Light-induced de Novo Synthesis of Ribulose 1,5-Diphosphate Carboxylase in Greening Leaves of Barley

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ABSTRACT

An antibody specific for ribulose 1,5-diphosphate carboxylase was used to isolate the enzyme from greening barley (Hordeum vulgare L.) leaves. Increases in enzymatic activity during greening were due to de novo synthesis of the enzyme. Increases in enzymatic activity were accompanied by corresponding increases in enzyme protein and in incorporation of radioactive leucine, all of which were inhibited by low concentrations of cycloheximide. 18C-Labeled amino acids were incorporated into the enzyme by covalent peptide bonding.

Light, an important environmental factor affecting several physiological processes of plants, is required both for development of the photosynthetic apparatus and for the chemical act of photosynthesis. When etiolated tissues are exposed to light, chlorophyll is formed, protein is synthesized, chloroplasts develop an ordered lamellar form, and net incorporation of CO2 begins. Ribulose 1,5-diP carboxylase activity increases during the greening of several species of plants (2-4, 6, 10). The light-induced increase in activity in barley leaves is closely analogous to increases in CO2 fixation in greening wheat leaves as reported by Tolbert and Gailey (11).

Kupke (8) used analytical ultracentrifugation techniques to show that an 18S component (probably fraction I protein) increases during the greening of etiolated bean leaves. Present evidence indicates that ribulose 1,5-diP carboxylase and fraction I protein are probably identical (12). Furthermore, the increase in enzymatic activity can be prevented by inhibitors of protein synthesis (6, 10). These studies indicate that increases in ribulose 1,5-diP carboxylase activity during greening may be due to protein synthesis. The alternative, light-induced activation of the enzyme, cannot be ignored. Chen et al. (2) reported that a 3-min illumination of dark-grown corn plants increased the activity of the carboxylase enzyme. Wildner and Criddle (13) recently isolated a factor involved in light activation of this enzyme from tomato chloroplasts. Determination of whether the carboxylase is synthesized or activated during greening is a key step toward elucidating the role of light in cellular control of this enzyme. This study reports that the light-induced increase of ribulose 1,5-diP carboxylase activity in greening barley leaves was due to de novo synthesis of the enzyme.

MATERIALS AND METHODS

Plant Materials. Hordeum vulgare L., var. 'Blanco Mariout' was grown in vermiculite in 28 × 33-cm plastic pans. Moisture was supplied to the developing seedlings by cotton wicks connecting the vermiculite with a full strength nutrient solution (4). The seedlings were grown 7 days in the dark at 27 C and 55% relative humidity.

Treatment Solutions. Preliminary experiments showed that light induction of ribulose 1,5-diP carboxylase was greater and of more reproducible magnitude when the excised leaves were placed in 30 mM MgCl2 and 50 mM NH4NO3 instead of water. Accordingly, these salts were included routinely in all solutions given the leaves.

1H-Leucine was supplied to the excised leaves at the beginning of treatments either in light or continued darkness. Each treatment received the same amount of 1H-leucine, 30 µc at a specific activity of 44 mc/µmole. For the inhibitor studies, cycloheximide at 10 µg/ml was supplied to the excised leaves in darkness for 2 hr, and 1H-leucine was then added prior to the induction period. The 2-hr pretreatment allowed the inhibitor to enter the tissue before light induction was started.

Preparation of Cell-free Extracts. Twelve centimeters (tip to base) of the first leaf of 10 seedlings were excised and placed base down in 10 ml of the treatment solutions in the light (21,000 lux) or in the dark for the specified times. The leaves were trimmed to 10 cm just before assay and were ground in a mortar and pestle in 0.2 ml tris-SO4 buffer, pH 8 (3 ml/g of leaf material). The homogenates were centrifuged for 15 min at 30,000g, and the supernatants were used as the source of enzyme, for soluble enzyme determinations, and for reaction with the antibody.

