Characterization of the 32,000 Dalton Chloroplast Membrane Protein

III. PROBING ITS BIOLOGICAL FUNCTION IN SPIRODELA

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ABSTRACT

The rapidly turning over, photoinduced thylakoid protein, P-32000, is the main pulse-labeled membrane polypeptide in the chloroplasts of Spirodea oligorrhiza, yet little is known of its physiological function. Two hypotheses are tested: that P-32000 synthesis is necessary for thylakoid biogenesis; that it directly participates in photosynthesis. Spirodea cultures were dissected into expanding and fully mature tissue. Fronds from both developmental stages transcribed a $0.5 \times 10^6$ dalton RNA likely to be the message for P-32000. As to the protein itself, synthesis occurred in both types of tissue but was considerably enhanced in the fully mature state. Thus, a purely transient, developmental function for P-32000 during thylakoid biogenesis appears ruled out. Low concentrations of d-threo-chloramphenicol severely suppressed P-32000 synthesis but not its turnover. As a result, fronds depleted in P-32000 were obtained. However, photosynthesis of CO₂ remained at 86% of normal in tissue > 80% depleted of P-32000. Thus, P-32000 did not appear to be rate-limiting, suggesting that it does not serve as a direct, integral part of the photosynthetic pathway.

A 32,000 d₁⁴ polypeptide, P-32000, is the major membrane protein synthesized within the chloroplasts of the duckweed Spirodea (11). An RNA, likely to be the message for P-32000, has a mol wt $0.5 \times 10^6$ d (29) turns over rapidly (33), is undermethylated (25) and lacks poly(A) (31, 34). Its appearance is enhanced during greening, forming about 85% of the discrete pulse-labeled RNA molecules produced shortly after transfer of steady-state, dark-grown fronds to the light (31). Low energy blue light is most efficient in stimulating its synthesis during greening (17). When Spirodea plants are deprived of a carbon source there is a relative accumulation of the $0.5 \times 10^6$ d RNA (30).

In cell-free protein-synthesizing systems, $0.5 \times 10^6$ d, poly(A)⁻, chloroplast RNA is translated to yield a precursor polypeptide, P-33500 (11). In intact plants and in isolated chloroplasts this precursor is quickly processed to P-32000. The processed product is an integral part of the thylakoid membrane, solubilized by ionic detergents but not by treatments with salts, chelating agents, or other procedures which remove more loosely bound proteins (11). P-32000 is itself relatively short lived, exhibiting an order of magnitude of more rapid decay than that of other major plastid polypeptides. Under many conditions there is a close relation between the level of P-33500 template RNA and P-32000 (11, 28). Under certain stress conditions this coordination can be uncoupled (11).

Plastid membrane proteins of about 32,000 d with properties overlapping those reported in Spirodea have been noted in other genera (5, 10, 13, 15, 23, 35). Several studies have also described chloroplast mRNA directed translation of P-32000-like polypeptides in cell-free protein-synthesizing systems (3, 20, 22). A few communications record the presence of rapidly labeled $0.5 \times 10^6$ d chloroplast RNAs not associated with RNA metabolism products in other plants (18, 19, 36, 38).

It is of interest to understand the biological function of the 32,000 d polypeptide. Why is the plastid investing so much of its resources in synthesizing a short lived mRNA and its ephemeral protein product? In this report we test two hypotheses on the function of P-32000: the first, that it is involved in thylakoid biogenesis; and the second, that it forms an integral part of the photosynthetic pathway within the thylakoids.

MATERIALS AND METHODS

Axenic Spirodea oligorrhiza (Kurtz) Hegelm. was cultured phototrophically under steady-state conditions (350 ft-c, 25 °C) in half-strength Hutner's medium (cf. 33). Conditions of labeling with [5,6-³²H]uridine (52 Ci/mmol; Amer sham) or [³⁵S]methionine (1,000 Ci/mmol; Amer sham) and incubation with d-threo-chloramphenicol (Sigma) are described in legends to the figures and tables. Labeled fronds were washed with sterile distilled H₂O and frozen on dry ice until extracted. In some experiments labeled and washed fronds were separated into the distal half of fully mature (completely expanded i.e., > 3 mm long) and expanding fronds (<1 mm long) by dissection, and frozen immediately, as outlined in reference 32.

