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Further Studies on Ribulose 1,5-Diphosphate Carboxylase from *Chromatium* Strain D†

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ABSTRACT: Ribulose 1,5-diphosphate carboxylase was isolated from autotrophically grown cells of purple sulfur photosynthetic bacterium, *Chromatium* strain D, and purified to a homogeneous state on the basis of ultracentrifugation and polyacrylamide gel disc electrophoresis at pH 7.5. Two bands detected in the sodium dodecyl sulfate–polyacrylamide gel disc electrophoresis indicated that the bacterial ribulose 1,5-diphosphate carboxylase consisted of two nonidentical polypeptide chains (subunits). The molecular weight of native enzyme was estimated to be 5.5×10^5, and that of each monomeric subunit (A and B) being 5.7×10^4 and 1.2×10^4, similar to that of spinach and *Chlorella* enzymes. Polyacrylamide gel electrophoresis at pH 8.9 indicated dissociation of the enzyme molecule as shown by the formation of a fast-moving band in addition to a slowly moving major band. These bands were considered to be A_m and B, respectively, where A_mB_n (A_8B_8 or A_8B_6) represents compositional subunit organization of this bacterial enzyme signifying that the enzyme molecule consists of *m* molecules of subunit A and *n* molecules of subunit B. Dissociation of the carboxylase molecule at the alkaline pH and its reassociation into the original molecule upon neutralization as accompanied by restoration of the enzyme activity were further ascertained by sucrose density gradient centrifugation.

In the series of our work on structure–function relationships of ribulose 1,5-diphosphate (RuP_2)[1] carboxylase [3-phospho-D-glycerate carboxylase (dimerizing), EC 4.1.1.39] from lower to higher photosynthetic organisms, the enzyme of the purple sulfur bacterium, *Chromatium* strain D, was of special interest. The *Chromatium* RuP_2 carboxylase was similar to the enzymes from spinach and a green alga, *Chlorella ellipsoidea*, in its large molecular size (Anderson *et al.*, 1968; Kieras and Haselkorn, 1968; Gibson and Hart, 1969; Akazawa *et al.*, 1970a), some enzymic properties (Akazawa *et al.*, 1970a), and immunological specificities (Kieras and Haselkorn, 1968; Akazawa *et al.*, 1970a), but was distinguishable from those of purple nonsulfur photosynthetic bacteria, *Rhodopseudomonas spheroides* and *Rhodospirillum rubrum* (Akazawa *et al.*, 1969; Akazawa *et al.*, 1970b). Previously we reported that the RuP_2 carboxylase isolated from either spinach or *Chlorella* consisted of two nonidentical polypeptide chains (Sugiyama and Akazawa, 1970; Sugiyama *et al.*, 1971). The amino acid composition of the large subunit (A) was similar, whereas that of the smaller subunit (B) was very different between the two plant species. Determination of the carboxyl-terminal amino acid of each subunit further revealed structural differences between the two enzymes. In this paper we report a rigorous purification of RuP_2 carboxylase from *Chromatium* cells and the studies made on the structure–activity relationships of the homogeneous enzyme preparation thus obtained. It is from these studies that we found that the *Chromatium* RuP_2 carboxylase consists of two nonidentical subunits, and that the native enzyme molecule is reversibly dissociated into subunits under an alkaline condition.

Materials and Methods

Bacterial Culture. Chromatium strain D was grown in the culture medium of Newton (1962) as described previously (Akazawa *et al.*, 1970a). The bacterial cells growing photo-

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[1] Abbreviations used are: RuP_2 carboxylase, ribulose 1,5-diphosphate carboxylase; TM buffer, 0.05 M Tris-HCl buffer (pH 7.0) containing 5 mM freshly distilled β-mercaptoethanol.

autotrophically under an anaerobic condition (2000 luxes, 30°) were harvested at the stationary growth phase. The collected cells washed once with 0.05 M Tris-buffer (pH 7.0) were used for the subsequent enzyme extraction. When a large amount of bacteria was needed for enzyme isolation the harvested cells were stored in a Deepfreeze (−20°) until enough cells were accumulated.

For the radioactive labeling of RuP_2 carboxylase, the method employed in our previous study was used (Akazawa and Miyagi, 1972). [^{14}C]$NaHCO_3$ (50 μCi) was added to a 5-l. culture flask at the logarithmic growth stage, and the harvested cells were combined with normally grown non-radioactive bacterial cells for the subsequent enzyme isolation.

Purification of RuP_2 Carboxylase. In our previous experiment, we stopped enzyme purification at the second ammonium sulfate precipitation (80%) after Sephadex G-200 gel filtration step. Although the purity of the *Chromatium* carboxylase appeared to be excellent even at this stage (from the sedimentation profile on the sucrose density gradient centrifugation) (*cf.* Figure 4 of Akazawa *et al.*, 1970a), further purification was accomplished as described below. Another modification employed was the addition of freshly redistilled β-mercaptoethanol just before use to 0.05 M Tris-HCl buffer (pH 7.0) in each purification step at the final concentration of 5 mM. This buffer was referred to as TM buffer.

