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Purification of structurally intact grana from plants thylakoids membranes

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Abstract Thylakoid membranes in higher plant chloroplasts are composed by two distinct domains: stacked grana and stroma lamellae. We developed a procedure for biochemical isolation of grana membranes using mild detergent to maintain membrane structure. Pigment and polypeptide analyses of membrane preparation showed the preparations were indeed enriched in grana membranes. The method was shown to be effective in four different plant species, although with small changes in detergent concentration. Electron microscopy analyses also showed that the preparation consisted of large membrane patches with roughly round shape and diameter comparable with grana membranes in vivo. Furthermore, protein complexes distribution was shown to be maintained with respect to freeze fracture studies, demonstrating that the protocol was successful in isolating membranes close to their in vivo state.

Keywords Photosystem · Grana partitions · Thylakoids membrane · Electron microscopy · Detergent solubilization

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Introduction

Thylakoid membranes are folded into a physically continuous three dimensional network enclosing an aqueous space called lumen. They are structurally inhomogeneous and two distinct domains are distinguishable: 80 % of the membrane is included into structures called grana, which are composed by stacked membrane disks with a diameter of about 0.5 µm; individual grana are connected by non stacked membrane called stroma lamellae (Mustardy and Garab 2003; Shimoni et al. 2005). Grana can be further differentiated into the "grana partitions", inner regions of the stacked membranes, segregated from the stromal solution, and the grana margins, the peripheral, curved, regions connecting two partitions on opposite sides of the lumen, which are in contact with the stromal compartment. Protein complexes of photosynthesis are found non homogenously distributed within thylakoid membranes: Photosystem II (PSII) and its associated light harvesting system (LHCII) are concentrated in the grana partitions, while Photosystem I (PSI) and ATPase are localized in the stroma lamellae (Albertsson 2001; Dekker and Boekema 2005). Physical separation of PSI and II has been proposed essential for efficient photosynthesis: in fact, since trapping of excitation energy at the PSI reaction centers is much faster than at the PSII, in case of a close contact there would be uncontrolled energy flow from PSII to PSI (Trissl and Wilhelm 1993). Instead, cytochrome (Cyt) b_6f can be found in both stacked and non-stacked membranes (Albertsson 2001; Dekker and Boekema 2005) and can change its partition between the two domains depending on physiological state (Vallon et al. 1991). Although lateral distribution of membrane proteins is probably the result of multiple factors, steric hindrance of the extramembrane portions of complexes has been proposed to play a major

role; in fact, unlike PSII, PSI and ATPase have large stroma exposed domains which are excluded from tightly appressed grana partitions (Borodich et al. 2003; Dekker and Boekema 2005). Besides stroma lamellae, PSI was suggested to be present also in grana margins, where there is no size limitation for stromal exposed domains (Albertsson 2001; Danielsson et al. 2004). This view was however challenged by the observation that the strong membrane curvature in the margins might be incompatible with insertion of large protein supercomplexes (Murphy 1986; Dekker and Boekema 2005).

Structural information is a necessary pre-requisite for the elucidation of function and regulation of photosynthetic complexes and a growing set of data becomes available at molecular and supramolecular level (see (Nelson and Ben Shem 2004; 2005; Dekker and Boekema 2005; Barber 2006) for recent reviews); instead the overall architecture of the thylakoid membrane and its dynamics during transitions between physiological states largely needs to be elucidated. As an example, the distribution of PSII supercomplexes within grana membranes has been shown to be regulated and depends from growth light conditions, thus suggesting a functional role (Kirchhoff et al. 2004; Kirchhoff et al. 2007; De Bianchi et al. 2008). This hypothesis found very recently experimental support as it was shown that PSII distribution in grana is altered upon light treatment in a process closely correlated with the activation of a photoprotection mechanism called NPQ (Non Photochemical Quenching) (Betterle et al. 2009). Further interest in thylakoid organization has been raised by the observation that grana stacking was reported to be generally absent in most algal species which, although showing some degree of thylakoid appression, do not have the complex organization found only in more recently evolved taxa of the Streptophyta line (Coleochaetales and Charales) and in higher plants (Larkum and Vesk 2003; Gunning and Schwartz 1999). Same organisms were also shown to present differences in the mechanisms for photosynthesis regulation, suggesting that lateral heterogeneity is fundamental for an adaptation to aerobic environment (Horton 1999; De Bianchi et al. 2008; Alboresi et al. 2008).

