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Further Studies on the Subunit Structure of *Chromatium* Ribulose-1,5-bisphosphate Carboxylase†

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ABSTRACT: Upon alkali exposure *Chromatium* ribulose-1,5-bisphosphate carboxylase dissociates into constituent subunits, a catalytic oligomer of the larger subunit, A_8, and monomeric form of the small subunit B. By sedimentation equilibrium molecular weights of the native enzyme and the catalytic oligomer produced by an alkali treatment were estimated to be 5.11×10^5 and 4.29×10^5, respectively. To provide information on reversibility of the dissociation by determining whether the enzymically inactive small subunit B of the whole enzyme molecule did indeed exchange with exogenously added subunit B a radioisotopic method was used. After initial alkaline dialysis at pH 9.2 of a mixture of a nonlabeled native enzyme preparation and ^{14}C-labeled subunit B, and the subsequent dialysis at pH 7.0, incorporation of ^{14}C into the recovered native enzyme was determined. Without the alkaline treatment there was no detectable exchange, while after alkaline dialysis for 5 and 10 hr the subunit B exchange was 89 and 82%, respectively. Rabbit antiserum prepared against the catalytic oligomer of the spinach ribulose-1,5-bisphosphate carboxylase, anti-[A] (spinach), inhibited the *Chromatium* carboxylase and oxygenase activities. This result together with the identical immunoprecipitation lines on an agar plate formed between the antiserum and the *Chromatium* carboxylase and between the antiserum and the catalytic subunit of the *Chromatium* enzyme strongly indicated structural near identity of the catalytic subunits of the spinach and *Chromatium* carboxylase molecules. Results also show that the catalytic site of the *Chromatium* ribulose-1,5-bisphosphate carboxylase and oxygenase exists in the large polypeptide chain.

Ribulose-1,5-bisphosphate (RuP_2)[1] carboxylase (EC 4.1.1.39) from a photosynthetic purple bacterium, *Chromatium* strain D, dissociated reversibly into constituent subunits upon brief exposure to alkali (eq 1) (Akazawa *et al.*, 1972). The oligomeric form of the larger subunit was shown to retain partial enzyme activities of RuP_2 carboxylase and oxygenase reaction without the aid of the small subunit B (Takabe and Akazawa, 1973a,b). The molecular weights of the catalytic oligomer determined by Sephadex G-200 gel filtration and polyacrylamide gel electrophoresis of different porosities were 4.4×10^5 and 4.2×10^5, respectively. Since a molecular weight value of 5.7×10^4 was obtained for the monomeric large subunit (A) of the enzyme (Akazawa *et al.*, 1972), it has been proposed that the catalytic unit is composed of eight large subunits (A_8) (Takabe and Akazawa, 1974). In spinach leaf RuP_2 carboxylase the octamer of the larger subunit produced by alkaline treatment in the presence of *p*-mercuribenzoate was shown to be the catalytic entity (Nishimura *et al.*, 1973). The absence of cross-contamination between the catalytic oligomer and the small

$$A_{m(=8)}B_n \underset{\text{pH 7.0}}{\overset{\text{pH 9.0}}{\rightleftharpoons}} A_{m(=8)} + nB \qquad (1)$$

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[1] Abbreviations used are: RuP_2, ribulose-1,5-bisphosphate; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; ammediol, 2-amino-2-ethyl-1,3-propanediol.

subunit was evidenced by the analytical results of their C-terminal amino acids as well as the immunochemical tests (Nishimura and Akazawa, 1973, 1974a,b). In this article, we report results obtained from tests for an exchange reaction between the small subunit moiety of the nonlabeled *Chromatium* RuP_2 carboxylase and exogeneously added ^{14}C-labeled subunit B. Results from these experiments as well as the results of immunochemical studies of the enzyme and its subunits support our previous conclusion that the catalytic site of the *Chromatium* RuP_2 carboxylase and oxygenase exists in the large polypeptide chain, namely subunit A.

