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Biosynthesis of Ribulose-1,5-Bisphosphate Carboxylase in Spinach Leaf Protoplasts[1]

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ABSTRACT

Spinach leaf (*Spinacia oleracea* L. var. Kyoho) protoplasts sustain protein-synthesizing activity as measured by the incorporation of [^{14}C]-leucine into the protein fraction both in the light and in the dark. By the immunoprecipitation of ribulose-1,5-bisphosphate (RuP_2) carboxylase with rabbit antibody raised against the purified spinach enzyme preparation, it was found that approximately 7% of the total radiocarbon incorporated into the protein fraction in the light was in the carboxylase molecules. However, there was no measurable net increase observed in the content of the enzyme protein in the experimental conditions employed. It was found that both chloramphenicol and cycloheximide inhibited the incorporation of [^{14}C]leucine into RuP_2 carboxylase and its constituent subunits, as measured by the immunoprecipitation of the enzyme molecule and its subunits, A and B.

RuP_2[2] carboxylase comprises the major portion of the soluble chloroplastic proteins essential for autotrophic carbon assimilation in photosynthetic organisms. Much attention has been given to its biosynthesis (5, 6, 11, 12). Considerable experimental evidence has now shown that the two constituent subunits of the enzyme are synthesized in different cell sites: the large subunit A on the 70S chloroplastic ribosomes, and the smaller subunit B on the 80S cytoplasmic ribosomes, respectively (2, 5, 9, 10, 19, 20). Although remarkable research achievements have been made on the topic using the *in vitro* experimental system, the regulation mechanism(s) of the biosynthesis of these subunits is obscure. The use of isolated chloroplasts for analysis of the regulation mechanism(s) is at a disadvantage because metabolic channeling between chloroplasts and cytoplasm is cut off. The use of protoplasts in which this relationship is maintained offers some advantages (8, 16, 17, 21).

We report here experiments on the biosynthesis of RuP_2 carboxylase and its regulation in spinach protoplasts using immunochemical methods.

MATERIALS AND METHODS

Protoplasts. Protoplasts were prepared from freshly harvested spinach leaves (*Spinacia oleracea* L. var. Kyoho) essentially following the method reported previously (16). The preparations used in the study exhibited photosynthetic activities of 35 to 70 μmol of CO_2 fixed/mg of Chl · hr under the standard assay system (16).

Incorporation of [^{14}C]Leucine into Protein. The basic experimental methods employed were those reported by Mans and Novelli (14). Unless otherwise described, the standard reaction mixture contained the following components (μmol) in a total volume of 0.5 ml: β,β′-dimethylglutaric acid-NaOH buffer (pH 5.5), 50; $NaHCO_3$, 1; mannitol, 350; L-[U-^{14}C]leucine (2 μCi) (330 mCi/mmol) (Radiochemical Centre, Amersham), and protoplast preparations (75–100 μg of Chl). The assay mixture, except [^{14}C]leucine, was preincubated in darkness for 5 min at 25 C, and the reaction was started by adding [^{14}C]leucine; incubation was carried out either in the light (30 klux, white tungsten light) or in the dark. At selected reaction intervals, 0.1-ml aliquots of the assay mixture were withdrawn and added to 0.5 ml of 5% (w/v) trichloroacetic acid solution. The resulting precipitate was collected by the filtration through a Whatman No. 3MM paper disc, which was washed twice with 10 ml of 5% trichloroacetic acid, followed by washing twice with 10 ml of acetone. The discs were dried and radioactivity measurements were made in a Packard liquid scintillation spectrometer.