Protocols for isolation of grana membranes and stroma lamellae have been largely employed in biochemical and spectroscopic studies. These methods are particularly challenging because they aim to isolate pure membrane particles while maintaining as much as possible their native state. In this work we present an optimization of the well known and largely employed method of grana isolation described by (Berthold et al. 1981), also named BBY after the authors names. One major modification in our protocol is the use of a very mild detergent, α-dodecylmaltoside (α-DM), which was already proved suitable for solubilization of photosynthetic complexes in their native state (van Roon et al. 2000; Dekker et al. 2002). Detergent solubilization was also optimized for different plant species, which showed slightly different behavior, probably because of small differences in thylakoids architecture, such as grana size. Another relevant modification is a decreased speed of centrifugation in a specific step, which allows selection for larger membrane patches. Biochemical characterization of purified membranes shows enrichment in PSII and depletion in PSI and ATPase polypeptides as expected for grana partitions. Membranes also showed PSII oxygen evolution activity very similar to the one observed with the Triton X 100 based method. This preparation is thus substantially equivalent with the previous but for one main advantage, its capacity of maintaining membrane intactness: EM analyses, in fact, showed that isolated grana membranes have sizes comparable to grana in leaves. Furthermore, PSII distribution within the membrane was shown to be maintained as observed in vivo by freeze fracture analyses, making this method very suitable to analyze protein distribution in mutants and plants treated with different light conditions.

Material and methods

Grana partitions isolation. Arabidopsis thaliana, maize (*Zea mays*), barley (*Hordeum vulgare*, WT and viridis *zb63* mutant) and spinach (*Spinacia oleracea*) plants were grown at 100 μE m^{-2} s^{-1}, 19 °C, 90% humidity and 8 h of daylight. Thylakoids were isolated from intact chloroplasts as in (Bassi et al. 1988) and washed twice with respectively 150 mM NaCl and 5 mM $MgCl_2$ 20 mM HEPES pH 7.5 and 15 mM NaCl and 5 mM $MgCl_2$ 20 mM HEPES pH 7.5 in order to maintain the granal stacking (Berthold et al. 1981). Thylakoids were resuspended in the latter solution at 1 mg Chl/ml and solubilized at 4 °C for 20 min in slow agitation with different amounts of α-DM ranging from 0.32 to 0.95 % (w/v), always in the presence of 15 mM NaCl, 5 mM $MgCl_2$ and 20 mM HEPES pH 7.5. Spinach control samples were solubilized at 2 mg Chl /ml with 25 mg Triton X-100 / mg Chl as in (Berthold et al. 1981). In the case of other species, lower Triton concentrations (10 mg /mg Chl) were employed to avoid over-solubilization. Unsolubilized thylakoids were pelleted by centrifugation at 3500×g for 5 min. Partially solubilized grana membranes were instead pelleted with a further 30′ centrifugation at 30000 × g (40000 × g in the case of Triton solubilization). Solubilized complexes and small membrane patches remained instead in the supernatant. Membrane pellet was washed with 15 mM NaCl and 5 mM $MgCl_2$ 20 mM HEPES pH 7.5, centrifuged for 30′ at 30000 × g and finally resuspended in 0.4 M sorbitol, 15 mM NaCl and 5 mM $MgCl_2$ 20 mM HEPES pH 7.5.

Biochemical characterization. SDS-PAGE analysis was performed with a Tris—Glycine buffer system as in (Laemmli 1970) with 8 M urea and acrylamide concentration of 12 %. Pigment composition was determined by HPLC analysis (Gilmore and Yamamoto 1991) and by fitting the acetone extract absorption spectra with those of the individual pigments, as described in (Croce et al. 2002). Spectra were recorded using an SLM-Aminco DW 2000 spectrophotometer, in 80 % acetone. Oxygen evolution was measured with an Clark electrod as in (Teardo et al. 2005; Pagliano et al. 2009). Total P700 content was determined from the ferricyanide oxidized minus ascorbate reduced difference spectrum as in (Jensen et al. 2002).

Electron microscopy. Electron microscopy (EM) on isolated grana membranes was conducted using a FEI Tecnai T12 electron microscope operating at 100 kV accelerating voltage. Samples were applied to glow-discharged carbon coated grids and stained with 2% uranyl acetate. Images were recorded using a CCD camera.

Results and discussion

Isolation of grana partitions

Thylakoids membranes were isolated in stacking conditions from four different plant species: spinach, *Arabidopsis*, maize and barley. These membranes were then utilized as starting material for purification of grana partitions. In our protocol stacked thylakoid membranes were treated with α-DM, which already proved suitable for isolation of photosynthetic complexes (van Roon et al. 2000; Dekker et al. 2002). Different detergent concentrations, ranging from 0.32 to 0.63% (w/v) were initially employed with the aim of partially solubilizing thylakoids membranes. In these conditions, detergent concentration is limiting with respect to the membrane lipids and only the solvent exposed regions of the membrane are expected to be accessible to detergent. In stacked thylakoids, grana partitions are largely masked from the stromal solution and thus stroma lamellae and grana margins will be preferentially subjected to the detergent action. After 20 min solubilization at 4 °C, unsolubilized thylakoids were spun out by centrifugation. A second centrifugation, run at higher speed, was employed to precipitate the largest membrane patches, whereas smaller membrane fragments and solubilized complexes remained in the supernatant.

In order to find the best compromise between solubilization of the stroma exposed portions of the membrane and maintenance of the grana native state, we tested different detergent concentrations.