Enzymatic Assay. Ribulose 1,5-diP carboxylase was assayed at 28 C by following the conversion of KH14CO3 into acid-stable products. The reaction mixture (0.1 ml) contained the following, in µmoles: ribulose 1,5-diP, 0.5; KH14CO3, 2.5 (with a specific activity of 0.455 µc/µmole); MgCl2, 3.0; tris-SO4 buffer (pH 8.0) 6.0. The reaction was initiated by adding to the reaction mixture 0.1 ml of an appropriately diluted extract. After 10 min the reaction was stopped by adding 50 µl of 1 N HCl. Aliquots of 0.1 ml were dried on strips of filter paper, placed in a toluene-base scintillation counter. Ribulose 1,5-diP was prepared as described previously (4).

The soluble protein content of the cell-free extracts was precipitated with trichloroacetic acid (final concentration of trichloroacetic acid, 5%) and was assayed by the method of Lowry et al. (9). The standard was bovine serum albumin, fraction V.

Antigen–Antibody Precipitation. The amount of a substance present in a mixture can be determined precisely by the quantitative precipitin method, provided that the substance is antigenic and can be obtained in a highly purified form for immunization.

4 Abbreviation: 1,5-diP: 1,5-diphosphate.
of rabbits (1). Rabbits were used because, up to the present time, precipitation of any antigen, either protein or carbohydrate in nature, with its specific rabbit antibody extrapolates to zero in the region of antibody excess (5). This method offers significant advantages over other analytical chemical methods (1, 5): (a) It is highly specific and permits the analysis of a constituent in a mixture without chemical fractionation; (b) very small amounts of antigen (10 μg) are required for protein analysis by the Folin-Ciocalteau colorimetric method; and (c) precipitates of antigen and antibody can be washed, dissolved and analyzed for radioactive label in the antigen (14).

Before being used, the antiribulose 1,5-diP carboxylase serum (7) was incubated for 1 hr at 36 C to inactivate complement. A series of dilutions of purified ribulose 1,5-diP carboxylase (7) were made containing 0 to 80 μg/0.2 ml. The antiserum was diluted 3-fold with 0.85% NaCl solution, and 0.2 ml was added to each of the antigen dilutions (0.2 ml), and the mixture was incubated 1 hr at 37 C. The precipitates were centrifuged and resuspended in 1-ml portions of saline solution twice, and the protein was assayed by the method of Lowry et al. (9). Figure 1 shows the resulting standard curve, which was used to determine the amount of ribulose 1,5-diP carboxylase in the antibody-antigen precipitates.

The validity of this method was established by showing that, at equal enzymatic activities, equal amounts of ribulose 1,5-diP carboxylase from purified and from crude extracts of both light and dark treatments were precipitated by the antibody. Also at equal enzymatic activities, equal amounts of antibody-antigen precipitate were formed.

The cell-free extracts were diluted so that the total precipitated protein was 50 to 200 μg. Equal volumes (0.2 ml) of diluted cell-free extract and antiserum were combined, and the precipitates were treated as above. Complete precipitation of the enzyme from the cell-free extracts was assured by conducting a ring or interfacial test with an aliquot each of the supernatant and antibody. Also, any ribulose 1,5-diP carboxylase activity remaining in the supernatant after centrifugation was detectable.

**Peptide Mapping.** Ten grams of excised leaves were given 100 μC of a 14C-amino acid algal hydrolysate (1.5 μc/μg) in the light. Ribulose 1,5-diP carboxylase was isolated from the leaves and was purified as previously described (7). Three milligrams of the enzyme protein were digested with trypsin (50:1, w/w) for 4 hr at 37 C in 0.1 M (NH4)2CO3, pH 8.5. The lyophilized product was dissolved in 0.2% ammonia and was chromatographed on Whatman No. 1 filter paper for 20 hr in butanol-acetic acid-water (4:1:5). After chromatography, the paper was turned 90 degrees and electrophoresis was conducted for 1 hr at 1500 v and 135 ma. The map was dried and used to make an autoradiogram, and then sprayed with 0.25% ninhydrin.

