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Light-Induced Nuclear Synthesis of Spinach Chloroplast Fructose-1,6-bisphosphatase[1]

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ABSTRACT

Etiolated spinach (*Spinacia oleracea* L. var Winter Giant) seedlings show a residual photosynthetic fructose-1,6-bisphosphatase activity, which sharply rises under illumination. This increase in activity is due to a light-induced *de novo* synthesis, as it has been demonstrated by enzyme labeling experiments with ^2H_2O and [^{35}S]methionine. The rise of bisphosphatase activity under illumination is strongly inhibited by cycloheximide, but not by the 70S ribosome inhibitor lincocin, which shows the nuclear origin of this chloroplastic enzyme.

FBPase[2] is a key enzyme in the regulation of reductive pentoses-phosphate cycle, and as such it has been profusely studied from structural and regulatory view points (6, 8, 17, 22, 28). This enzyme, as some others of the Calvin cycle and related processes, show changes in activity after the dark-light transition, which are concerned with an increase in the stromal pH and Mg^{2+} concentration (21), but also to a light modulation by reduction of essential –S–S–groups of the enzyme molecule. Such activation-deactivation processes act in a reversible way, and are mediated either by the Fd-thioredoxin system of the stromal solution (26), or the LEM factors bound to the thylakoid lamellae (1). Both mechanisms show some degree of enzyme specificity, which takes place because the existence of different thioredoxins as well as membranal LEM factors (7). Concerning FBPase, its light activation by the Fd-thioredoxin system occurs specifically through thioredoxin-f (27), whereas the LEM_{II} is the only LEM operative in this process (2).

However, little attention has been paid to the possible existence of a second light regulatory mechanism of FBPase activity, by way of a photoregulated induction of enzyme synthesis, such it has been found for the $NADP^+$-MD (25), the 32 to 34 kD polypeptide which shields the B acceptor of PSII (23), and for the assembly as a whole of ribulose-1,5-bisphosphate carboxylase (5). In this work, we study the role of light on the biosynthetic rate of photosynthetic FBPase from spinach leaves, as well as the site in which its synthesis takes place.

MATERIALS AND METHODS

Spinach (*Spinacia oleracea* L. var Winter Giant) seeds were germinated in moistened sand in the dark, at 22°C for 2 weeks; the etiolated seedlings were then transferred to hydroponic cultures in ⅓ diluted Hewitt medium, at 25°C under 100 $w \cdot m^{-2}$ irradiance (Grolux-Sylvania tubes and Osram incandescent lamps). After 50 h growth, the leaves were blended (1:10, w/v) with 25 ml Tris-HCl buffer (pH 7.5), 5 mM $MgCl_2$, filtered through four layers of nylon cloth, and the homogenate centrifuged at 30,000g for 20 min at 4°C. The supernatant was used for protein and enzymic analysis, and acrylamide gel electrophoresis.

In the inhibition assays the etiolated 2 week seedlings were grown after addition to the hydroponic media of 200 μg/ml chloramphenicol, 1 μg/ml cycloheximide, or 100 μg/ml lincocin. Other experimental data were as above.

For ^2H labeling experiments the above 2-week seedlings were grown in the same conditions, but with ⅕ ^2H_2O dilution of the original medium (Bureau des Isotopes Stables, Saclay, France). The leaves were processed as above, and the supernatant of centrifugation adjusted to 80% $(NH_4)_2SO_4$ saturation; the pellet was dissolved in 50 mM acetate buffer (pH 5.5), and the solution filtered through a Sephadex G-50 column (2 × 10 cm). The eluate was used for enzyme determinations and for ultracentrifugation experiments according to Chrispeels and Varner (9). A volume of 3.5 ml was carried out at 34% CsCl saturation in a final volume of 5 ml, and centrifuged at 250,000g for 65 h at 4°C in an L5-65 Beckmann ultracentrifuge equipped with a SW-65L swinging-bucket rotor. An internal standard of 1 mg of yeast glucose 6-P dehydrogenase (Calbiochem) was added to each tube before centrifugation. The gradient was drained upward at a flow rate of 0.36 ml/min (56 fractions with about 90 μl each), and tested for enzyme activities.

For [^{35}S]methionine labeling, 0.4 g leaves of the etiolated 2-week seedlings were incubated in a Petri dish with 20 ml of the diluted Hewitt solution, at 25°C under 100 $w \cdot m^{-2}$ irradiation. After 3.5 h incubation, the medium was changed with 5 ml of fresh medium containing 0.4% Tween 80 and 160 μCi of [^{35}S]methionine (100 Ci/mol, Amersham) mixed with carrier to a final amount of 0.25 nmol. After labeling for additional 3.5 h, the leaves were washed with water and homogenized and filtered as above. After centrifugation at 30,000g for 20 min, the supernatant was heated at 60°C for 30 min and clarified by centrifugation in the same conditions, being used for enzymic and electrophoretic analysis.

FBPase, $NADP^+$-MD, NAD^+-MD, and glucose 6-P dehydrogenase activities were determined by standard methods (12, 16, 24). The incubation mixture for FBPase activity contained 5 mM substrate concentration, at which the cytosolic FBPase was fully inactive (29). Proteins were measured according to Lowry *et al.* (20), and Chl by Arnon's procedure (3).