A further optimization with respect to the original protocol (Berthold et al. 1981) was the use of a reduced centrifugation speed for the separation of grana membranes, 30000 g instead of 40000. At this speed, in fact, only the largest membrane patches were found in the pellet, while the smaller ones remained in the supernatant. The relevance of this point was evidenced when we compared our results with those obtained by solubilization of thylakoids with Triton X-100 as in (Berthold et al. 1981). In the latter case, the pellet after centrifugation at 30000 g was almost absent, suggesting that membrane particles obtained with Triton solubilization were too small to be spun out in these conditions. Thus, our preparation is selective for large membrane patches as confirmed below.

Grana partitions are depleted in PSI and ATPase polypeptides

Membranes obtained with this modified protocol were first analyzed biochemically in order to test their actual enrichment in grana partitions. We first evaluated polypeptide composition of the different fractions on SDS PAGE. In Fig. 1a, the polypeptide profile of preparations from spinach using different detergent concentration are shown and compared with unfractionated thylakoids used as a starting material. In the upper part of the gel bands corresponding to PSI core (PsaA and B) and ATPase subunits, as identified by western blotting, are clearly visible. Interestingly, these bands are progressively reduced increasing detergent concentrations during solubilization. At 0.63 % α-DM, ATPase subunits are not detectable anymore, while PSI core subunits are largely depleted. As the detergent concentration increases, a relative enrichment in the bands corresponding to PSII core and LHCII is clearly observed, indicating an increased relative amount of these complexes in our preparation as compared with thylakoids. In the last lane, the supernatant of the first centrifugation at 30000 g is also shown, containing the part of the sample which was better solubilized and did not precipitate at this speed. In this fraction, PSI and ATPase polypeptides are clearly enriched demonstrating the preferential solubilization of stroma with respect to grana partitions. In Fig. 1b sample obtained with α-DM solubilization are compared with those obtained with Triton X-100 solubilization and a very similar depletion of ATPase and PsaA/B polypeptides is observed. In order to further confirm this point we also measured PSI content spectroscopically and found 22.4±6.0 and 17.4±4.6 fold depletion in grana membranes solubilized with α-DM and Triton X-100 with respect to thylakoids.

Pigment content of the same samples was analyzed by fitting of the acetone extract spectra (Croce et al. 2002), which allows accurate determination of Chl a/b and Chl/Car

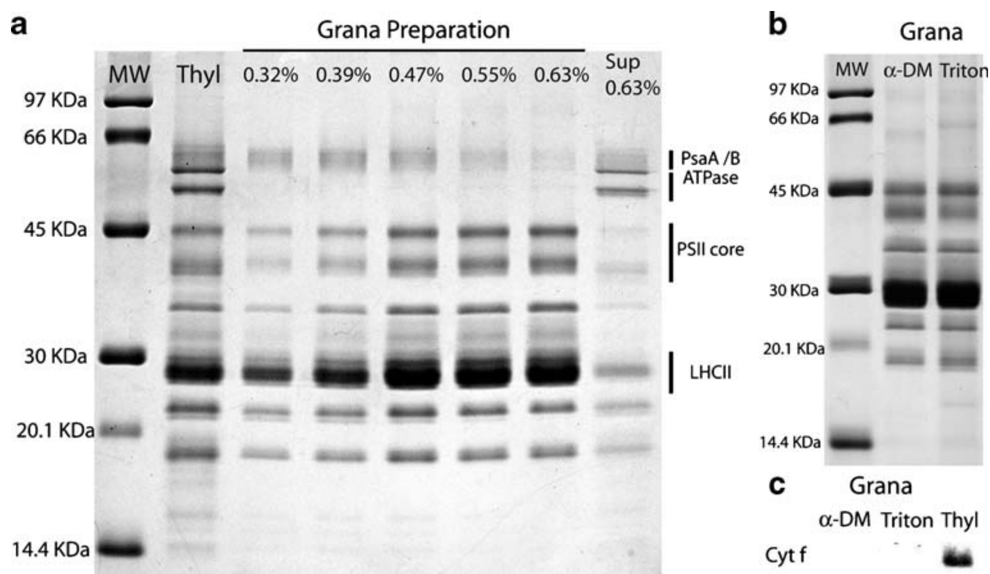

Fig. 1 Polypeptide composition of grana membranes isolated from spinach. **a** SDS PAGE profiles of different grana preparation obtained with variable detergent solubilization are compared with purified thylakoids (Thyl) used as starting material. The supernatant fraction, representing the solubilized material is also shown. 5 µg of Chls were loaded for each sample. Bands corresponding to PSI, PSII core polypeptides, ATPase and LHCII antenna polypeptides are indicated. Molecular weights reference (MW) is also shown. **b** Comparison of SDS-PAGE profile of grana preparation obtained with α-DM (0.63%) and Triton X-100. **c** Cytochrome f content in grana membranes (obtained with α-DM and Triton X-100 solubilization) and thylakoids analyzed by western blotting using antibody raised against *Chlamydomonas reinhardtii* Cytochrome f

ratios. As shown in Table 1, Chl a/b ratio decreases with the increasing detergent concentration. This is consistent with the observed decrease in PSI and increase in LHCII polypeptides content. In fact, given the Chl a/b ratio of 10 for the PSI-LHCI complex and of 1.4 for LHCII (Ballottari et al. 2004), a lower Chl a/b ratio is expected for grana enriched membranes as compared to intact thylakoids.

