RECONSTITUTION OF GRANA THYLAKOIDS IN SPINACH CHLOROPLASTS

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1. Introduction

The electron microscopy study of Izawa and Good [1] showed that the grana of spinach chloroplasts suspended in low-salt media disappear and their lamellae dissociate without loss of chlorophyll (Chl). High-salt addition to these plastids results in reassociation of the lamellae in grana-like structures. The electron microscopy data offered no answer as to whether the high-salt induces really a reassociation of the single lamellae of the opened granum, and, moreover, if this reassociation takes place at specific or random binding sites.

To find an answer to these questions we studied the mechanism of grana reconstitution in digitonin disrupted spinach chloroplasts, and agranal proto-chloroplasts of young flashed bean leaves that contain primary thylakoids [2,3].

As is known, differential centrifugation of digitonin disrupted spinach chloroplasts results in separation of heavy and light subchloroplast thylakoid fractions [2]. The heavy fraction, sedimenting between 1000 and 10 000 *g* (10 K), consists primarily of grana and end membranes of grana stacks [4], contains high amount of Chl *b* (*a*/*b* = 2–2.5) [2], has photosystem II (PSII) activity [2,4], and is enriched in the Chl-Protein Complex II [3]. The light fraction, sedimenting between 50 000 and 140 000 *g* (140 K) consists primarily of stroma lamellae [4], contains mainly Chl *a* (*a*/*b* = 7) [2], does not have any PSII activity [2,4], and is enriched in the Chl–Protein Complex I [3].

Digitonin under the conditions of these experiments seems to act preferentialy on single lamellae and not on stacked ones (grana). Addition, therefore, of digitonin to chloroplasts suspended in low-salt media would result in the disruption of the dissociated lamellae of the grana, and the formation of an increased amount of the 140 K fraction. In addition all subchloroplast fractions would be expected to have similar Chl *a* to Chl *b* ratios and the light fractions to be active in PSII reactions. On the contrary, if the high salt induces really a reassociation of the lamellae of the granum, then addition of digitonin to chloroplasts suspended first in low-salt and then in high-salt media would result in the formation of 10 K fractions in amounts similar to those obtained from chloroplasts suspended in control medium. Moreover, if the reassociation takes place at specific binding sites then the reconstituted grana fraction would be expected to have the same amount of Chl *b* (same *a*/*b* ratio), and PS II activity as the control 10 K subchloroplast fraction.

Our results show that reconstitution of grana takes place at specific binding sites which are absent from primary thylakoids.

2. Methods

Chloroplasts were isolated from spinach *(Spinacea oleracea)*, obtained from a local market, according to Anderson and Boardman [2]. The washed chloroplasts pellet obtained from 16–24 g fresh wt tissue, were resuspended in a small volume of the homogenization buffer, separated into three parts (A, B, C), and then recovered again as pellets. The chloroplasts of the sample A were resuspended in control medium (0.05 M phosphate, 0.01 M Kcl, pH 7.2) and those of the samples B and C in low-salt medium (0.05 M Tricine,

pH 7.3). The Chl concentration was adjusted at about 350–400 μg/ml. The suspensions were left in crushed ice for 30 min with occasional stirring. Following this treatment the chloroplasts of the sample C were recovered from the low-salt medium as a pellet after centrifugation at 10 000 *g* for 10 min, and then resuspended in high-salt medium (0.05 M Tricine, 0.1 M NaCl, pH 7.3). The Chl concentration was again adjusted between 350 and 400 μg/ml, and the chloroplasts were allowed to stand for 30 additional min in the high-salt medium. Thereafter, digitonin (2%) was added slowly with stirring to the chloroplast suspensions of the three samples (final digitonin concentration 0.5%). Incubation with digitonin was for 30 min at 0°C.

Protochloroplasts, (chloroplasts of the early stages of greening) were isolated according to Anderson and Boardman [2], from 6 day-old etiolated bean leaves (*Phaseolus vulgaris*, var. red kidney) after exposure to 42 light-dark cycles (2 min white light–98 min dark) as previously described [3,5,6]. Ten gram fresh weight leaves were homogenized at a time with 50 ml homogenization buffer. The protochloroplasts, isolated from 60 g fresh weight tissue, as pellets at 3000 *g* for 10 min were divided in three parts (A, B, C) and treated as the spinach chloroplasts.