Materials and Methods

Bacterial Culture and Purification of RuP_2 Carboxylase. The experimental procedure of culturing *Chromatium* cells under photoautotrophic conditions (2000 lx) and the subsequent procedure for isolating a pure RuP_2 carboxylase preparation were reported previously (Akazawa *et al.*, 1972). The purified homogeneous enzyme had a specific activity of 0.80 μmol of CO_2 fixed/mg of protein per min (pH 7.8). In order to obtain a labeled RuP_2 carboxylase preparation, [^{14}C]$NaHCO_3$ (200 μCi) was added to a 1-l. culture at the late logarithmic growth phase, and the cells were harvested after 10 hr and mixed with nonlabeled bacterial cells before enzyme purification.

Alkali Dissociation of ^{14}C-Labeled RuP_2 Carboxylase. Approximately 9.5 mg of the purified ^{14}C-labeled *Chromatium* carboxylase (2×10^4 dpm/mg of protein) dissolved in 0.5 ml of 0.025 M Tris-HCl buffer (pH 7.5) was loaded on a column of Sephadex G-200 (2 × 65 cm), preequilibrated with 0.025 M Tris-HCl buffer (pH 9.2) containing 0.1 mM EDTA and 1 mM DTT, and eluted with the latter at a flow rate of 40 ml/hr at 4°. To avoid formation of higher aggregates of the enzyme (*cf.* Figure 3 in Takabe and Akazawa, 1973a), elution time was reduced by using a short column containing coarse Sephadex G-200 particles ($d > 74$ μ). Fractions collected were subjected to (i) ultraviolet (uv) absorbancy measurement at 280 nm, (ii) enzyme assay at pH 8.3 (see below), and (iii) radioactivity measurement (*cf.* Figure 1). Two fractions, one containing subunit A and the other containing subunit B, were clearly separated, and the absence of cross-contamination was confirmed by the SDS–polyacrylamide gel electrophoresis (Weber and Osborn, 1969). The fraction containing subunit B (1.91×10^4 dpm/mg of protein) was concentrated in a collodion membrane bag for reconstitution experiments described later.

Another enzyme preparation was treated with 1% SDS–1% β-mercaptoethanol, and the subunits formed were separated by Sephadex G-100 column chromatography. The mass ratio of the two subunits determined by the colorimetric analysis of Lowry *et al.* (1951) was 78:22, in agreement with the value reported by Rutner (1970) for spinach leaf RuP_2 carboxylase.

Ultracentrifugation Studies. The determination of the molecular weights of the native enzyme and the catalytic oligomer was performed by sedimentation equilibrium as described by Yphantis (1964) in a Hitachi UCA-1A ultracentrifuge. The native enzyme (0.06 ml) dialyzed in 0.05 M Tris-HCl buffer (pH 7.0) containing 0.1 mM EDTA, 5 mM DTT, and 0.1 M NaCl at a concentration of 0.5 mg/ml was pipetted into a sample channel, and 0.06 ml of the buffer was added to the complementary sector. To estimate the molecular weight of the catalytic oligomer produced by alkali treatment, 1 part of the native enzyme preparation dialyzed against 0.01 M Tris-HCl buffer (pH 7.0) containing 0.1 mM EDTA, 5 mM DTT, 0.1 M NaCl and 10% glycerol was added to 9 parts of 0.05 M Tris-HCl buffer (pH 9.0) containing 0.1 mM EDTA, 5 mM DTT, 0.1 M NaCl, and 10% glycerol. The protein sample (0.06 ml) at concentrations of 0.3 and 0.5 mg/ml was pipetted into the sample channels, and 0.06 ml of the same buffer mixture devoid of enzyme protein was added to the complementary sectors. Centrifugation was carried out for 20 hr (with native enzyme) and 16 hr (with alkali-treated enzyme) respectively, at 10,591 rpm at 20°.

