Biosynthesis of Grana and Stroma Lamellae in Spinach

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ABSTRACT

Park and co-workers (Annu. Rev. Plant Physiol. 22: 395–430) have suggested that stroma lamellae, which perform only photosystem I, contain a developing photosystem II which becomes functional upon the folding of these membranes to form grana stacks.

The present study was undertaken to test this hypothesis. The lipid and protein constituents of chloroplast membranes were pulse-labeled radioactively with 14C, and the specific activity of stroma and grana components were studied in a pulse-chase experiment. The components of both grana and stroma lamellae become labeled and decay at similar rates which suggest that the Park and Sane hypothesis is not correct. The results also show large differences in the turnover rates of some chloroplast membrane components supporting the multistep model for membrane synthesis and maintenance in this organelle.

Stroma and grana lamellae of higher plant chloroplasts constitute an elaborate complex of membranes where the conversion of light into chemical energy takes place. These two types of membranes have been separated physically and comparative studies of their structure, gross chemical composition, pattern of development, and enzymatic capabilities have been made (1, 5, 9, 15, 32–34, 36, 44). However, the biogenetic relationships between the two types of membranes, if any, are not established clearly.

In 1971 Sane and Park (37) studied a stroma lamella fraction from spinach chloroplasts which contained only PSI and found a decline in the quantum yield for NADP photoreduction at wavelengths shorter than 700 nm. This result indicated the presence of a pool of Chl in stroma lamellae which was ineffective in the PSI reaction. Subsequent fluorescence studies of the stroma lamella preparations (30) and reports in the literature of similar observations on PSI fractions obtained by Triton (45) or digitonin (39, 47) treatments, provided evidence that the inactive Chl was not due to damaged PSI caused by the fractionation procedure. Support for the notion that the presence of uncoupled Chl was not a fractionation artifact also came from the work of Homann and Schmid (18) on intact chloroplasts of the tobacco mutant NC95. Plastids from the yellow regions of the leaves of NC95 contain mainly stroma lamellae and perform primarily the PSI reaction. Homann and Schmid (18) showed, with intact chloroplasts from the yellow regions, a similar decline in quantum yield of PMS cyclic photophosphorylation at short wavelengths. All these results strongly suggest that the presence of photochemically inactive Chl is an inherent characteristic of stroma lamellae, and Sane and Park (30) proposed that this Chl might be related to sites of membrane biogenesis.

Further characterization of the stroma lamellae indicated that some components of PSII are present in the unstacked membrane and the use of DPC as electron donor revealed incipient PSII activities in this fraction (29). Interestingly, this electron transport from DPC to DCIP showed characteristics very similar to those observed for PSII in developing bean chloroplasts (2), namely a large insensitivity to DCMU, and all these observations led Sane and Park to generalize their concept and hypothesize that the stroma lamellae contain a developing PSII which is completed upon the folding of these lamellae to form grana stacks (37).

Such a scheme, although agreeing with the wealth of data on development of structure and function during the greening process of chloroplasts (10–12, 15, 16, 25, 31, 34, 49) and supporting the view that PSII activities are closely associated with grana appearance, is based only on circumstantial evidence and has received, so far, no direct experimental support.

The present study was undertaken to test this hypothesis. Our approach was the following. Two spinach plants were allowed to photosynthesize with 14CO2 for 1 hr; immediately after this period, the stroma and grana lamellae of one plant were separated, and the specific radioactivity of their lipid and protein components was analyzed. The other plant was allowed to grow in the presence of atmospheric CO2 for 7 days more and after that subjected to identical treatment. The 7-day cold chase period was initially selected because the data of Shlyk, as reported by Kutiurin (21), showed that the half-life of Chl in tobacco leaves is 13.2 days.

If the hypothesis is correct, one would expect to find a considerable amount of radioactivity in the stroma components immediately after the labeling period compared with the grana region. After 7 days, labeled stroma membranes would become folded into grana and if reformation of stroma membranes from cold precursors occurred, then, an increase in the relative amounts of radioactivity of the grana regions should accompany a decrease in radioactivity of the stroma.