In the electrophoretic analysis, 10-μl samples were fractionated on refrigerated horizontal polyacrylamide gels (7.5% acrylamide, 'Cyanogum' type), at 40 mamps for 3 h, and then the plates (25 × 11 cm) divided in five pieces. The first and fifth were fixed

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[2] Abbreviations: FBPase, fructose-1,6-bisphosphatase, EC 3.1.3.11; LEM, light effector mediator; $NADP^+$-MD, $NADP^+$-malate dehydrogenase; NAD^+-MD, NAD^+-malate dehydrogenase.

Table I. *Effect of Protein Synthesis Inhibitors on Chl, Protein Content, and Enzyme Activities of Spinach Leaves during Greening*

Chl was determined in intact leaves, and protein and enzyme activities analyzed in supernatants after centrifugation of leaf homogenates. One enzyme unit (U) is the amount of enzyme which reduces 1 μmol of oxalacetate (NAD^+-MD and $NADP^+$-MD), or hydrolyzes 1 μmol of fructose-1,6-bisphosphate (FBPase), per min in the assay conditions.

	Chl	Protein	FBPase		NAD^+-MD	$NADP^+$-MD
	μg/mg protein	*μg/seedling*	*mU/seedling*	*mU/mg protein*	*mU/mg protein*	
Green leaves	59	813	167	155	1305	5
Etiolated leaves	0	417	50	77	1426	0.3
Greening in the presence of:						
Cycloheximide (1 μg/ml)	20	593	53	75		
Chloramphenicol (200 μg/ml)	46	645	100	127		
Lincocin (100 μg/ml)	47	645	177	203		

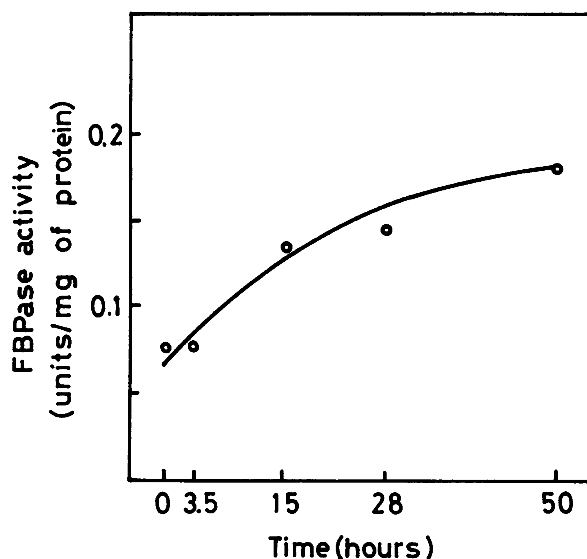

FIG. 1. Rise of FBPase activity with the illumination time of etiolated spinach seedlings. Activities were determined in crude leaf extracts.

and stained for proteins with Coomassie Blue. The second and third were enzymically stained for FBPase (16) and $NADP^+$-MD activities, using for the latter the $NADP^+$-linked phenazine methosulphate-nitroblue tetrazolium system (13), with L-malate as substrate. In the ^{35}S-labeling experiments, a fourth piece was fixed with methanol-acetic acid-H_2O (3:1:4, v/v), dried on vacuum at 60°C on a filter paper piece, and autoradiographically exposed to X-Omat Kodak film, for 6 d at −20°C.

RESULTS AND DISCUSSION

The function of photosynthetic cells requires a close cooperation between the protein synthesizing systems of cytoplasm and chloroplast. Many chloroplast proteins are known to be formed in the cytoplasm, from which they reach the chloroplast through the organelle double envelope by a mechanism still unknown in some aspects (10). This is the case of the low mol wt subunit of ribulose-1,5-bisphosphate carboxylase (11), plastocyanin, and Fd-$NADP^+$-reductase (15). In addition, some other organelle proteins are synthesized in the chloroplast, but frequently their synthesis is in a way under nuclear control (18).

The results of Table I show that the synthesis of photosynthetic FBPase during the greening of spinach seedlings is strongly inhibited by a cycloheximide concentration of 1 μg/ml, a known inhibitor of the 80S ribosomes mechanism. On the contrary, lincocin concentrations up to 100 μg/ml did not show any significant effect, whereas this inhibitor of the chloroplast 70S ribosomes strongly inhibited Chl synthesis by 23%. With chloramphenicol the results are not so clear, but this inhibitor has been often questioned about its specificity and secondary effects