Extraction and polyacrylamide gel electrophoresis of [³²H]uridine-labeled RNA, gel scanning, slicing, radioactive counting, and apparent mol wt determinations were as previously described (33). The [³⁵S]methionine-labeled membrane fraction was prepared by centrifugation from tissue homogenates essentially as described in reference 10 and soluble protein was prepared according to reference 27. Electrophoresis of proteins on SDS slab gels containing a 10 to 20% gradient of polyacrylamide with a 3% stacking gel is described in reference 21. Gels were stained with Coomassie brilliant blue, destained, and prepared for fluorography according to reference 4, dried, and exposed on CURIX RP 2 x-ray film (Agfa). Fluorograms were scanned densitometrically using the linear transport attachment of a Gilford spectrophotometer.

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Photosynthetic CO₂ assimilation was determined by a modification of reference 39. Twenty fronds per treatment were floated in medium in 25-mm-diameter containers and placed in a sealed plastic box with a center well containing 100 μCi NaH¹⁴CO₃ (60.3 Ci/mmol; Amersham). Tartaric acid was added by remote control to release ¹⁴CO₂, and the container was placed in 350 ft-c fluorescent light. After 1 h, the atmosphere in the container was filtered through a KOH trap to remove unused ¹⁴CO₂, and the tissue removed and dried at 100°C overnight. The dry tissue was oxidized in a Packard Tri-Carb sample oxidizer and the ¹⁴CO₂ automatically collected in a Carbosorb (Packard) which was then added to Permafluor (Packard) scintillation fluid and counted. Preliminary experiments with this procedure showed negligible dark fixation.

Chl was solubilized in 80% acetone from young fronds and distal halves of mature fronds following an overnight incubation in darkness at room temperature. Chl content was then estimated following centrifugation (2).

RESULTS AND DISCUSSION

RELATIONSHIP TO THYLAKOID BIOGENESIS

A growing colony of Spirodela plants contains both expanding and mature fronds. The expanding or developing fronds are capable of both cytoplasmic and chloroplast rRNA synthesis and actively amass thylakoid membranes in the light. The distal halves of the fully expanded fronds represent mature tissue. They do not synthesize measurable amounts of plastid RNAs, although synthesis of cytoplasmic rRNA continues (33). Net biosynthesis of thylakoid components is not to be expected at this stage of development.

We tested the possibility that P-32000 function was related to thylakoid membrane biogenesis by comparing patterns of precursor incorporation into RNA and protein of expanding versus mature fronds.

0.5 x 10⁶ d RNA. Our previous results had indicated that Spirodela 0.5 x 10⁶ RNA could be readily identified in whole tissue extracts from light-grown cells labeled for short periods of time (34). Employing this finding we fractionated pulse-labeled RNA extracts from expanding and mature fronds on polyacrylamide gels and compared the relative levels of incorporation into 0.5 x 10⁶ RNA. The profiles in Figure 1 clearly indicate a discrete peak of incorporation into plastid RNA at 0.5 x 10⁶ both in expanding and mature tissue. Analysis of the gel patterns revealed that in both instances ~4% of the total [³H]uridine incorporated into the RNA on the gels was located in the 0.5 x 10⁶ d peaks. When these data were normalized on the basis of radioactivity per unit A₅₅₀ of cytoplasmic rRNA (32), the 0.5 x 10⁶ d peaks from both tissues gave values of ~700 cpm/μg rRNA. We thus conclude that in addition to synthesis of 0.5 x 10⁶ d RNA in young, expanding tissue, production of this plastid mRNA continues in the distal halves of fully expanded fronds. Recently reported patterns of 0.5 x 10⁶ d chloroplast RNA synthesis in young and aged tobacco leaves (38) complement the above conclusion with Spirodela plastid RNA.