Reconstitution of RuP_2 Carboxylase. The exchange reaction between the ^{14}C-labeled subunit B and a small subunit moiety in unlabeled native enzyme molecule was examined by sucrose density gradient centrifugation. A mixture of the ^{14}C-labeled subunit B (0.27 mg) and nonlabeled native enzyme (0.81 mg) in a total volume of 0.45 ml was dialyzed against 0.025 M Tris-HCl buffer (pH 9.2) containing 0.1 mM EDTA and 1 mM DTT at 4° for 5 or 10 hr. The mass ratio of subunit B to the larger catalytic oligomer was 2.5 times greater in this mixture than that in the native enzyme. During the alkaline dialysis, the native enzyme molecule was expected to dissociate into the catalytic subunit oligomer, A_8, and the monomeric small subunit B, the latter being in equilibrium with the labeled sample. The mixture was then dialyzed against 0.025 M Tris-HCl buffer at pH 7.0 containing 0.1 mM EDTA and 1 mM DTT at 4° for 4 hr. During this dialysis, reassociation of native enzyme from the two constituent subunits was expected to occur. An aliquot (250 μl) withdrawn from the dialyzed mixture was then layered on the top of the sucrose gradient (5–20%, w/v) containing 0.025 M Tris-HCl buffer (pH 7.0), 0.1 mM EDTA, and 1 mM DTT, and centrifuged at 5.8×10^4 rpm in an SW 65 Ti rotor of a Beckman Spinco Model 65-B ultracentrifuge (2.5 hr at 4°). To each fraction (0.25 ml) collected, 0.1 ml of 0.025 M Tris-HCl buffer (pH 7.0) was added, and an aliquot from that sample analyzed for (i) protein content using the method of Lowry *et al.* (1951), (ii) enzymic assay at pH 7.0, and (iii) radioactivity.

Enzyme Assay. (i) RuP_2 CARBOXYLASE. The assay method was essentially the same as that reported previously (Akazawa *et al.*, 1972). The following reaction mixture was used in a total volume of 0.5 ml (in micromoles): Tris-HCl buffer (pH 7.0 or 8.3), 100; $MgCl_2$, 5; RuP_2, 0.7; and [^{14}C]$NaHCO_3$, 25 (2.0 μCi). The reaction was started by adding RuP_2. At the end of incubation (25°, 10 min), 0.05 ml of glacial acetic acid was added to terminate the reaction. $^{14}CO_2$ fixed was counted with a Packard liquid scintillation spectrometer.

(ii) RuP_2 OXYGENASE. The standard reaction mixture contained the following components in a total volume of 1.0 ml (in micromoles): ammediol-HCl buffer (pH 9.0), 200; $MgCl_2$, 10; RuP_2, 1.4. The reaction was started by adding RuP_2 and O_2 consumption was measured using a Rank Bros. oxygen electrode (Bottisham, U.K.) at 25° (Akazawa and Osmond, 1974).

Immunochemical Methods. Experimental details of immunochemical methods were essentially the same as those employed in our previous work on spinach leaf RuP_2 carboxylase (Nishimura and Akazawa, 1974a). Rabbit antisera developed against native *Chromatium* RuP_2 carboxylase and the catalytic oligomer of the spinach enzyme (anti-[A]) (Nishimura and Akazawa, 1974a) were used to examine their inhibitory effect on *Chromatium* RuP_2 carboxylase and oxygenase activities. The double immunodiffusion on

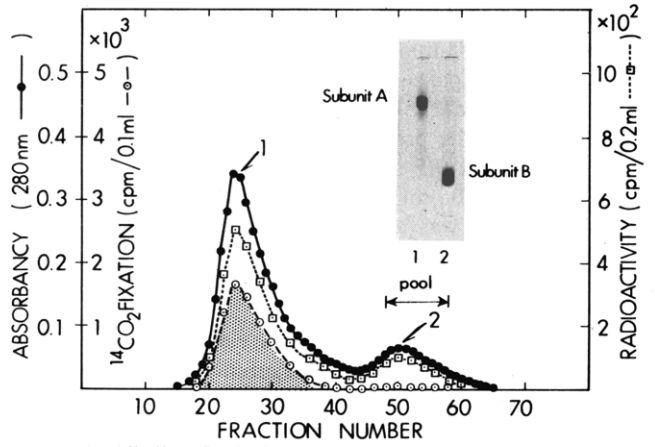

FIGURE 1: Alkaline Sephadex G-200 gel filtration of ^{14}C-labeled RuP_2 carboxylase. Experimental procedures are described in the text. Enzymic activity of each selected fraction was determined by adding 100 μl of the fraction to the standard assay system (pH 8.3), and incubating the mixture at 25° for 10 min. The inset shows a SDS–polyacrylamide gel electrophoretogram of two fractions (tubes 26 and 50), one containing subunit A and another subunit B.