The subchloroplast fractions were isolated by differential centrifugation of the digitonin disrupted chloroplasts or protochloroplasts as described in [2], except that 240 000 *g* were used instead of 140 000 *g*, and in the case of protochloroplasts 3000 *g* instead of 1000 *g*. Chl was determined in 80% acetone solution according to MacKinney [7].

PSII activity was determined by following the reduction of dichlorophenol indophenol (DPIP) in the presence or absence of 1,5-diphenylcarbazide (DPC) according to Vernon and Shaw [8].

3. Results

Table 1 shows the distribution of Chl between the various subchloroplast fractions obtained from digitonin disrupted chloroplasts treated with low-salt or low-salt followed by high-salt media. Comparison of the values found for these two samples with those of the control shows that the low-salt induces formation of light thylakoid fractions, while the high-salt reverses this effect. The distribution of Chl between the heavy (1 K + 10 K) subchloroplast fractions and the light (50 K + 240 K) subchloroplast fractions was constant in all the experiments done, although the distribution of Chl between the 1 K and 10 K or between the 50 K and 240 K was not always constant.

Table 1
Distribution of Chl between heavy and light subchloroplast fractions obtained from digitonin disrupted spinach chloroplasts

Subchloroplast fraction	Control	Low-salt	Low + high salt
1 K + 10 K	85	50	92
50 K + 240 K	15	50	8

The chloroplasts were preincubated in (A) control medium, (B) low-salt medium, and (C) low-salt followed by high-salt medium. The values shown are average of 6 experiments, and represent percentages of Chl based on total recovered Chl in all subchloroplast fractions.

Table 2 shows the Chl*a* to Chl*b* ratios in the various subchloroplast fractions. It is evident that all subchloroplast fractions of the low-salt chloroplasts have similar Chl*a* to Chl*b* ratio (2.9–3.3). On the contrary, the heavy fractions obtained from chloroplasts incubated in control medium, have lower Chl*a* to Chl*b* ratio than the light fractions in agreement with the values found by Anderson and Boardman [2]. In the case of the chloroplasts treated first with low-salt and then with high-salt the light fraction recovers its high Chl*a* to Chl*b* ratio, and the values for both heavy and

Table 2
Chl*a* to Chl*b* ratios in subchloroplast fractions of digitonin disrupted spinach chloroplasts

Sample	Control	Low-salt	Low + high salt
Chloroplasts	3.1	–	–
1 K	2.6	2.9	3.0
10 K	2.7	2.9	2.9
50 K	6.2	3.0	6.5
240 K	6.0	3.3	5.5

The chloroplasts were pretreated as in table 1.

light fractions are similar to those of the control. Therefore, it seems that the low-salt disagregated grana thylakoids are disrupted by digitonin so that subchloroplast fractions containing mixed parts of membranes derived from grana and stroma lamellae are produced. In addition, the reconstitution of the grana by high-salt is followed by a return of the Chl*a* to Chl*b* ratio to values close of those found for the grana fraction of the control chloroplasts. In all the experiments done the Chl*a* to Chl*b* ratio returns to a greater or lesser extent to the values found for the respective subchloroplast fractions of control samples.

This is also evident from the absorption spectra of the subchloroplast fractions after suspension in 0.5 M sucrose-0.005 M phosphate, pH 7.0, which are shown in figs. 1–3. The absorption band at 677 nm is due to Chl*a*, and that at 651 nm in due to Chl*b*. Thus, the 240 K fraction of the control, low-salt, and low-high salt chloroplasts had a ratio of OD_{677}/OD_{650} of 2.76, 2.39 and 3.02 respectively. This shows that Chl*b* is present in higher proportion as compared to Chl*a* in the 240 K fraction of the low-salt treated chloroplasts than in that of the other two samples. Moreover, the 240 K fraction of the low-salt, high-salt treated chloroplasts, contain even less Chl*b* as compared to Chl*a* than the respective fraction of the control. Similarly the absorption spectra of the 50 K subchloroplast fractions of these 3 samples showed that the low-salt fraction contained more Chl*b* (in comparison to Chl*a*) than the respective control fraction, (fig. 2). Thus the OD ratio 677nm/650nm was 3.78, 2.66 and 3.45 for the 50 K of the control, low-salt and low-high salt chloroplasts respectively. These differences were less dramatic in the absorption spectra of the 10 K fractions shown in fig. 3.