Polypeptide composition and pigment data allow concluding that our preparation was successful in isolating PSII enriched membranes with reduced PSI and ATPase content. In spinach, the best results were obtained with 0.63 % α-DM solubilization. Higher detergent concentration were also tested (0.71, 0.79 and 0.95 %), but no increase in purity was observed; on the contrary, a strong decrease in grana membranes yield was obtained, suggesting an excessive solubilization.

In order to further verify the quality of our best grana preparation (0.63 % sample) we quantitatively evaluated PsaA, β subunit of ATPase by using specific antibodies. Western blotting for the case of ATPase is reported in Fig. 2a: as shown, in order to obtain an antibody signal close to the one obtained loading 0.35 µg Chl from thylakoids, about 12 Chl µg from the grana preparation were needed. This is clearly shown by the densitometric analysis of the antibody signal, also reported in Fig. 2. The good linearity of the antibody response within the range of loaded amounts allowed us to estimate a 34 fold depletion of ATPase polypeptides in our grana preparation with

Table 1 Pigment binding properties of grana preparations. Chl a/b and Chl / Car ratios of thylakoids (Thyl) and grana membranes from spinach, obtained after different detergent treatments, are reported above. In the lower part, Chl a/b ratios are compared between thylakoids and the best grana purification in different species. Standard deviation in all data is below 0.1 for Chl a/b and 0.2 for Chl/ Car

Spinach	Thyl	α-DM 0.32%	α-DM 0.39%	α-DM 0.47%	α-DM 0.55%	α-DM 0.63%	Triton X-100
Chl a/Chl b	2.6	2.4	2.4	2.2	2.2	2.1	2.0
Chl/Car	3.7	3.8	4.0	4.1	3.9	4.1	3.2

Chl a/Chl b	Spinach	*Arabidopsis*	Maize	Barley
% alpha DM	0.63	0.47	0.39	0.39
Thylakoids	2.6	2.8	3.5	3.0
Grana	2.1	2.6	2.4	2.0

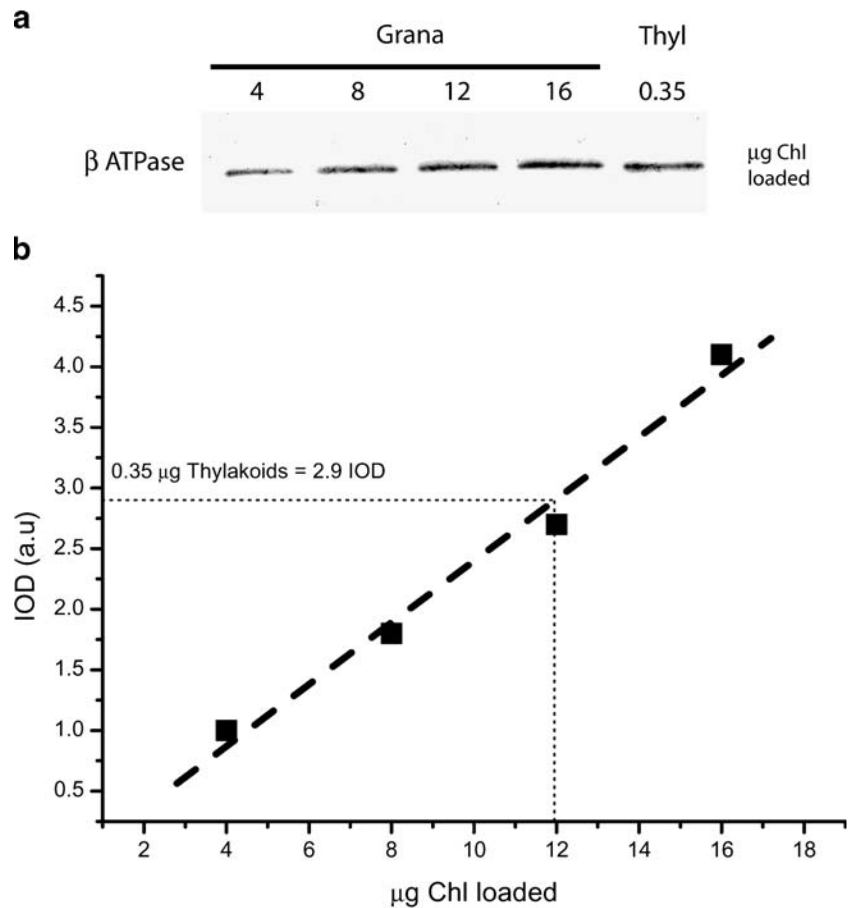

Fig. 2 ATPase beta subunit quantification in spinach grana preparation. **a** ATPase content in spinach grana preparation obtained with 0.63 % α-DM solubilization was quantified by using antibodies recognizing specifically β subunit. Different amounts (respectively 4, 8, 12 and 16 µg of Chls) were loaded in the same gel together with 0.35 µg of thylakoids. **b** Densitometric quantification of antibody signal in the western blotting. As shown, in the observed range antibody signal is linear with loading amounts. As indicated, signal obtained from 0.35 Chl µg of thylakoids is equivalent to approximately 12 Chl µg of grana preparation, allowing the calculation of ratios reported in Table 2