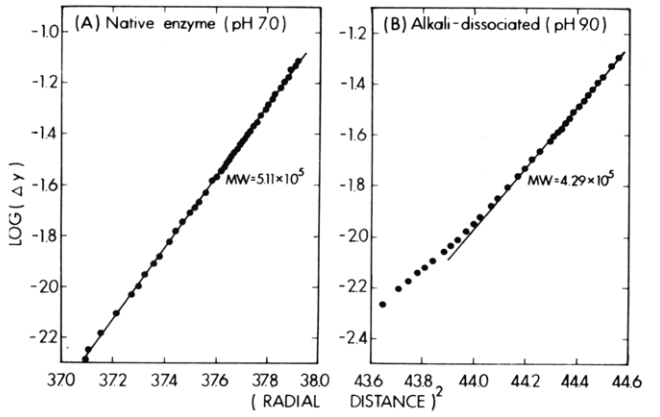

FIGURE 2: Equilibrium sedimentations of RuP_2 carboxylase and alkaline treated RuP_2 carboxylase. Experimental procedures are described in the text. (A) Purified RuP_2 carboxylase was centrifuged at 10,591 rpm and 20° for 20 hr. Initial protein concentration was 0.5 mg/ml. For this analysis, meniscus depletion occurred, and a base line was directly obtained. (B) Alkaline treated enzyme was centrifuged at 10,591 rpm and 20° for 16 hr. Initial protein concentration was 0.5 mg/ml. The base line was determined by overspeeding to 60,000 rpm for 4 hr. In these figures, fringe shift (Δy) and radial distance are expressed in centimeters.

an agar plate was carried out using the anti-[A] according to the method of Ouchterlony (1949).

Results

Clear separation of the larger catalytic subunit A_8 and the small subunit B produced upon alkaline treatment of the ^{14}C-labeled *Chromatium* RuP_2 carboxylase was achieved by Sephadex G-200 column chromatography at pH 9.2 (Figure 1). The absence of cross-contamination was supported from the result of SDS–polyacrylamide gel electrophoresis (inset). The specific enzyme activity of the fast-eluting catalytic oligomer was 0.085 μmol of CO_2 fixed/mg of protein per min (pH 8.3), which was approximately 14% of the specific activity of the native enzyme assayed at pH 7.0. This relative activity was comparable with that reported previously (Takabe and Akazawa, 1973a,b).

The native enzyme and the alkali-treated preparation were subjected to equilibrium centrifugation. By assuming