Though the results reported here do not support the sequential stroma lamellae → grana lamellae biogenetic scheme hypothesized by Sane and Park (37), they do show that the protein components of the chloroplast membranes turn over with different rates, and this observation is discussed in relation to the assembly of the thylakoid membranes.
MATERIALS AND METHODS

\(^{14}\text{CO}_2\) Assimilation. The \(^{14}\text{CO}_2\) assimilation was conducted in a closed system, described by Bassham and Kirk (6), incorporating an infrared gas analyzer, an ionization chamber for \(^{14}\text{CO}_2\) measurements, and an oxygen analyzer, connected with a multipoint recorder. The illumination was provided by two 250w photoflood lamps operated at 110 v and the light was passed through filters to prevent excessive heating and injury of plants.

Spinach (Spinacia oleracea L.) plants approximately 1 month old with four fully expanded leaves were used in all experiments. The plants were allowed to photosynthesize for 1 hr in the presence of 250 mCi of \(^{14}\text{CO}_2\) and then either immediately processed (1-hr plant) or grown for 7 days more (7-day plant) in the presence of atmospheric \(^{14}\text{CO}_2\). Subsequent treatment was identical in both cases.

Isolation of Subchloroplast Fractions. Plants exposed to \(^{14}\text{CO}_2\) were diluted with market-purchased spinach leaves to give a final weight of 100 g, necessary for the subsequent fractionation procedure. Subchloroplast fractions were prepared as described by Sane and Park (37). Chloroplasts were suspended in 0.05 M potassium phosphate (pH 7.4)-0.15 M potassium chloride, passed through the French pressure cell, and the resulting brei was submitted to fractional centrifugation. The heavy grana stacks were pelleted at 10,000g (hereafter called 10K fraction) and the lighter unpressurized stroma lamellae were sedimented at 160,000g (160K fraction). The fractions were washed with EDTA (1 mM, pH 8.0) for 30 min at C. Total Chl and the ratio of Chl a/Chl b were determined by Arnon's method (4).

Extraction and Separation of the Pigments. The pigments were extracted with 80% acetone and separated by two-dimensional TLC on maninit plates, according to Smith et al. (43). In these experiments 2% (v/v) methanol in petroleum ether (b.p. 30-60 C) in the first direction and 5% (v/v) acetone in petroleum naphtha (b.p. 60-70 C) in the second direction were used. The amounts of individual Chl a and b after separation were calculated from the Mackinney coefficients (23) and the ratio of Chl a/Chl b was used to assess the degree of resolution obtained in the chromatogram. The recovery of total Chl from the plates averaged 96%.

Preparation and Determination of Protein. The membrane protein fraction, recovered by centrifugation after lipid extraction in 80% acetone, was suspended at a concentration of 1 mg of protein/ml in 10 mM sodium phosphate buffer (pH 7.0)-1% SDS-1% \(\beta\)-mercaptoethanol and was incubated at 45 C overnight. The suspension then was heated at 100 C for 20 min and dialyzed against 10 mM sodium phosphate buffer, pH 7.0-0.1% SDS-0.1% \(\beta\)-mercaptoethanol for 6 hr. The amount of protein was estimated using the method of Lowry et al. (22) with BSA suspended in the same buffer as a standard.

Electrophoresis Technique. Acrylamide gel electrophoresis, in the presence of SDS, was carried out in cylindrical tubes (10 x 0.6 cm) containing 7% acrylamide and 0.2% methylene-bisacrylamide (24). The electrophoretic buffer consisted of 0.05 M sodium phosphate (pH 7.0)-0.1% SDS-0.1% \(\beta\)-mercaptoethanol (48). Approximately 70 \(\mu\)g of protein were layered on the top of the gel and subjected to electrophoresis at 8 mamp/tube until the tracking dye (pyronine y) was about 1 cm from the end of the tube. Electrophoresis was completed in 5 to 6 hr. The gels were fixed and stained with 0.25% Coomassie brilliant blue R in 7.5% acetic acid-50% methanol (48); the destaining was performed by several changes in Weber and Osborn solution. The destained gels were scanned at 560 nm using a Gilford gel-scanner accessory on a Beckmann DU spectrophotometer with 0.1-mm slit.

Molecular weights were determined from a standard plot using phosphorylase A (93,000 daltons), BSA (68,000 daltons), ovalbumin (43,000 daltons), chymotrypsinogen (26,000 daltons), myoglobin (17,000 daltons) and Cyt c (11,700 daltons) as standard proteins.