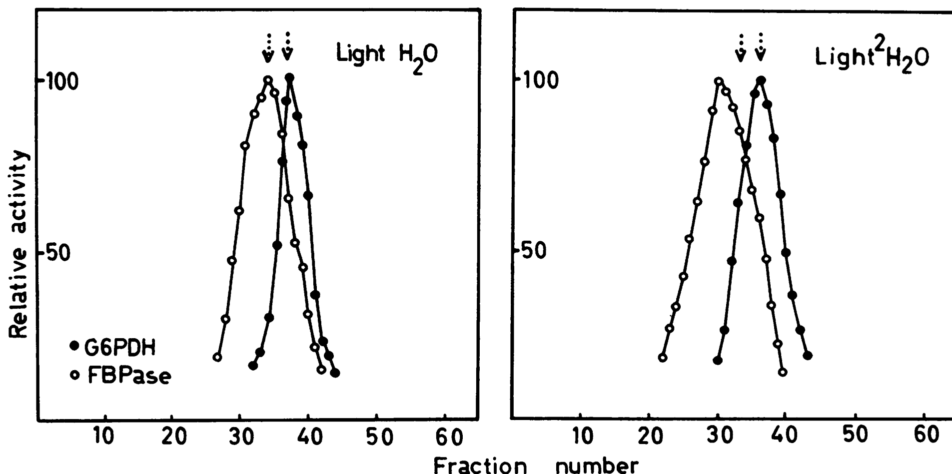

FIG. 2. CsCl density gradient ultracentrifugation of partially purified leaf extracts of etiolated spinach seedlings grown in the light in the presence of H_2O or ^2H_2O. An internal standard of yeast glucose 6-P dehydrogenase (G6PDH) was added to the purified extracts before centrifugation. Activities are expressed as percentages of those of the fractions with highest values; these fractions are marked in the diagram with arrows.

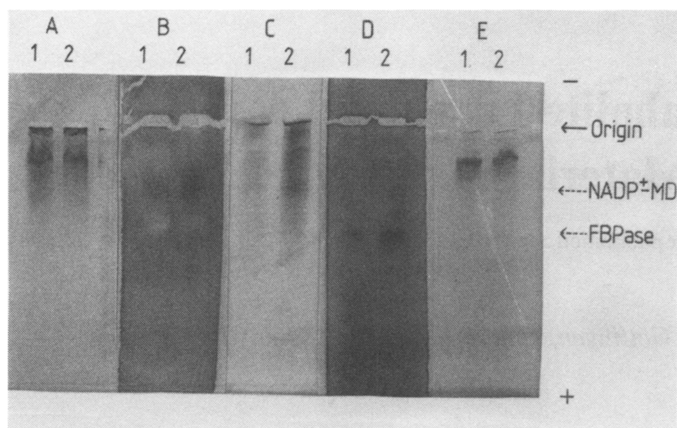

FIG. 3. Acrylamide gel electrophoresis of leaf extracts of etiolated spinach seedlings grown in the dark (1) or at light (2) in the presence of [^{35}S]methionine. A and E, Coomassie Blue stain; B and D, $NADP^+$-MD and FBPase specific stainings, respectively; C, autoradiography of ^{35}S-labeled proteins.

on chloroplast metabolism (19). Our results are consistent with the notion that the chloroplastic FBPase is synthesized in the cytosol, either as the native enzyme, or as a higher mol wt precursor. Indeed Grossman *et al.* (15) earlier showed the possibility of protein transport system in isolated pea plastids, even though the authors could not detect any translation product related to FBPase.

It has been known for a long time that light drives the synthesis of the large subunit of the ribulose-1,5-bisphosphate carboxylase (4) and some other chloroplast synthesized proteins. Moreover, the transfer of some cytoplasm synthesized proteins into the chloroplast is light dependent (14), as well as the assembly of some oligomeric plastid components, such as the ribulose-1,5-bisphosphate carboxylase (5). The outstanding role of light in building up the chloroplast enzyme equipment, moved us to study the incidence of illumination in the FBPase synthesis. FBPase activity sharply rises with the time when etiolated spinach seedlings were illuminated (Fig. 1).

The occurrence of a light induced *de novo* synthesis was demonstrated by enzyme labeling experiments. Etiolated seedlings grown at light with ^2H_2O incorporated ^{2}H in the FBPase, supplying enzyme molecules with a greater density. As other authors found in similar experiments concerned with $NADP^+$-MD synthesis (25), the heavy enzyme showed a shift in the elution profile when compared with the position of glucose 6-P dehydrogenase, used as internal standard (Fig. 2). Moreover, the symmetric narrow peak of normal FBPase changed to a wider and asymmetric one, mixture of the light and heavy enzyme.

Similarly, etiolated leaves incubated under illumination with [^{35}S]methionine incorporated ^{35}S in *de novo* synthesized FBPase. After acrylamide gel electrophoresis, a stronger activity was found in the illuminated leaves, as well as a close coincidence between the enzymically stained FBPase and one of the autoradiographically labeled bands (Fig. 3). As found by Vidal and Gadal (25), a rise in the $NADP^+$-MD activity was also detected in the illuminated samples, with incorporation of radioactive material in the corresponding band.

In conclusion, these results show that the light regulation of FBPase activity in plants may be exercised by a double mechanism: a faster one by activation-deactivation carried out through a reduction-oxidation process of the enzyme molecule, and a second slower brought about by an induction-repression of the enzyme synthesis. If the light acts in a direct way on the expression of FBPase gene, or indirectly through a metabolic induction linked to the greening, is now a matter of work in progress in our laboratory.

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