respect to the starting thylakoids (Table 2). The same method was employed to quantify PsaA polypeptides which resulted to be depleted by a factor 28, in good agreement with results from spectroscopic quantification of P700.

Cytochrome b6f is believed to be more or less evenly distributed in both stroma and grana membranes (Olive et al. 1986). Nevertheless, cytochrome b6f polypeptides have been shown to be depleted in grana membranes purified upon Triton X-100 solubilization, differently from the ones obtained from sonication (Albertsson 1995; Albertsson et al. 1991). We tested the presence of Cyt b6f in our preparations by western blotting analysis and, as shown in Fig. 1c, we observed a large depletion of Cyt f in purified membranes, confirming that detergent treatment with both Triton X-100 and α-DM is causing its specific depletion, as already suggested (van Roon et al. 2000). In the case of Cyt f we did not reach a precise estimation of its content, but we can estimate this polypeptide is at least 100 times depleted in grana purifications, as reported in Table 2. The same pigment and western blotting analyses have been performed for grana partitions solubilized with Triton X-100: results in terms of purity were substantially equivalent, as judged both from pigment and western blotting data which are reported in Table 1.

Finally, in order to prove effectiveness of our preparation we also tested the capacity of maintaining oxygen evolution activity, as shown in the case of Triton solubilized membranes (Berthold et al. 1981). Grana particles isolated

Table 2 Quantification of PsaA, β ATPase and Cyt f depletion in different preparations. PsaA and β ATPase polypeptides have been quantified in grana membranes purified from different species and compared with the thylakoids content using quantitative western blotting, as shown in Fig. 2

Species	Spinach	Spinach (Triton X-100)	*Arabidopsis*	Maize	Barley
% alpha DM	0.63	–	0.47	0.39	0.39
ATPase fold depletion	34.2 ± 4.5	> 50	26.0±7.2	27.2 ± 3.5	20.8 ± 2.3
PsaA fold depletion	28.0 ± 1.4	27.2 ± 1.5	6.4 ± 2.4	14.7 ± 1.6	10.3±0.8
Cyt f depletion	>100	>100	41 ± 8	>100	>100

with both detergent treatments showed similar oxygen evolution activity, respectively 90.7±7.7, 84.9 ± 16.8 and 107.3 ± 13.1 μmol h^{-1} mg Chl^{-1} for grana solubilized with Triton X-100, with α-DM and thylakoids, showing both methods have equivalent capacity of maintaining PSII activity. Concerning functionality, it is worth mentioning that PSII within grana membranes prepared with this protocol were also proved to be functional and in particular to present induction of fluorescence curves close to the one measured in leaves (De Bianchi et al. 2008).

Each plant species requires individual optimization of detergent concentration

As mentioned, the same method was applied to three other plant species largely employed in plant research studies: *Arabidopsis*, maize and barley. The analysis of different species is interesting because these species are characterized by slightly different optimal growth conditions, grana sizes, number of stacked membrane layers and Chl to lipids ratios. This is therefore a test for the robustness of the protocol and its adaptability to different thylakoid morphologies. In fact, detergent concentrations reported in (Berthold et al. 1981) are optimal for the case of spinach but in other species such as Arabidopsis they were too harsh and causes a very low yield in grana membranes. For this reason, in the case of *Arabidopsis*, maize and barley, reference membranes purified with Triton X-100 solubilization were obtained using a lower detergent concentration.

In all cases we tested the same range of α-DM concentrations and analyzed the preparations for their polypeptide composition by SDS PAGE, western blotting and pigment content to evaluate the residual content in PSI, Cyt f and ATPase polypeptides, as shown in the case of spinach. Interestingly, optimal detergent concentration was found to differ among species: while solubilization with 0.47 % α-DM worked reasonably well for all species and can be considered as the best overall, the optimal solubilization was found with 0.47 % in *Arabidopsis* and 0.39 % in maize and barley, as compared with 0.63 % for spinach. Also with α-DM solubilization, thus, spinach appears to be peculiar with respect to other species. Data from the best preparation for each species are reported in Tables 1 and 2. Pigment and polypeptide quantifications demonstrate that in all species the procedure was effective in enriching PSII and LHCII while reducing PSI and ATPase content, although with slightly different performances. In fact, we observed significant reduction in Chl a/b indicating enrichment in PSII-LHCII particles with respect to PSI, confirmed also by the spectroscopic quantification of P700 content. For all species, SDS PAGE profiles also showed reduction in PSI and ATPase polypeptides bands (not shown). Quantitative evaluation of these polypeptides by Western blotting, reported in Table 2, also confirms the effectiveness of the procedure. For all species we also compared these results with the one obtained with the Triton X-100 solubilization. Results in terms of purity and functionality were similar, as shown in more detail in the case of spinach. Membranes treated with the two detergents were also verified to be capable of oxygen evolution.