Counting Procedures. The radioactivity of the pigments was determined with a Geiger-Müller gas flow counter (Nuclear Chicago, Model 47D), the samples being counted for sufficient time to accumulate 2500 counts. For measurements of radioactivity in proteins, the gels were frozen in solid CO\(_2\) and sliced into 1-mm pieces. The slices were incubated with 0.1 ml of 30% H\(_2\)O, for 8 hr at 60 C. After solubilization 12 ml of aquosal solution were added and the radioactivity was measured in a Packard Tri-Carb automatic liquid-scintillation spectrometer, Model 3375.

RESULTS AND DISCUSSION

Lipids. The selective disruption of spinach chloroplasts by the French pressure cell followed by fractional centrifugation permits the separation of grana stacks, pelleted at 10,000g, from the unstacked stroma lamellae pelleted at 160,000g. The biochemical characterization of these fractions showed that the grana stacks were enriched in PSI and had a lower Chl a/Chl b ratio than intact chloroplasts, whereas the stroma lamellae, containing only PSI, were characterized by a much higher Chl a/Chl b ratio (36). The values of these ratios for our preparations, obtained by the French pressure technique, are presented in Table 1; the Chl a/Chl b ratio of the initial chloroplasts was 2.95 for the 1-hr plant and 2.88 for the 7-day plant, and the Chl a/Chl b ratios of the fractions fall within the range expected for grana and stroma lamellae.

Also presented in Table 1 are Chl a/Chl b ratios obtained after separation of the different pigment components by TLC on maninit plates. The differences found are minor, indicating that an adequate separation of the Chl was obtained in the chromatogram. In all the cases, distinct spots for carotenones, lutein, violoxanthin, and neoxanthin were also visible, the carotenones being well resolved from the origin.

The data on \(^{14}\text{CO}_2\) incorporation into the Chl a and b of 10K and 160K are reported in Table 1. Although the individual carotenones were analyzed, the amounts of radioactivity were too low for accurate measurement with the exception of \(\beta\)-carotene. The carotenones accounted for a major portion of the radioactivity of the pigment fraction in the 1-hr plant. During the 7 days of chasing with atmospheric \(^{14}\text{CO}_2\), the specific radioactivity of the carotenones decreased and was accompanied by an

<p>| Table 1. Specific Radioactivity of Chlorophylls a and b after a 1-Hr Labeling Period and after a 7-Day Chase with Atmospheric (^{14}\text{CO}_2) |
|-----------------|-----------------|-----------------|-----------------|
| Fraction | Chl a (chl a) Ratio | Chl a (chl b) Ratio | Specific Radioactivity of Chlorophylls |</p>
<table>
<thead>
<tr>
<th>Chl a</th>
<th>Chl b</th>
<th>cpm/ ug</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Hr plant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10K</td>
<td>2.52</td>
<td>2.37</td>
</tr>
<tr>
<td>160K</td>
<td>5.16</td>
<td>4.98</td>
</tr>
<tr>
<td>7-Day plant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10K</td>
<td>2.48</td>
<td>2.53</td>
</tr>
<tr>
<td>160K</td>
<td>5.32</td>
<td>5.51</td>
</tr>
</tbody>
</table>
increase of that of the xanthophylls. These findings, previously reported by other workers for several different organisms, have been used to suggest that \( \beta \)-carotene can act as a precursor for the more oxygenated forms of carotenoids (8, 19). Our observations are consistent with those of Roux and Husson (35) who found that \( \beta \)-carotene has a much faster turnover rate than any other of the chloroplast pigments.

The data presented in Table I for specific radioactivity of Chl \( a \) and Chl \( b \) agree quite well with published values (40). The higher specific radioactivity of Chl \( a \) compared with Chl \( b \) in the short term experiment and the decrease of this ratio during the 7 days supports the generally accepted view that Chl \( b \) arises from Chl \( a \), as first proposed by Shlyk et al. (40, 41). The 7-day cold chase in our experiments led to a 30% decline in specific radioactivity of Chl \( a \), indicating a Chl turnover time not greatly different from that reported for tobacco (21).