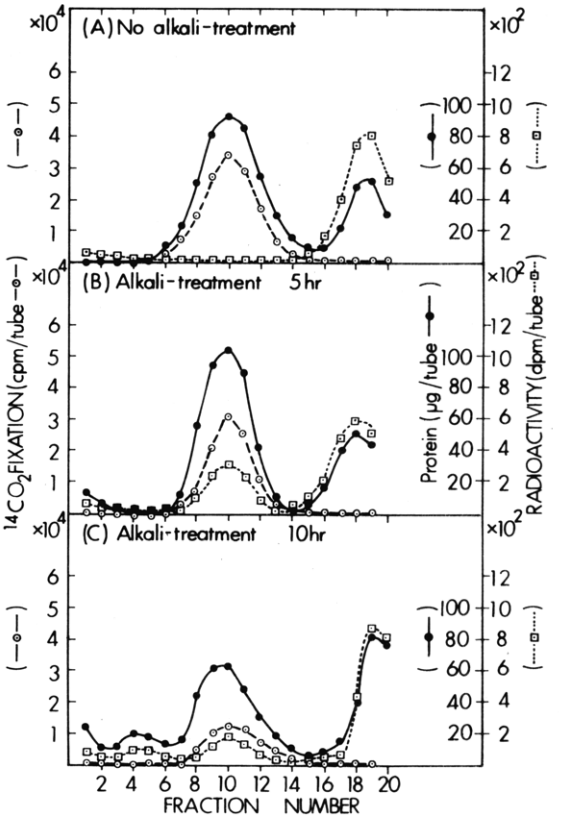

FIGURE 3: Exchange of subunit B moiety in RuP_2 carboxylase. Experimental details are described in the text. Enzyme samples mixed with ^{14}C-labeled subunit B were preliminarily dialyzed at an alkaline pH (9.2) (A, 0; B, 5 hr; C, 10 hr) and again dialyzed at pH 7.0 and subsequently applied to sucrose gradient centrifugation.

a partial specific volume of the enzyme of 0.74 ml/g, the molecular weight was estimated to be 5.11×10^5 (Figure 2A). The addition of 10% glycerol to alkaline buffer (pH 9.0) prevents the further dissociation of the catalytic oligomer formed into smaller molecular species. Catalytic oligomer is stable for 20 hr. The results presented in Figure 2B show that the plot of log Δy *vs.* (radial distance)^2 is definitely nonlinear, and the molecular weight calculated near the bottom of the sample column was 4.29×10^5 taking 0.74 ml/g as partial specific volume. The value was in good agreement with that reported separately (Takabe and Akazawa, 1974), supporting the octameric form of the catalytic subunit of *Chromatium* RuP_2 carboxylase.

Exchange of subunit B of the *Chromatium* carboxylase with added ^{14}C-labeled subunit B can be expressed in the following equation (2), where asterisks indicate relative ap-

$$aA_8B_n + bB^{**} \xrightarrow{\text{pH } 9.2} aA_8 + (an + b)B^* \qquad (2)$$
$$\xrightarrow{\text{pH } 7.0} aA_8B_n^* + bB^*$$

proximate specific radioactivities. Results of the exchange experiments are presented in Figure 3. Without the alkali treatment (control), there was no detectable radioactivity in the native enzyme fractions (tubes 8–12 in Figure 3A). Upon alkali treatment for 5 and 10 hr, followed by an annealing process for 4 hr at pH 7.0, about 96 and 72% of the native enzyme protein were recovered, respectively, and measurable ^{14}C incorporation into the reassociated enzyme fractions (B and C) was observed. From the mass per cent of subunit B in the native molecule (22%), it could readily

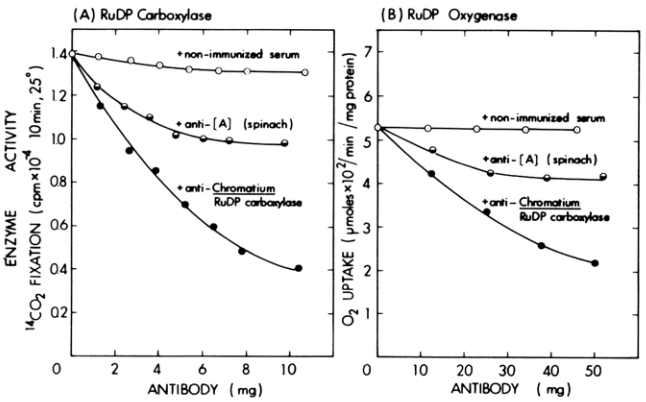

FIGURE 4: Inhibitory effect of rabbit antisera on *Chromatium* RuP_2 carboxylase (A) and RuP_2 oxygenase (B) activities. Experimental procedures were the same as those reported previously for spinach RuP_2 carboxylase (Nishimura and Akazawa, 1974a). Each respective antiserum of various amounts was added to the native enzyme (30 μg with RuP_2 carboxylase and 150 μg with RuP_2 oxygenase reaction, respectively), and incubated for 30 min at 25° before starting the enzyme assay. Enzyme assay methods are described in the text.