Sucrose Density Gradient Centrifugation. In order to determine the radioactivity distribution in the subcellular fractions, protoplast preparations (75 μg of Chl) exposed to [^{14}C]leucine for 3 hr in light (30 klux) were suspended in 1 ml of 0.05 M Tricine-NaOH buffer (pH 7.5) containing 0.5 M sucrose and 0.1% BSA, and mechanically ruptured in a syringe using the method reported previously (17). One ml of the ruptured preparation was then applied to sucrose density gradient centrifugation (35–60%, w/w) using an SW 25-3 rotor in a Beckman L-2 preparative ultracentrifuge. At the end of the centrifugation (24,000 rpm/3 hr at 4 C), 1-ml fractions were collected from the bottom of the tube, and subsequently used for the analysis of Chl and the ^{14}C incorporation into the trichloroacetic acid insoluble fraction exactly as described above. The peak fraction was subjected to the standard polyacrylamide gel electrophoresis (7.5%, pH 8.9) for protein staining as reported previously (18).

Quantitative Immunoprecipitation Analysis. For the preparation of rabbit antisera against the native RuP_2 carboxylase (anti-[N]) as well as the two constituent subunits (anti-[A] and anti-[B]), fractions obtained by $(NH_4)_2SO_4$ precipitation (50% saturation) of the specific antisera prepared according to our previous report (15) were used. The specific immunological cross-reactivity of each antiserum was confirmed. For the purpose of measuring ^{14}C incorporation into RuP_2 carboxylase and its subunits, a 0.5-ml aliquot of the reaction mixture containing protoplasts incubated with [^{14}C]leucine for appropriate periods was homogenized in a Teflon homogenizer. The resulting whole homogenate was passed through a small column of Sephadex G-25, which had been equilibrated with 0.05 M Tris-HCl buffer (pH 7.5), and the eluate obtained was made up to 1 ml with the same buffer solution. A 0.5-ml sample of the resulting eluate was then mixed with 0.1 ml

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[2] Abbreviation: RuP_2: ribulose 1,5-bisphosphate.

of the rabbit antisera, *e.g.* anti-[N] (11 mg/ml), anti-[A] (44 mg/ml), or anti-[B] (46 mg/ml) and the whole mixture was left overnight at 4 C. The precipitates collected by centrifugation were repeatedly washed with 0.5 ml of 0.5% NaCl at 4 C, and finally dissolved in 0.2 ml of 0.02 M phosphate buffer (pH 7) containing 1% SDS and 1% DTT. The resulting mixture was then subjected to radioactivity measurements, analysis of protein content, and SDS gel electrophoresis (see below).

SDS-Polyacrylamide Gel Electrophoresis. The basic method employed was that of Weber and Osborn (22), and a 0.1-ml protein sample was used for electrophoresis at 10% gel concentration. After the electrophoresis, gels were stained with Coomassie brilliant blue, and destained gels were scanned at 620 nm using a densitometer (Toyo Digital Densitorol DMU-33C). Subsequently, the sliced gel pieces (1 mm) were solubilized using 0.5 ml of H_2O_2-NH_4OH (99:1, v/v) at 50 C for 8 hr. Then 0.5 ml of distilled H_2O and 10 ml of Triton-toluene scintillator were added to the digested samples, and the radioactivities determined in a Packard liquid scintillation spectrometer.

Analysis. Chl content was analyzed according to the method of Arnon (1), and protein content was determined according to Lowry *et al.* (13) using BSA as a standard.

RESULTS AND DISCUSSION

Under the standard reaction system employed, it was found that [^{14}C]leucine was incorporated into the total protein fraction at a linear rate up to 5 hr, and the rate in darkness was about 70% of that in the light. When N_2 was substituted for air, the rate in darkness was reduced by 76% (data not shown, but see Fig. 3). On the basis of the specific radioactivity of [^{14}C]leucine supplied, the rate of incorporation in the light was calculated to be 0.04 to 0.1 nmol of leucine/mg of Chl·hr.

To determine the specific incorporation into RuP_2 carboxylase, mechanically ruptured protoplast samples were treated with the rabbit antisera against RuP_2 carboxylase (anti-[N]), and the antigen-antibody precipitate was subjected to radiocarbon measurements. Figure 1 shows that there occurred a time-dependent labeling of the immunoprecipitate, demonstrating the incorporation of [^{14}C]leucine into the enzyme molecule; the rate in the light was much greater than in the darkness. We consistently observed a certain lag period of the ^{14}C incorporation in the initial 1 hr or so. In the light, the total incorporation of [^{14}C]leucine into RuP_2 carboxylase during 3 hr of incubation was approximately 7% of that into the total trichloroacetic acid-insoluble fraction. However, there was no measurable net increase in the content of the enzyme protein in either light or darkness (Fig. 1) or in enzyme activity (data not shown).