The heavy 10 K subchloroplast fraction is known to be highly active in PSII activity in comparison to the light 144 K fraction [2]. It therefore is considered to be enriched in PSII particles [4]. The distribution of PSII activity among the heavy and light subchloroplast fractions, was used as a check of the grana reconstitution. Table 3 shows the results of a representative experiment. The rate of DPIP reduction by the heavy subchloroplast fractions (1 K and

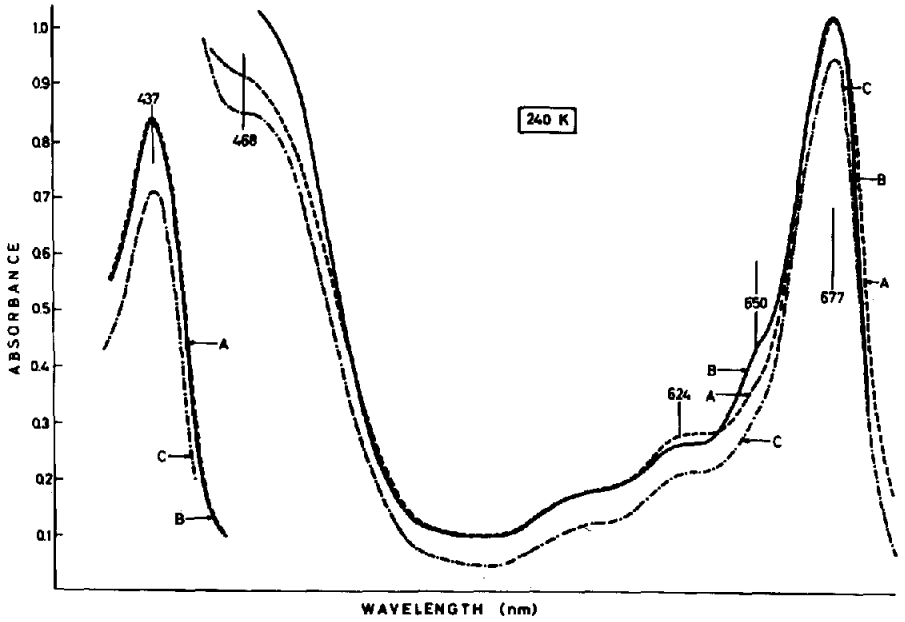

Fig. 1. Absorption spectra of the 240 K subchloroplast fractions obtained from spinach chloroplasts after digitonin disruption. Chloroplasts incubated in control medium (A), low-salt medium (B), or low-salt followed by high-salt medium (C). For details see methods. The subchloroplast fractions were suspended in 0.5 M sucrose, 0.005 M phosphate, pH 7.0.