**RESULTS AND DISCUSSION**

Increases in enzymatic activity with time of illumination were accompanied by concurrent increases in concentration of ribulose 1,5-diP carboxylase protein, and by incorporation of labeled leucine into the enzyme (Fig. 2). The specific activities (enzymatic activity and 3H-leucine incorporation per unit of newly synthesized protein) remained constant during treatment. Little increase occurred in the incorporated leucine, in the enzymatic activity, or in the enzyme protein in leaves remaining in the dark.

The incorporation of label into total soluble protein increased with time of illumination (Fig. 3), whereas incorporation into ribulose 1,5-diP carboxylase reached a maximum at 18 hr (Fig. 2). The label in the carboxylase protein accounts for about 12% of the total incorporation into soluble protein after 18 hr of light. More 3H-leucine was incorporated into total soluble protein per milligram than into ribulose 1,5-diP carboxylase. This may be a reflection of the differential leucine content of the protein constituents or leucine pool sizes in the cytoplasm and chloroplasts. Of the 30 μc taken up by the plants, 1.2% was found in ribulose 1,5-diP carboxylase after 18 hr. Three milligrams (grams fresh weight)−1 of ribulose 1,5-diP carboxylase were synthesized during 18 hr of illumination. During this same period, total soluble protein increased 4.3 mg (grams fresh weight)−1 (Fig. 3). Thus, 70% of the protein synthesized was in the form of one enzyme.

**Fig. 2.** The effect of light on the synthesis of ribulose 1,5-diP carboxylase. □, ■: 3H-leucine incorporation in light and dark; ○, ●: ribulose 1,5-diP carboxylase protein in light and dark; △, ▲: ribulose 1,5-diP carboxylase activity in light and dark. Enzyme protein was determined by precipitation with a specific antibody.

**Fig. 3.** Incorporation of 3H-leucine into total soluble protein, □, ●: light and dark; △, ▲: total soluble protein light and dark. See "Materials and Methods" for experimental procedure.
If the enzyme were synthesized de novo, the increases in protein, activity, and incorporation of label should be decreased by inhibitors of protein synthesis. Cycloheximide (10 μg/ml) prevented any increase in enzymatic activity or enzyme protein and also prevented any incorporation of label (Fig. 4). The enzyme was inactivated between 18 and 24 hr, apparently by the cycloheximide treatment because enzymatic activity decreased while enzyme protein remained constant at 24 hr.

A peptide map of the enzyme protein was prepared to determine whether the incorporation of label into the protein was due to covalent bonding or to nonspecific binding of amino acids. Incorporation of label into the peptides of ribulose 1,5-diP carboxylase was widely distributed (Fig. 5). Ninety percent of the ninhydrin positive spots were radioactive; hence, the incorporation of labeled amino acid into the enzyme protein during greening was due to covalent bonding in the newly synthesized protein.

We tested for an activator of ribulose 1,5-diP carboxylase, according to the method of Wildner and Cridle (13), by running the enzyme reactions (extracts of both light and dark treatments) in the presence of light at 325 nm. No activation was observed here. We have recently learned that the barley enzyme can be activated in vitro by 550 nm of light (personal communication, L. W. Peterson and W. R. Anderson), and we have verified a 30% activation of the enzyme from greening barley leaves. The in vitro activity of the enzyme in this study was not affected by the light-activating factor because light of 550 nm was not supplied during the incubation period.

That this enzyme was synthesized de novo during greening of barley leaves was shown by the incorporation of label into the protein of ribulose 1,5-diP carboxylase and corresponding increases in actual enzyme protein, both of which corresponded to the observed light-induced increase in enzyme activity.

LITERATURE CITED

Fig. 4. Effect of cycloheximide on the synthesis of ribulose 1,5-diP carboxylase. □, □: light and dark ribulose 1,5-diP carboxylase protein; △, △: light and dark ribulose 1,5-diP carboxylase activity; •: incorporation of 3H-leucine into the enzyme. Arrow denotes time of application of inhibitor.

Fig. 5. Peptide map of labeled ribulose 1,5-diP carboxylase protein. A: developed x-ray film from B, 18-× 22-inch chromatogram. Ninhydrin-positive spots 49, five of which were nonradioactive. Radioactive spots 46, three of which were ninhydrin-negative. See "Materials and Methods" for experimental procedure.