To substantiate further the radiocarbon labeling of the carboxylase molecule, the antigen-antibody precipitate obtained after 3 hr of incubation was subjected to separation by SDS gel electrophoresis, followed by radioactivity measurements of the sliced gel segments. Figure 2 clearly shows the labeling of the two constituent subunits, A and B. After subtracting the background level of ^{14}C, the ratio of ^{14}C in A and B was 5:1, approximately the same as that of the molar ratio of leucine in the two subunits of the enzyme molecule.

Our current knowledge of the biosynthesis of RuP_2 carboxylase in eukaryotic plant cells has indicated that the large (A) and small (B) subunits of the enzyme molecule are synthesized on chloroplastic (70S) and cytoplasmic (80S) ribosomes, respectively (2, 5, 9, 10, 19, 20). In order to examine the possible interaction of mechanisms underlying the biosynthesis of the two subunits, the effect of antibiotics was tested. From the results of preliminary experiments, the lowest concentrations of each reagent causing the maximal inhibitory effect were determined. Thus, either chloramphenicol (0.5 mg/ml) or cycloheximide (5 μg/ml) or both were added to the reaction mixture. Figure 3 shows that the incorporation of [^{14}C]leucine into the total protein fraction in the light was strongly inhibited by cycloheximide; chloramphenicol also exhibited a prominent inhibitory effect (about 54%). The labeling of chloroplast proteins was markedly suppressed by treatment

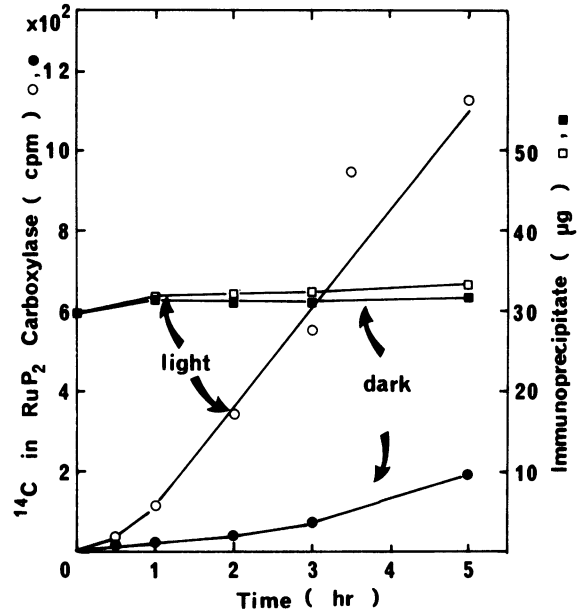

FIG. 1. Incorporation of [^{14}C]leucine into RuP_2 carboxylase and analysis of immunoprecipitate content using anti-[N]. Basic experimental procedures are described in the text, and the reaction mixture (pH 5.5) was incubated with [^{14}C]leucine for up to 5 hr in the light or in the dark. At the selected reaction intervals, aliquots were withdrawn, burst in a Teflon homogenizer, and subsequently treated with 0.1 ml of the rabbit anti-[N] solution (11 mg/ml) for quantitative immunoprecipitation analysis. The antigen-antibody precipitates were thoroughly washed with NaCl solution, followed by measurements of radioactivities and analysis of protein content.