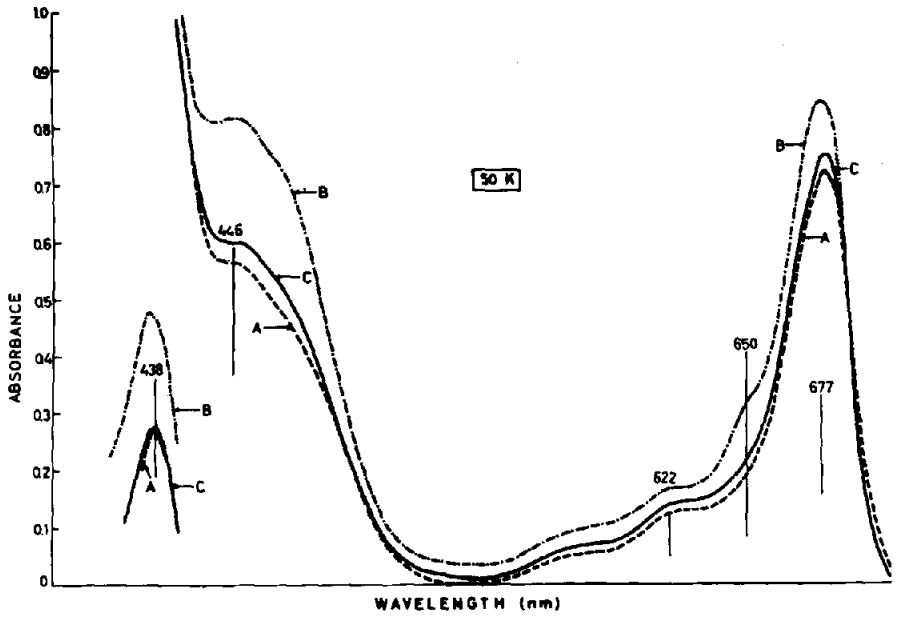

Fig. 2. Absorption spectra of the 50 K subchloroplast fractions obtained from spinach chloroplasts after digitonin disruption. A,B,C, as in legend to fig. 1.

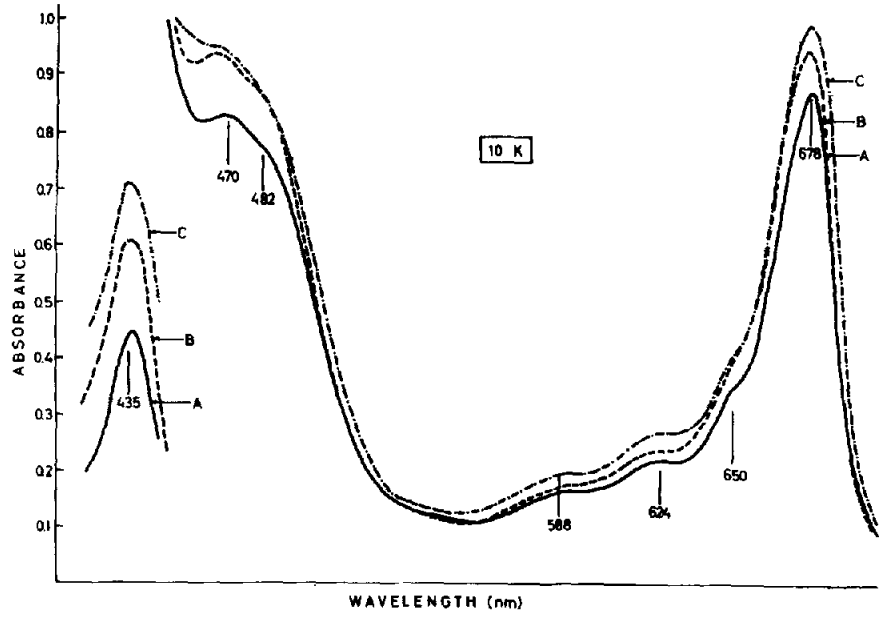

Fig. 3. Absorption spectra of the 10 K subchloroplast fractions obtained from spinach chloroplasts after digitonin disruption. A,B,C, as in legend to fig. 1.

Table 3
Rate of DPIP reduction by spinach chloroplasts or subchloroplast fractions of digitonin disrupted spinach chloroplasts

Sample	μmoles DPIP reduced/mg Chl per hr					
	+ DPC			− DPC		
	Control	Low-salt	Low+high salt	Control	Low-salt	Low+high salt
Chloroplast	120	112	120	130	90	97
1 K	135	−*	144	120	−*	120
10 K	97	100	72	80	80	60
50 K	15	96	21	0	70	10
240 K	0	40	5	0	10	0

The reaction mixture contained in a final volume of 1 ml: 0.1 mM DPIP; 0.25 M sucrose; 50 mM phosphate, pH 6.4, and chloroplasts or subchloroplasts fractions containing 15–20 μg Chl. Wherever DPC was used as the electron donor its concentration was 0.5 mM. Illumination was done with a spot reflector lamp coupled with a water filter at a light intensity of 1.14×10^6 ergs/cm^2 × sec. The DPIP reduction was followed at 590 nm in a Beckman DU spectrophotometer [8]. The values were calculated from the absorption change during the first 30 sec of illumination, and after substraction of the insensitive to 0.01 mM 3-(3,4-dichlorophenyl)-1,1-dimethyl ureau (DCMU) DPIP reduction. The chloroplasts were pretreated as in table 1.