Finally, we tested the effect of membrane freezing on the procedure performances. This was done by solubilizing spinach thylakoids previously frozen in the presence of 0.4 M sorbitol as cryo-protectant. Even in this case, the preparation yielded PSII enriched membranes with significant depletion in PSI and ATPase. Results, however, were slightly poorer with respect to freshly isolated thylakoids. It is also worth noticing that in spinach we observed better results with a 0.47 % α-DM respect to the 0.63% for freshly prepared thylakoids, suggesting that frozen membranes are somehow more accessible to detergent.

PSII supercomplexes distribution is maintained during membranes isolation

Membranes prepared by the method presented here were proven to be enriched in Photosystem II particles and have similar levels of purity and functionality with respect to ones with Triton X-100 solubilization. As discussed previously, the aim of the protocol optimization, however, was to purify grana membranes with protein complexes organization as close as possible to that in vivo.

To this aim, we verified by EM analysis the morphology of grana membranes isolated by this method. The first observation was the size of isolated membranes: in all species we detected several large sized patches, with a diameter of several hundreds of nm (Fig. 3a) which compares favorably with the measured size of 300 – 600 nm for the grana in intact chloroplasts (Kaftan et al. 2002; Mustardy and Garab 2003). In addition to size, it was also common to observe distinct connected grana discs, which still maintained part of the grana margins connecting different layers (Fig. 3b and c). If grana margins are not completely solubilized but they are partially retained in the preparation, we may expect that grana partitions are even less affected by the solubilization procedure and may also account for the observed presence of residual PSI and ATPase contaminations. This finding, however, also suggests that the preparation guarantees good preservation of the in vivo organization of the complexes within the grana partitions.

As mentioned, other protocols with α-DM solubilization have been described. These differ with the present because they used a higher detergent concentration and purified membrane patches by size exclusion chromatography (van Roon et al. 2000). EM images of