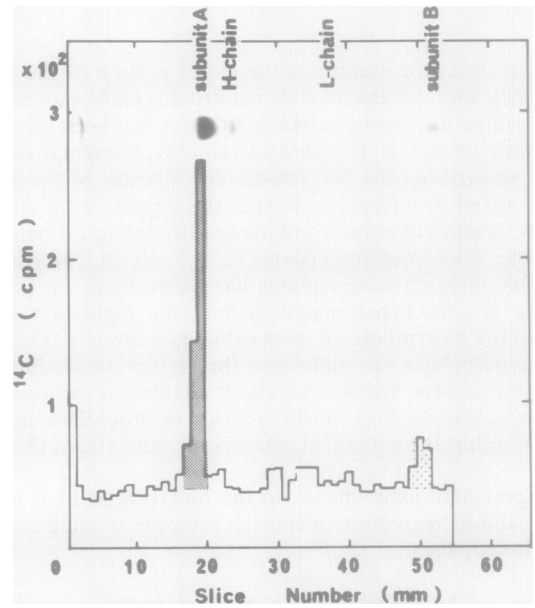

FIG. 2. Radiocarbon labeling of subunits A and B of RuP_2 carboxylase. Experimental conditions using the standard reaction mixture (pH 5.5) were the same as for Figure 1. After incubation for 3 hr in the light, the antigen-antibody precipitate was applied to SDS gel electrophoresis. Sliced gel segments (1 mm) were solubilized and radioactivity measurements were made using an aqueous scintillator system.

with chloramphenicol (80% inhibition at 3 hr), while labeling of soluble proteins appeared to be less sensitive to this treatment (Fig. 4). Electrophoretic analysis of the chloroplast fraction from the sucrose gradient (Fig. 4) showed that a major component of this fraction was RuP_2 carboxylase. The proportion of the chloroplast label in this protein was 18%.

Under comparable experimental conditions, the effect of two antibiotics on the radiocarbon labeling of RuP_2 carboxylase and its subunits was examined by means of immunoprecipitation techniques. From the results in Figure 5A, it is evident that by

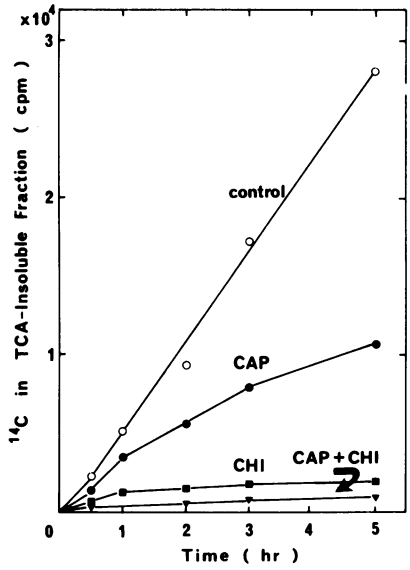

FIG. 3. Effect of antibiotics on [^{14}C]leucine incorporation into the protein fraction in the light. Experimental conditions were basically the same as for Figure 1, except that chloramphenicol (CAP) (0.5 mg/ml), cycloheximide (CHI) (5 μg/ml), and CAP (0.5 mg/ml) + CHI (5 μg/ml) were added to the assay mixture.

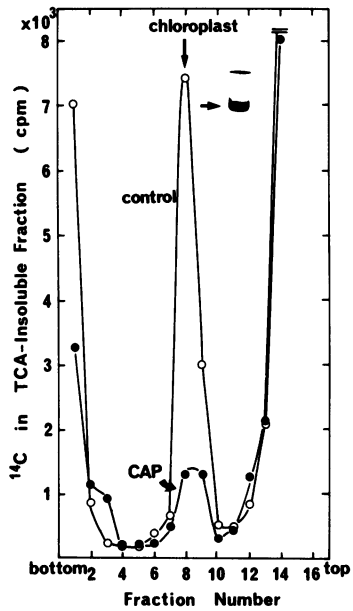

FIG. 4. Effect of chloramphenicol on [^{14}C]leucine incorporation into the chloroplastic protein fraction in the light. Basic experimental procedures are described in the text, except that the effect of chloramphenicol (CAP) (0.5 mg/ml) was tested during 3-hr incubation in the light. Subsequently, sucrose density gradient centrifugation was employed to determine the ^{14}C distribution in various fractions. A portion of the top fraction was applied to polyacrylamide gel electrophoresis; arrow indicates RuP_2 carboxylase band (inset).