* Small concentration of Chl in relation to digitonin present in the 1 K of low-salt chloroplasts rendors the assay mixture turbid.

10 K) of the control chloroplasts, both in the absence or presence of DPC is high (per mg Chl). The 240 K fraction has either no activity at all (in the absence of DPC) or only a small fraction of the chloroplast activity. On the contrary, the rate of DPIP reduction is considerable in the light subchloroplast fractions obtained from low-salt grana-free chloroplasts. Resuspension of low-salt treated chloroplasts in high-salt, results in reconstitution of the membranes in such a way that only the heavy subchloroplast fractions obtained after digitonin treatment are active in the DPIP reduction. The value found for the high-salt reconstituted grana thylakoids are only slightly lower than those found for the control chloroplasts. Therefore, it seems in this case that the high-salt reconstituted heavy fractions contain membranes associated again at specific 'grana forming' sites. Table 4 shows the distribution of Chl between the heavy and light subplastid fractions of digitonin disrupted protochloroplasts which were pretreated the same way as the chloroplasts. As it is evident addition of low-salt to protochloroplasts or addition of high-salt to low-salt preincubated protochloroplasts had no effect on the distribution of Chl between the subplastid fractions. This suggests that the primary thylakoids are unable to associate and form heavy fractions under the influence of high-salt.

Table 4
Distribution of Chl between heavy and light subplastid fractions obtained from digitonin disrupted bean protochloroplasts

Subplastid fraction	Control	Low-salt	Low + high salt
3 K + 10 K	48	34	31
50 K + 240 K	52	66	69

The protochloroplasts were pretreated as the chloroplasts in table 1. The values represent percentages of Chl based on total Chl recovered in the fractions.

4. Discussion

The results presented are in agreement with those

of Izawa and Good [9] and Anderson and Vernon [10] as far as the low-salt treatment of chloroplasts is concerned. All the subchloroplast fractions separated after digitonin treatment are active in PSII reactions and possess approximately the same Chl composition as do the chloroplasts.

What becomes of importance in this study in the fact that after high-salt addition in the grana-free low-salt treated chloroplasts, most of the chloroplast Chl is recovered in the heavy fractions (95%). These heavy fractions as well as the light fractions contain Chl*a* and Chl*b* in ratios similar to those found for the respective fractions of control chloroplasts. In addition, the light subchloroplast fractions lose their ability to photoreduce DPIP, while the reconstituted heavy fractions retain their activity.

This evidence can be explained by assuming reconstitution of the grana thylakoids at specific 'grana forming sites' in the presence of the high-salt medium. These sites may involve the Chl*b* molecules which are known to be present in high amounts in the grana stacks. The question, therefore, arises why the Chl*a* and Chl*b* attach themselves to domains of the membrane having different properties.

Our results also imply that part of the grana thylakoids—containing high amount of PSII particles—are disrupted by digitonin in low-salt media forming light-single lamellae, which retain at least part of their PSII activity. On the contrary high-salt addition to the low-salt treated agranal chloroplasts induces reaggregation of only those single lamellae which were originated from grana stacks. Thus the reconstituted grana are active in the photoreduction of DPIP both in the presence or absence of the electron donor DPC, and the light fraction stroma lamellae are inactive.

These results, therefore, also support the notion that reconstitution of grana thylakoids takes place at specific binding sites. This concept is also supported by the results found on the distribution of Chl in the subplastic fractions of digitonin disrupted protochloroplasts. The primary thylakoids of the early stages of greening seem to be unable to aggregate in high salt media. This may indicate that compounds necessary for stacking are absent from such primary thylakoids. Since these thylakoids are deficient in Chl*b* and the Chl*b*-rich Chl-Protein Complex II [3,5, 6], it is tempting to propose that these lamellar compounds may be involved in the stacking.

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