P-32000 Thylakoid Protein. The relative amounts of [³°Smethionine incorporation into P-32000 thylakoid protein in developing versus mature tissue were then investigated. The distribution of rapidly labeled membrane polypeptides extracted from expanding and mature fronds was compared by SDS-gel electrophoresis (Fig. 2). Equal amounts of radioactivity were applied to each slot. It is immediately apparent that relatively more P-32000 was synthesized in mature than expanding tissue. Densitometric analysis of the fluorographic patterns revealed that incorporation of [³°Smethionine into P-32000 was 5 and 25% of the total short term label in the membrane fractions of developing and mature tissue, respectively. The opposite trend held for P-26000, the apoprotein of the light-
harvesting Chl a/b complex (Fig. 2). This relatively stable, nuclear coded and cytoplasmically translated polypeptide is the main protein accumulating in the thylakoids (1, 11). In expanding fronds (where chloroplast membrane biosynthesis is a major function) > 10% of the total short term label incorporated into the membrane proteins is found in P-26000. Conversely, in the distal half of fully expanded fronds, where chloroplasts already contain a mature complement of thylakoid lamellae (32), the incorporation into P-26000 was 1.2% of that incorporated into newly synthesized membrane protein.

Among the rapidly labeled soluble proteins (Fig. 2) are the large (LS) and small (SS) subunits of RuBPCase, whose bands dominate the fluorogram patterns. The vigorous incorporation into RuBPCase both in developing and fully mature tissue is clearly evident. Active synthesis of RuBPCase in the distal section of mature leaves has also been reported in wheat (7). These two cases stand in contrast to the marked reduction in incorporation of labeled precursors into carboxylase in a number of other plant species at about the time that leaf expansion ceases (8, 9, 24).

An interesting finding emanating from our experiments is that in fully expanded fronds two chloroplast-translated proteins (P-32000 and LS-RuBPCase) were made in large quantities in the apparent absence of chloroplast rRNA synthesis (32). Thus, in mature photosynthetic tissue, plastid protein synthesis takes place on "old" ribosomes, a situation similar to that occurring in the cytoplasm of mature enucleate mammalian reticulocytes.

In conclusion, fronds of both developmental stages transcribed 0.5 × 10⁶ d RNA and translated P-32000 in vivo. In the case of the protein, its synthesis compared to that of other proteins was enhanced in mature tissues. The relative rates of labeling of plastid mRNA and proteins in developing versus mature tissues have not been methodically investigated. Still, preliminary experiments suggest that the differences, if any, are minor. Our data thus appear sufficient to discount a purely developmental or transient function for P-32000 during thylakoid biogenesis. Support for this conclusion comes from the persistent production of P-32000-like molecules, at all stages, in chloroplasts isolated from green corn (16), peas (35), and expanding spinach (14) leaves.

**Relationship to Photosynthesis**

The objective of subsequent experimentation was to explore a possible functional relation between P-32000 and the complete photosynthetic apparatus as measured by CO₂ fixation. More specifically, does the 32,000 d polypeptide operate as an integral rate-limiting part anywhere in the steps leading from light to CO₂ assimilation in the chloroplast?

**Photosynthetic Competence.** We first demonstrated that both expanding and mature fronds were quite capable of photosynthetic CO₂ fixation. This was ascertained in two ways to preclude the effects of possible artifacts. One group of fronds was incubated as intact plants with ¹⁴CO₂ in the light and then dissected, to prevent any effects of tissue damage on photosynthetic activity. In this case there is always the possibility that the expanding tissue acts as a metabolic sink. Thus, a second group was first dissected and the frond pieces then incubated with ¹⁴CO₂ in the light to exclude the effects of assimilate transport on the measurements. In both cases the expanding and mature half-fronds were photosynthetically competent (Table 1), the lighter green developing fronds having a greater capacity for photosynthesis when Chl served as the common denominator. These data indicate that young, developing Spirodela fronds (at <10% of their fully expanded size) are capable of producing their own photosynthates and need not serve as a "sink" for the photosynthates in the fully expanded fronds. This is in contrast to 10% expanded leaves of beans and several other plants which serve as sinks (37). Thus, the presence of P-32000 and photosynthetic capacity in the expanding and mature tissues neither negated or supported a direct link between P-32000 and photosynthesis. Therefore, we chose a technique which would selectively reduce the amount of P-32000 in the tissue to differentiate between the two.