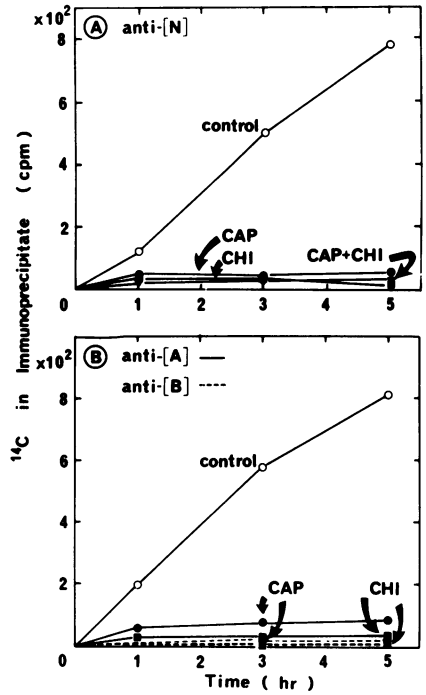

FIG. 5. Effect of antibiotics on [^{14}C]leucine incorporation into RuP_2 carboxylase (A) and its constituent subunits (B). A: experimental conditions were basically the same as for Figure 3, except that the effect of chloramphenicol (CAP) (0.5 mg/ml), cycloheximide (CHI) (5 μg/ml), and CAP (0.5 mg/ml) + CHI (5 μg/ml) on the time-dependent labeling of RuP_2 carboxylase in the light was examined. For the radioactivity measurements, the enzyme protein was precipitated by treating with 0.1 ml of anti-[N] (11 mg/ml). B: same as for A, except that 0.1 ml each of either anti-[A] (44 mg/ml) or anti-[B] (46 mg/ml) was added to collect the antigen-antibody precipitate. In both A and B, assay mixture without addition of the antibiotics served as the control, and the enzyme protein was precipitated either by anti-[N] (A) or by anti-[A] (B). Other experimental details are described in the text.

treating the protoplast preparation with either chloramphenicol or cycloheximide the labeling of the whole RuP_2 carboxylase molecule precipitable by anti-[N] is completely suppressed. Figure 5B shows that radioactivity is barely detectable in immune precipitates formed with anti-[A] and anti-[B] when the antibiotics are present.

Our previous experiments (15) using rabbit antisera against each individual subunit molecule of RuP_2 carboxylase have revealed that both anti-[A] and anti-[B] bind with the native enzyme as well as the dissociated free form of each subunit. It is therefore assumed that under the experimental conditions presently employed, any free subunit will be precipitated along with native enzyme. The results in Figure 5B therefore demonstrate that the inhibition of synthesis of 1 subunit molecule of RuP_2 carboxylase is accompanied by the abolition of the synthesis of the other subunit molecule. This suggests the operation of a coordinated coupling mechanism in the biosynthesis of the whole enzyme molecule. However, we must reserve our final conclusion on this observation in view of the fact that some side effects may be exerted by the antibiotics (6) and the size of the free subunit pool may be regulated by endogenous proteases (either naturally, or artifactually after homogenization of the protoplasts). Similar results showing that the biosynthetic mechanisms of the two subunits are tightly coupled were reported by Cashmore (3) in pea leaf tissues and Iwanij *et al.* (12) in synchronously growing cells of *Chlamydomonas reinhardtii*. On the other hand there is substantial experimental evidence that the synthesis of the two subunits can be partially (4) or completely uncoupled in barley (7).

In the present study, we have been unable to detect measurable

net increase of the enzyme content during the assay periods in the light, although [^{14}C]leucine was incorporated into the RuP_2 carboxylase molecules. In order to answer a crucial question of whether this incorporation reflects the turnover of the enzyme protein, further exploitation of pulse-label and/or chase experiments is clearly needed. Such an approach with the protoplast system may help answer the question of whether appreciable amounts of subunits accumulate during carboxylase biosynthesis. In this connection it is of interest that protein precursors of subunit B have been detected in *C. reinhardtii* (5) and in pea (11, 19).

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