig. 5). Laser densitometry (not shown) confirms that the 180sT3 fraction contains more of the 27 and 25 kDa LHC II polypeptides than do the T3 (or stroma lamellae). We interpret this to mean that the extra amount of LHC II found in the margins over and above the amount normally encountered in the stroma lamellae is functionally coupled to PS I and not to PS II, since the sizes of the antennae attached to PS II of margins and stroma lamellae do not differ whereas the sizes of the antennae attached to PS I do (180sT3 slightly larger, Fig. 3). Further support for this reasoning comes from a study by Andreasson and Albertsson, who showed that PS Iα is coupled to a special pool of LHC II and that this special pool was enriched in the margin domain [32].

Finally, the initial report by Svensson and Albertsson concerning the domain organization of the grana compartment indicated that those regions of the membrane enriched in PS I were also enriched in the coupling factor (CF1) [8]. In return, others have used immuno-electronmicroscopy to show that in spinach thylakoids the coupling factor is present on all the exposed surfaces of the membrane including the grana margins [30,31]. Consolidating these data, one is led to the conclusion that both the ATPase complex and PS I reaction center are present in the grana margins. The SDS-PAGE analysis of our fractions shown in Fig. 5 fits this model.

In summary, the unique composition of the enriched margin fraction speaks against the possibility that it is a mixture of vesicles originating from both the grana and stroma lamellae. Rather it suggests that the enriched margin fraction (180sT3) originates from a distinct domain of the thylakoid.

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