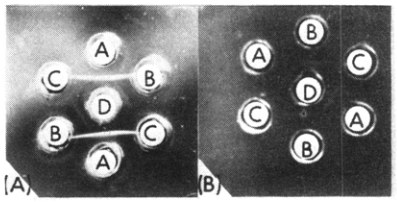

FIGURE 5: Ouchterlony immunodiffusion experiment. Agar plates were made by dissolving 1% Difco special agar noble in 15 ml of 0.025 M Tris-HCl buffer (pH 8.5) containing 1% NaN_3. The antibody well contained *ca.* 200 μg of the γ-globulin fraction of anti-[A] (spinach) serum, and antigen wells contained *ca.* 25 μg of protein samples. (A) Well D contained anti-[A] (spinach) serum. A, B, and C contained spinach RuP_2 carboxylase, *Chromatium* catalytic subunit A_8, and small subunit B, respectively. (B) Well D contained anti-[A] (spinach) serum. A, B, and C contained *Chromatium* RuP_2 carboxylase, catalytic subunit A_8, and small subunit B, respectively.

be shown that the specific radioactivity of the subunit B in the assay mixture had been diluted to 1.14×10^4 dpm/mg of protein, assuming a complete equilibrium was established. The specific radioactivity of the reconstituted enzyme molecule (A_8B_n*) could also be calculated to be 2.53×10^3 dpm/mg. The observed radioactivity in whole enzyme fractions (tubes 8–12 in Figure 3B,C) was 89% for the 5-hr treatment and 82% for the 10-hr treatment, showing a nearly complete exchange of subunit B under the experimental conditions employed. The specific enzyme activity of reassociated enzyme was determined to be 79% (5 hr) and 52% (10 hr) of the native enzyme activity. Upon the longer alkali exposure (10 hr), more formation of higher aggregates (tubes 1–4 in Figure 3) and less recovery of enzyme activity are noted than for the shorter alkali exposure (5 hr).

The exchange experiments described above not only showed that the added radioactive subunit B was indeed exchanged with subunit B in the unlabeled native enzyme, but also demonstrated that subunit B alone did not show the enzyme activity (Figure 3). An immunochemical study gave further evidence that the catalytic site of the *Chromatium* carboxylase and oxygenase reactions is in the subunit A. Figure 4 clearly shows the inhibition of the enzyme reactions by the rabbit antiserum raised against the native *Chromatium* RuP_2 carboxylase. Since we have been unsuccessful in obtaining a sufficiently potent rabbit antiserum against the catalytic oligomer of the bacterial enzyme because of difficulty in obtaining a large amount of the catalytic oligomer, the effect of anti-[A] from the spinach enzyme was tested. The spinach anti-[A] did show definite, though not as much as that for the anti-*Chromatium* RuP_2 carboxylase, inhibitory effect on both RuP_2 carboxylase and oxygenase activities. The immunoprecipitation lines on the Ouchterlony agar plate further lent support to the probable structural homology of the catalytic subunits in the spinach and *Chromatium* RuP_2 carboxylases. When spinach RuP_2 carboxylase and the catalytic oligomer (A_8) and the small subunit (B) from the *Chromatium* enzyme were tested against anti-[A] (spinach RuP_2 carboxylase) immunodiffusion, partially identical precipitation lines were detected between the spinach enzyme and *Chromatium* A_8 (Figure 5A). As shown in Figure 5B, a complete fusion occurred in the precipitation lines with *Chromatium* RuP_2 carboxylase and its catalytic oligomer, A_8.

Discussion

Results of our present studies clearly demonstrate that reversible dissociation–association occurs with the bacterial enzyme in accordance with eq 1. This is well supported by nearly complete exchange of the subunit B moiety (Figure 3). The discrepancy between the theoretical and observed values may partly be attributable to experimental errors. However, other explanations may be considered. For example, alkaline treatment of the enzyme molecule perhaps causes, even in the presence of DTT, nonspecific conformational changes of the subunits, which may prevent reassociation of the subunit A_8 with the subunit B. In this connection it is significant that the longer the alkaline treatment, the greater is the amount of higher aggregates formed as shown in Figure 3C (also Figure 5 of Akazawa *et al.*, 1972), and the less enzyme activity remains in the reconstituted enzyme after longer alkaline treatment (79%, 5 hr; 52%, 10 hr). A possibility also exists that there is a small undissociable segment in the native enzyme molecule.