An important observation for our purpose is that the distribution of \(^{13} \text{C} \) into the Chl of grana and stroma is very similar, immediately after the labeling period. These results argue against the hypothesis of the stroma membrane as being more actively implicated in the biosynthesis of the Chl. The observation that in the 7-day plant the ratios

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\frac{\text{specific radioactivity Chl } a_{10K}}{\text{specific radioactivity Chl } b_{10K}}
\]

remain constant, supports the “1-hr” data and definitely rules out the hypothesis of preferential localization of chlorophyll biosynthesis centers in the stroma lamellae with later migration of the new pigment molecules to the grana region by stacking of the single membranes. Contrarily, it suggests that both stroma lamellae and grana stacks have their own centers for the formation of pigments and these incorporate \(^{13} \text{C} \) at about equal rates. These results are in apparent disagreement with the extensive work done by Shlyk and co-workers (42) on centers of Chl biosynthesis. These authors repeatedly reported the existence of “light particles” in chloroplasts where Chl synthesis preferentially takes place. However, by reasons of differences in the procedures followed and by an absence of a correspondence between the light particle and its counterpart in intact chloroplasts, it is not easy to compare their results with ours, and we will not attempt it here.

The results of Goldberg and Ohad (14) with the \( y^{-1} \) mutant of the green algae \( \text{Chlamydomonas reinhardtii} \) are of comparative interest. These authors investigated the distribution of labeled lipids between the stacked and unpaired photosynthetic membranes of this organism and found that the radioactive pattern obtained after a short pulse with acetate-\( \text{H}^+ \) is not changed by a chase period with cold acetate, suggesting that no migration of membrane material occurs in the mutant chloroplast, in agreement with our conclusions. The observation, also made by the authors, that unpaired membranes incorporate acetate-\( \text{H}^+ \) twice as fast as the stacked membranes, apparently in contradiction with our results, may depend on the fact that the paired membranes of \( \text{Chlamydomonas} \) are relatively isolated from the chloroplast matrix.

**Peptides.** The proteins of the photosynthetic membranes, solubilized by SDS as described under “Material and Methods,” show a complex electrophoretic pattern discussed in detail elsewhere (26). Comparison of their electrophoretic mobilities with those of known proteins gives the mol wt estimates in kilodaltons shown in Figures 1 and 2; no mol wt are assigned to peaks below 18 kd since the plot relating mol wt with relative mobilities is not linear at these low values. A comparison of the densitometric tracings of the 10K and 160K fractions shows a great similarity in the polypeptide composition of both fractions; however, minor qualitative differences can be seen. Prominent differences are the absence of the 36 kd peak in the 10K fraction and the presence of a 39 kd peak in this fraction which is lacking in the 160K. The peak with mol wt 54 kd in the stroma-membrane preparation represents two unresolved peaks with 56 kd and 54 kd (shown by the use of improved electrophoretic technique).

Quantitatively, the stroma and grana lamellae show important differences. The 23 kd peak, representing the major polypeptide component of the 10K fraction and accounting for approximately 30% of the total membrane protein in the grana, is much smaller in the 160K fraction where the two high mol wt components, 60 kd and 54 kd, clearly predominate. The 160K fraction is comparatively enriched in the 19 kd and 21 kd components, whereas the 10K fraction, on the other hand, contains more of the 30 kd peak.

Some of these peaks have been tentatively identified with known components of the photosynthetic membranes (26, 46). However, no reliable identification has been presented so far with possible exception for the peaks 60 kd and 56 kd, presumed to be two subunits of the coupling factor.

The pattern of \(^{13} \text{C} \) incorporation immediately following the
assimilation period into the protein of stroma and grana lamellae is also shown in Figures 1 and 2. The radioactivity measurements, in a qualitative way, closely match the densitometric tracings of the membrane polypeptides. It is interesting to note that the radioactivity measurements appear to resolve the smooth peak in the lowest portion of the gel into a number of different peaks. This indication of several components in this region of the gel was subsequently demonstrated by increasing the acrylamide concentration to 10% which resolved distinct peaks.

Though there is a qualitative match between radioactivity and densitometric tracings in Figures 1 and 2, the amount of radioactivity does not parallel the density. In the 10K fraction, although the 23 kd polypeptide is the most radioactive peak, it is clear that the 30 kd and 25 kd peaks are relatively more labeled. Also, in the 160K fraction, more radioactivity is proportionally incorporated in the 30 kd and 24 kd peaks than in the 60 kd and 54 kd components. In both membrane fractions the two high mol wt components show a slow rate of carbon incorporation suggesting a lower turnover of these components.