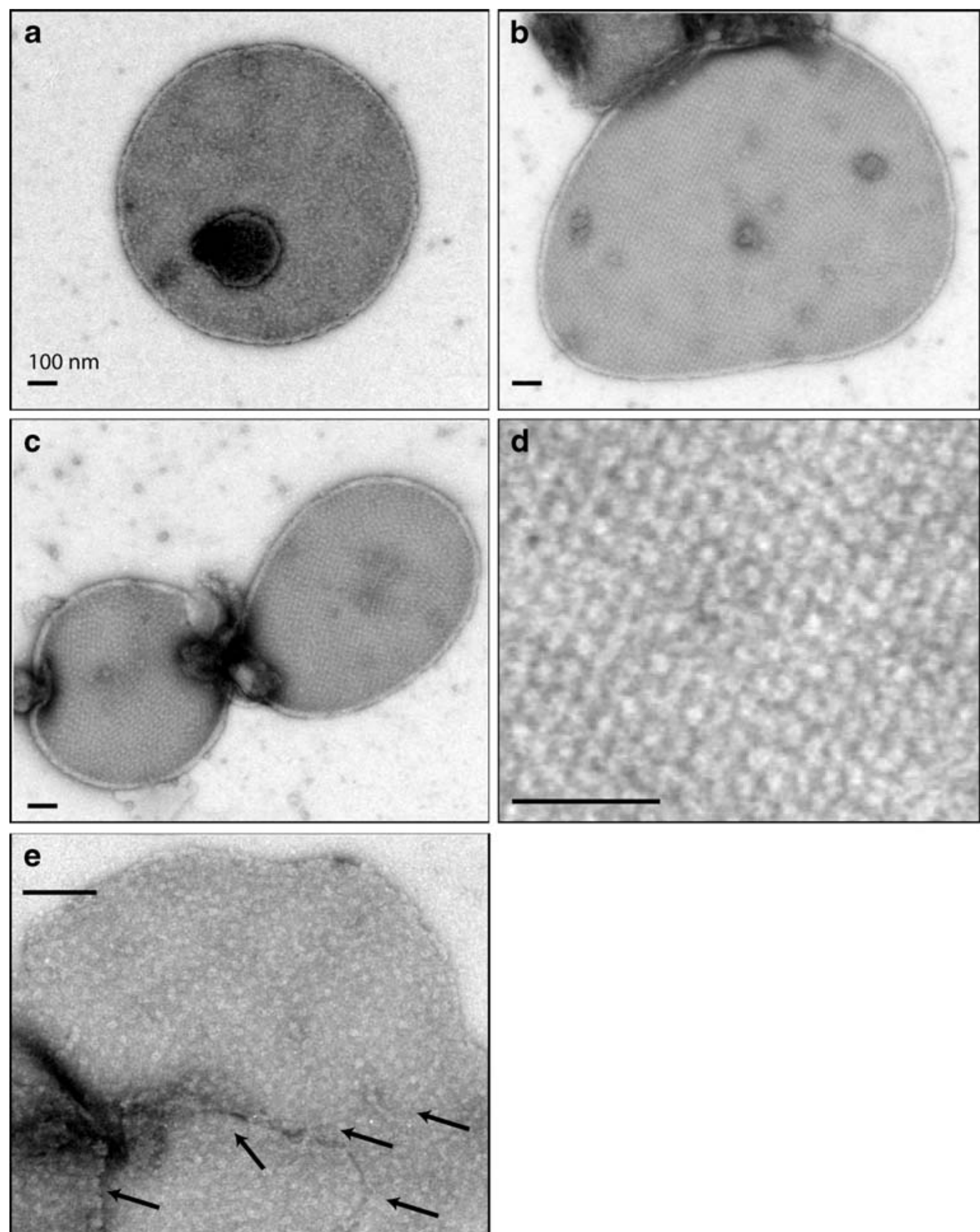

Fig. 3 Electron microscopy of grana particles isolated with the described method. **a** Example of grana patch purified from *Arabidopsis* WT. **b** and **c** Examples of grana isolated from the mutant *viridis Zb63* which conserved their regular organization observed in freeze fracture. **d** Particular of membrane shown in b. **e** Grana membranes isolated with Triton X-100 as in (Berthold et al. 1981) Arrows indicate discontinuities between different fused membrane patches. In all figures space bars represent 100 nm; figures a, b and c are shown with the same magnification while for d and e this is respectively 5 and 2.5 times higher

membranes purified with this method also show some differences with respect to the one shown here. In Fig. 3, in fact, it can be seen that membrane edges are stain excluding, as it is expected for closed vescicles. On the contrary, membranes purified with the protocol by (van Roon et al. 2000) are in general smaller and showed unconnected margins, probably the result of the stronger solubilization (Boekema et al. 2000).

In order to verify the capacity of our procedure to maintain the PSII distribution in grana, we chose to analyze the PSI-less barley mutant *viridis Zb63* which is known from freeze fracture studies to present regular arrays of PSII-LHCII supercomplexes in vivo (Simpson 1983; Morosinotto et al. 2006). Thus, grana membranes from this mutant were isolated, in order to verify if the native regular arrangement of the complexes could be preserved. As shown in Fig. 3b and c, huge membranes patches were obtained from this mutant containing stain-excluding particles with a clear tetrameric structure. These particles, identifiable as PSII cores, are still organized in regular arrays, as in the native membranes (Fig. 3d). It is worth pointing out that, in this case, solubilization conditions required further optimization because the mutation altered the Chl / lipids and grana/stroma membranes ratios. In the case of barley mutant, optimal solubilization was obtained by using only 0.15 % α-DM. The preservation of PSII distribution was also proven true in the case of other plants presenting regular structures, as is the case for *Arabidopsis*

plants depleted in Lhcb6 or Lhcb5 and Lhcb6 (De Bianchi et al. 2008).

As a comparison, we observed by EM also grana partitions isolated with Triton X-100 solubilization. In this case, membranes observed were smaller, consistent with the observation that reduced centrifugation speed was not sufficient to precipitate them efficiently. When grana partitions were re-suspended with a high Chl concentration in the case of spinach we could observe larger membranes, which however showed clearly discontinuities indicating they were rather the result of smaller membrane fusions than representative of intact grana partitions (Fig. 3e). Furthermore, Triton solubilised membranes also are shown to be missing stain excluding membrane edges, confirming a stronger solubilization with respect to α-DM membranes.

Finally, we should mention that this protocol, here presented in detail, has been very recently applied to *Arabidopsis* plants treated with different illuminations (Betterle et al. 2009). For the first time this allowed isolating grana membranes where the PSII distribution was shown to respond to a light treatment, showing in particular that upon light treatment one domain of the membrane is enriched in reaction centers and the other in antenna. These results would not be achievable without a protocol allowing for the isolation of large membrane patches, and thus support the potential of the present protocol for functional studies.

Conclusions

In this work we present an improved protocol for grana membrane purification. This method is based on a very mild detergent solubilization of thylakoids membranes in stacking conditions which is able to solubilize stroma lamellae and grana margins without affecting grana partitions. This preparation was shown to be depleted of PSI and ATPase polypeptides, reaching levels of purity similar to the one of the protocol by (Berthold et al. 1981) as well as similar conservation of PSII oxygen evolution activity. The major point of strength of our preparation, however, is the capacity of isolating large membrane patches with a size comparable to the one observed in intact leaves by freeze etching experiments and, even more importantly, to preserve the arrangements of the protein complexes within the purified membrane patches. We believe that this method may represent a new opportunity to study PSII regulation in a state very close to the actual in vivo condition, as demonstrated by a very recent application (Betterle et al. 2009).