It may be argued that the inhibition of the bacterial enzyme activities exhibited by the anti-[A] raised against the subunit A of the spinach enzyme cannot be considered as evidence for the subunit A of the bacterial enzyme being the catalytic segment, simply because bacterial anti-[A], which could not be produced for this study, may not be identical with the spinach anti-[A]. However, a number of immunochemical studies have shown that there seems to be an interspecies homology among RuP_2 carboxylases from various plant sources, which resides in subunit A (Dorner *et al.*, 1958; Sugiyama *et al.*, 1969; Kawashima and Wildman, 1970; Matsumoto *et al.*, 1969; Akazawa *et al.*, 1972; McFadden, 1973; Akazawa, 1974; Osmond *et al.*, 1974). Results of amino acid compositions strongly indicate that the larger subunit might in fact be the structurally homologous segment in these different enzymes. Indeed the catalytic subunit of the *Chromatium* carboxylase has an amino acid composition very similar to the catalytic subunits of plant enzymes (T. Takabe and T. Akazawa, unpublished results). Therefore, it is reasonable to conclude that the inhibition of the enzyme reactions elicited by the rabbit anti-[A] produced against the spinach enzyme (Figure 4) as well as the result of immunodiffusion (Figure 5) can be considered as supporting evidence that the subunit A_8 of the bacterial enzyme does play the catalytic role and that there is structural similarity (perhaps near identity) between the

catalytic subunits of *Chromatium* and spinach carboxylases.

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Crystallization and Partial Characterization of Prenyltransferase from Avian Liver†

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ABSTRACT: Prenyltransferase (EC 2.5.1.1) has been obtained from chicken liver in a stable crystalline form. The enzyme has been shown to be homogeneous by polyacrylamide gel electrophoresis at pH 8.4, and by electrophoresis in sodium dodecyl sulfate containing gels. Electrofocusing of the crystalline enzyme results in a single sharp protein peak with a p*I* of 5.72. The protein is a dimer of molecular weight 86,000 whose subunits were not resolved by gel electrophoresis in sodium dodecyl sulfate. Michaelis constants of 0.5 μM for both isopentenyl pyrophosphate and geranyl pyrophosphate are 3–20-fold lower than those found for prenyltransferase from yeast or pig liver (Eberhardt, N., and Rilling, H. C. (1974), *J. Biol. Chem.* (in press); Dorsey, J. K., Dorsey, J. A., and Porter, J. W. (1966), *J. Biol. Chem. 241,* 5353; Holloway, P. W., and Popjak, G. (1967), *Biochem. J. 104,* 57). The enzyme primarily synthesizes farnesyl pyrophosphate from dimethylallyl or geranyl pyrophosphate although some geranylgeranyl pyrophosphate is formed under certain conditions. This is the first preparation of a stable crystalline enzyme of sterol and terpene biosynthesis.

Prenyltransferase (EC 2.5.1.1) has been isolated in a substantially purified form from pig liver by Dorsey *et al.* (1966) and Holloway and Popjak (1967). More recently, Eberhardt and Rilling (1974) have reported the preparation of homogeneous prenyltransferase from *Saccharomyces cerevisiae.* In all instances the copurification of both dimethylallyl transferase and geranyl transferase activities was demonstrated. Thus, the dimethylallyl transferase reaction (the condensation of dimethylallyl pyrophosphate with isopentenyl pyrophosphate to form geranyl pyrophosphate) and the geranyl transferase reaction (the condensation of geranyl pyrophosphate with isopentenyl pyrophosphate to form farnesyl pyrophosphate) appear to be the combined functions of a single enzyme. Unfortunately, the homogeneous enzyme obtained from *S. cerevisiae* was insufficiently stable to permit an experimental approach to the deter-

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