**P-32000 Depletion with Chloramphenicol.** The 32,000 d thylakoid protein seemed to have the shortest half-life of all rapidly labeled Spirodela proteins resolved on polyacrylamide gel fluorograms (11). We thus designed experiments to determine whether P-32000 could be massively depleted by precubating tissue with an inhibitor of organelle protein synthesis before measuring photosynthesis. We chose D-threo-chloramphenicol which has been widely used to prevent translation on 70S chloroplast ribosomes (12, 26). Results from such an approach can be unambiguously interpreted only if chloramphenicol treatment does not also lead to serious reduction of photosynthetic competence in the tissue; for if a reduction of photosynthesis occurred, it could be the result of depletion of P-32000, or any other rate-limiting protein not visualized by our procedures.

The distributions of Spirodela proteins extracted from steady-state light-grown cultures, pulse-labeled for 3 h with [³⁵S]methionine following a 39-h preincubation with chloramphenicol, are shown in Figure 3, B and D. Among the membrane proteins (Fig. 3B) there was a severe and specific suppression of [³⁵S]methionine incorporation into P-32000 by chloramphenicol (compare slot 4 with slots 5 and 6). Densitometric analysis of fluorograms showed that chloramphenicol suppressed incorporation into P-32000 by >95% (Table II). Conversely, the cytoplasmically translated, light-harvesting Chl a/b protein (P-26000) as well as most other rapidly labeled Spirodela membrane components, was not noticeably affected at these inhibitor concentrations and incubation time.

Before the results in Figure 3B concerning P-32000 could be properly analyzed vis à vis photosynthesis, it was important to determine whether the long preincubation with the inhibitor was, perhaps, suppressing the rate of P-32000 turnover in the tissue. This could lead to a situation where, although synthesis was inhibited, depletion of the existing molecules was blocked. We checked for this by pulse-labeling cultures for 3 h with [³⁵S]methionine and then removing the label for a 39-h chase in the presence or absence of chloramphenicol. From the profiles in Figure 3A it is apparent that about the same level of radioactivity remained associated with P-32000 in the untreated (slot 1) and inhibitor-treated samples (slots 2 and 3).

An indication of the considerable extent of normal turnover of P-32000 during the elapsed 39 h can be had by comparing slots 4 and 1 which show the control pattern (no chloramphenicol) for the pulse and chase experiments, respectively. This was checked by densitometrically scanning the fluorograms. Following the chase only 12 to 16% of the counts incorporated in the 3-h period remained in P-32000 (Table II). This was irrespective of whether chloramphenicol was used during the chase period. We concluded that under the conditions used, chloramphenicol did not have a significant effect on P-32000 turnover and could be used to prevent its synthesis.

Another factor to be considered was the effect of chloramphenicol on photosynthetic enzymes such as RuBPCase in the soluble protein fraction. The inhibitor severely reduced incorporation of [³⁵S]methionine into LS-RuBPCase as well as several other rapidly labeled soluble proteins (compare slots 11 and 12 with control slot 10). Unlike the situation with short lived P-32000, the depletion

Table 1. Photosynthetic ¹⁴CO₂ Fixation in Expanding and Mature Spirodela Fronds

<table>
<thead>
<tr>
<th>Labeling Conditions</th>
<th>Expanding (d1 mm)</th>
<th>Mature Distal Half</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO₂ Fixation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact plants</td>
<td>430</td>
<td>1290</td>
</tr>
<tr>
<td>Dissected tissue</td>
<td>480</td>
<td>1740</td>
</tr>
<tr>
<td>Radioactivity fixed normalized per unit Chl</td>
<td>209</td>
<td>123</td>
</tr>
</tbody>
</table>


of the long lived LS protein and other major labeled soluble proteins did not take place during the 42 h of the experiment. This is seen by comparing slots 10 and 7 which are the chloramphenicol-free controls for the pulse and chase experiments, respectively. The inhibitory effects of chloramphenicol on the synthesis of the cytoplasmically translated SS-RuBPCase (compare slots 11 and 12 with 10) have been reported before and are apparently due to some type of coordinate control (10). Finally, we note that there was no apparent change in the Coomassie staining pattern of the soluble or membrane proteins following inhibitor treatment (not shown, indicating that our regime of chloramphenicol did not significantly reduce any of the proteins present in the largest absolute quantities. Thus, under the experimental conditions which we used, the combined effects of inhibition of synthesis and depletion appeared to be more or less specific for the membrane-bound 32,000 d protein.