A major factor determining the results of the preparation is Chl to detergent ratio optimization. We showed, in fact, different species behave differently in this respect probably owing slightly different thylakoid architecture. Also growth conditions have to be taken into account as they can influence grana size (Albertsson and Andreasson 2004). In general, in order to achieve the best results we suggest some optimization of the detergent concentration with reference not only to the plant species used but also to the growth conditions.

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References

Albertsson P (2001) Trends Plant Sci 6:349–358
Albertsson PA, Andreasson E (2004) Physiol Plant 121:334–342
Albertsson P-A (1995) Photosynth Res 46:141–149
Albertsson P-A, Andreasson E, Svensson P, Yu S-G (1991) BiochimBiophysActa 1098:90–94
Alboresi A, Caffarri S, Nogue F, Bassi R, Morosinotto T (2008) PLoS ONE 3:e2033
Ballottari M, Govoni C, Caffarri S, Morosinotto T (2004) Eur J Biochem 271:4659–4665
Barber J (2006) BiochemSocTrans 34:619–631
Bassi R, Rigoni F, Barbato R, Giacometti GM (1988) BiochimBiophysActa 936:29–38
Berthold DA, Babcock GT, Yocum CF (1981) FEBS Lett 134:231–234
Betterle N, Ballottari M, Zorzan S, De Bianchi S, Cazzaniga S, Dall’Osto L, Morosinotto T, Bassi R (2009) J Biol Chem 284:15255–15266
Boekema EJ, van Breemen JF, van Roon H, Dekker JP (2000) J Mol Biol 301:1123–1133
Borodich A, Rojdestvenski I, Cottam M, Oquist G (2003) BiochimBiophysActa 1606:73–82
Croce R, Canino G, Ros F, Bassi R (2002) Biochemistry 41:7334–7343
Danielsson R, Albertsson PA, Mamedov F, Styring S (2004) BiochimBiophysActa 1608:53–61
De Bianchi S, Dall’Osto L, Tognon G, Morosinotto T, Bassi R (2008) Plant Cell 20:1012–1028
Dekker JP, Boekema EJ (2005) BiochimBiophysActa 1706:12–39
Dekker JP, Germano M, van Roon H, Boekema EJ (2002) Photosynth Res 72:203–210
Gilmore AM, Yamamoto HY (1991) Plant Physiol 96:635–643
Gunning BES, Schwartz OM (1999) Aust J Plant Physiol 26:695–708
Horton P (1999) Aust J Plant Physiol 26:659–669
Jensen PE, Rosgaard L, Knoetzel J, Scheller HV (2002) J Biol Chem 277:2798–2803
Kaftan D, Brumfeld V, Nevo R, Scherz A, Reich Z (2002) EMBO J 21:6146–6153
Kirchhoff H, Haase W, Wegner S, Danielsson R, Ackermann R, Albertsson PA (2007) Biochemistry 46:11169–11176
Kirchhoff H, Tremmel I, Haase W, Kubitscheck U (2004) Biochemistry 43:9204–9213
Laemmli UK (1970) Nature 227:680–685
Larkum AWD, Vesk M (2003) Algal plastids: their fine structure and properties. In: Larkum AWD, Douglas SE, Raven JA (eds) 'Photosynthesis in algae'. Kluwer Academic Publishers, the Netherlands, pp 11–28

Morosinotto T, Bassi R, Frigerio S, Finazzi G, Morris E, Barber J (2006) FEBS J 273:4616–4630
Murphy DJ (1986) BiochimBiophysActa 864:33–94
Mustardy L, Garab G (2003) Trends Plant Sci 8:117–122
Nelson N, Ben Shem A (2004) NatRevMolCell Biol 5:971–982
Nelson N, Ben Shem A (2005) Bioessays 27:914–922
Olive J, Vallon O, Wollman FA, Recouvreur M, Bennoun P (1986) Biochim Biophys Acta 851:239–248
Pagliano C, La Rocca N, Andreucci F, Deak Z, Vass I, Rascio N, Barbato R (2009) AnnBot(Lond) 103:505–515
Shimoni E, Rav-Hon O, Ohad I, Brumfeld V, Reich Z (2005) Plant Cell 17:2580–2586
Simpson DJ (1983) Eur J Cell Biol 31:305–314
Teardo E, Frare E, Segalla De, De MV, Giacometti GM, Szabo I (2005) FEBS Lett 579:4991–4996
Trissl HW, Wilhelm C (1993) TIBS 18:415–419
Vallon O, Bulte L, Dainese P, Olive J, Bassi R, Wollman FA (1991) ProcNatlAcadSciUSA 88:8262–8266
van Roon H, van Breemen JF, de Weerd FL, Dekker JP, Boekema EJ (2000) Photosynth Res 64:155–166