The Sane and Park hypothesis suggested that components of stroma lamellae might become labeled more rapidly than those of grana lamellae; the data (Fig. 1 and 2) fail to show any preferential incorporation of the label into the stroma lamellae polypeptides. Although the establishment of a quantitative relationship is difficult because the amounts of different polypeptides present in the fractions are not easily measured, the specific activities of the different polypeptides in both fractions are similar after this short time of carbon incorporation. For example, in the 30 kd component of the 10K fraction, the absorbance is about 1 and the radioactivity is about 1500. In the 160K fraction, this same component has an absorbance of 0.6 and a radioactivity of about 800. The 7-day plant shows a parallel decrease in radioactivity in both fractions (Fig. 3 and 4), contrary to what would be expected if a precursor-product relationship existed between the stroma and grana lamellae. These data would appear to rule out the initial hypothesis that stroma contains some incomplete PSII components that are assembled into a functional PSII unit upon stacking of the single stroma lamellae.

A comparison of Figure 1 with Figure 3, and Figure 2 with Figure 4, indicates a heterogeneity in the rate of loss of radioactivity among the different membrane proteins. For example, the 30 kd component not only becomes labeled more rapidly, but decays much faster than other components whereas the 23 kd and the two high mol wt components, 60 kd and 56 kd, have slow decays. However, one has to be cautious in the interpretation of decay kinetics because of possible reutilization of some labeled material. We interpret these data as indicating a heterogeneity in the turnover rates of the different membrane polypeptides and this observation has a bearing on the concepts

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**Fig. 2.** Densitometric tracing (---) compared with distribution of radioactivity (-----) in the 1-hr 160K fraction after SDS-acrylamide gel electrophoresis.

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**Fig. 3.** Densitometric tracing (---) compared with distribution of radioactivity (-----) in the 7-day 10K fraction after SDS-acrylamide gel electrophoresis.
of thylakoid assembly. Kirk (20) has recently presented a critical review on the process of thylakoid assembly. Based on the observation of a time-course acquisition of the different photosynthetic activities, it seems that "there is a stepwise assembly process, by which first a membrane is built up containing a certain minimum number of components, with other constituents being added later..." (20). Indeed, extensive work done during the last few years on chloroplast development has provided much evidence for a gradual acquisition of the different photochemical capabilities throughout the greening process of this organelle. Such evidence favors a stepwise model for thylakoid membranes assembly of several higher plant chloroplasts and is supported by our observations of a marked asynchrony in the turnover rates of the different spinach chloroplast membrane polypeptides. A stepwise membrane-assembly model has also been postulated for the photosynthetic lamellae of the alga Chlamydomonas (13, 27, 38) and other membrane systems of eucaryotic cells, namely mitochondria (7) and endoplasmic reticulum (3, 28).

CONCLUSION

In this study we attempted to test the hypothesis proposed by Sane and Park that stroma lamellae contain a developing PSII which becomes completed upon the folding of these membranes to form grana stacks. The lipid and protein components of stroma and grana have been labeled with C and the specific radioactivity of these components was analyzed immediately following the labeling period and subsequent to a 7-day chase with atmospheric CO. According to the hypothesis, it was anticipated that a preferential incorporation of label into the stroma components compared with grana should occur in the short term experiment, and an inversion of this situation would be found 7 days later as a result of stacking of labeled stroma lamellae to form the grana and reformation of the former from cold precursors.

The data reported in this paper are not consistent with that biogenetic model for chloroplast membranes and favor the view that stroma and grana membranes have distinctive centers of biosynthesis, working independently and apparently at similar rates. We are, therefore, left with the intriguing problem of the presence of a large pool of photochemically inactive Chl and protein components in the stroma lamellae which apparently are not transformed into PSII functional elements. More work is necessary to clarify the significance of this "inactive Chl pool." When this problem is explored, it should not be forgotten that previous references to inactive Chl (17) were merely an expression of our ignorance of its true function.

Our results are also of interest regarding the proposed models for thylakoid assembly. A stepwise assembly has been generally accepted, based mainly on the time-course appearance of the different photochemical activities. The heterogeneity found in our work on the turnover rates of the chloroplast membrane polypeptides favors this multistep model and reinforces the concept of the membrane as a very dynamic structure in continuous exchange with the medium through breakdown and resynthesis of its components.

LITERATURE CITED

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