It was now possible to ascertain the level of photoassimilation of CO$_2$ in tissue considerably depleted of P-32000 by chloramphenicol. At 10 µg chloramphenicol/ml, synthesis of P-32000 was inhibited >95% while depletion of the polypeptide present before treatment reached 84% (Table II). Under these same conditions, photosynthetic CO$_2$ fixation was maintained at >85% of the control value (Table II). There is the possibility that the depletion of a protein of the photosynthetic pathways will not be rate-limiting when light is above saturation levels. We thus performed similar photosynthesis experiments with fluxes between 400 and 11,500 ergs/cm$^2$·s. The chloramphenicol-treated tissue showed the same low percent reduction of CO$_2$ fixation at all light intensities, both above and below saturation. The calculated amounts of residual P-32000 in the chloramphenicol-treated samples may, in fact, be overestimated due to the probable presence of minor protein bands of similar electrophoretic mobility, whose synthesis was not affected. There was no total elimination of P-32000 from the fronds. Still the marked lack in stoichiometry between the levels of P-32000 and CO$_2$ assimilation in the treated samples suggests that this protein is not at all rate-limiting for the photosynthetic process. It is likely that any protein with a very high turnover rate would be rate-limiting well before depletion. Any P-32000 depletion then would decrease photosynthesis if there is a connection. As such, the data then indicate that P-32000 is probably not a direct, integral part of the photosynthetic pathway within the thylakoids of Spirodela.

Our results do not support a direct participation of P-32000 in two major and multifaceted plastid functions: thylakoid biogenesis, and light-mediated CO$_2$ fixation. The continuing search for function thus becomes harder and, in a sense, more intriguing. It has been observed that P-32000-like molecules from pea chloroplasts are immunoprecipitated from detergent-solubilized thylakoids (together with most of the other membrane proteins synthesized in isolated chloroplasts) by antisera to purified coupling factor. This has led to the speculation that P-32000 may be a component of the “membrane sector” of the chloroplast ATP-synthetase complex (14). We are in agreement with (13) that this supposition needs additional documentation before it can be further evaluated. Both the ephemeral nature of P-32000 and its being a minor plastid translation product synthesized in Spirodela (11) are suggestive of its having a possible role in transport or assembly of organelle proteins. Investigations along these lines are planned.

Acknowledgments — We very much appreciate the useful discussions with H. Hoffman, R. Fluhr, Z. Kahana, and K. M. Jakob.

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FIG. 3. Differential effects of chloramphenicol on incorporation into labeled proteins in Spirodela. Steady-state log phase phototrophic cultures were transferred to 2 ml of medium in culture dishes. Chloramphenicol was added during the 39-h chase period after a 3-h pulse of [35S]methionine (A and C) or as a 39-h preincubation before a 3-h pulse of [35S]methionine (B and D). In A and C the [35S]methionine was removed and the plants were rinsed five times with a solution containing 5 x 10^-8 M unlabeled DL-methionine. Chloramphenicol concentrations were 0, 10, 30, µg/ml; 38.5 µCi [35S]methionine was used for the 3-h pulse. Preparation of membrane and soluble fractions, gel electrophoresis, fluorography, and standards were as in Figure 2. Equal numbers of counts (80,000 cpm) were applied to all slots.

Table II. Relationship between the 32,000 d Thylakoid Protein and Photosynthetic CO$_2$ Fixation

<table>
<thead>
<tr>
<th>Chloramphenicol µg/ml</th>
<th>% 35S-labeled P-32000 Remaining after Treatment</th>
<th>3-h Pulse of ([35S]methionine then 39-h chase with chloramphenicol</th>
<th>Photosynthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100 (control)*</td>
<td>12</td>
<td>100b</td>
</tr>
<tr>
<td>10</td>
<td>4.6</td>
<td>16</td>
<td>86</td>
</tr>
<tr>
<td>30</td>
<td>3.1</td>
<td>14</td>
<td>76</td>
</tr>
</tbody>
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* 21,800 cpm (determined densitometrically).

b 4050 cpm/10 fronds-h.
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