After the second ammonium sulfate precipitation step, the precipitate was dissolved in TM buffer and dialyzed against TM buffer exhaustively overnight at 4°. The dialyzed protein solution was applied to a column of DEAE-cellulose, which was preequilibrated with TM buffer, and was eluted by the same buffer with a linear sodium chloride gradient, 0–1.0 M, and 10-ml fractions were collected (*cf.* Figure 1A). The enzymically active fractions were precipitated again by adding solid ammonium sulfate at 80% saturation, and (after dialysis as before) the dissolved enzyme was applied to a column of DEAE-Sephadex A-50, which was preequilibrated with TM buffer containing 0.1 M sodium chloride. The elution was carried out by the same buffer with a linear sodium chloride gradient, 0.1–0.6 M (*cf.* Figure 1B). The enzymically active fractions were collected and precipitated by addition of ammonium sulfate at 80% saturation. The final enzyme preparation was stored in a cold room (0°) after exhaustive dialysis against TM buffer. Under this condition the enzyme was found to be stable for more than 1 month without loss of activity. In the purification procedures employed, our aim was directed to remove as many impure contaminants from the final preparation as possible even at the sacrifice of enzyme yield. Starting from 200 g (fresh weight) of freshly harvested bacterial cells approximately 20 mg of the purified enzyme protein was obtained. The specific enzyme activity determined was 0.8 μmole/min per mg of protein, quite comparable to that of the spinach enzyme (Wishnick *et al.*, 1969).

Enzyme Assay. Unless otherwise indicated the assay method used was the one previously described by us (Sugiyama *et al.*, 1968a). The composition of the reaction mixture was: Tris-HCl buffer (pH 7.0), 100 μmoles–RuP_2, 0.7 μmole–$NaHCO_3$, 25 μmoles (2.0 μCi)–$MgCl_2$, 5 μmoles–0.05–0.1 ml of the enzyme preparation at an appropriate purification stage in a total volume of 0.5 ml. Under the assaying conditions, substrate and Mg^{2+} are saturated. After incubation at 25° for 10 min the enzyme reaction was stopped by adding 0.05 ml of glacial acetic acid and a 0.2-ml aliquot was used for radioactivity counting with 10 ml of aqueous scintillating fluid of Bray (1960).

Sucrose Density Gradient Centrifugation. The method employed was previously described (Akazawa *et al.*, 1970a; Sugiyama *et al.*, 1970; Akazawa and Miyagi, 1972) except that TM buffer was used as the medium. An appropriately treated enzyme sample was loaded on the top of the sucrose gradient (5–20%, w/v), and centrifuged at 30,000 rpm for 15 hr (4°) in an SW 50 rotor of a Beckman Spinco Model L-2 preparative ultracentrifuge. After the run, fractions consisting of 12 drops (approximately 0.2 ml) each were collected by puncturing the bottom of the tube. To each fraction 0.3 ml of TM buffer (pH 7.5 or 8.9) was added before measuring the uv absorption at 280 nm and the RuP_2 carboxylase activity.

Estimation of Molecular Weight of RuP_2 Carboxylase. The method originally reported by Andrews (1964), using an upward flow column (2.5 × 83 cm, Uppsala, Sweden) of Sephadex G-200, was adopted to determine the molecular weight of *Chromatium* RuP_2 carboxylase (*cf.* Akazawa *et al.*, 1969). The elution was carried out with TM buffer at a flow rate of 5 ml/hr at 2°. A calibration curve was made by determining the exclusion volume of marker proteins; apoferritin (4.8×10^5), γ-globulin (1.6×10^5), bovine serum albumin (6.7×10^4), and ovalbumin (4.5×10^4). The exclusion volume of the RuP_2 carboxylase was determined by assaying the carboxylase activities under standard conditions as described above.

Polyacrylamide Gel Electrophoresis. Analytical acrylamide gel electrophoresis (7.5% cross-linkage) described by Ornstein and Davis (1962) and Davis (1964) was carried out at pH 8.9 and 7.5 at the constant current of 4 mA per tube at 4°. It must be noted, however, that the electrode buffer was the same one used in electrophoresis at pH 8.9 (*cf.* Figures 3 and 5).

For sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the method described by Weber and Osborn (1969) was used as in our previous study with spinach and *Chlorella* enzymes (Sugiyama and Akazawa, 1970; Sugiyama *et al.*, 1971), except that the chemical modification of the enzyme molecule was omitted. For the estimation of molecular weight of each subunit, the native enzyme incubated with 1% each of sodium dodecyl sulfate and β-mercaptoethanol at 37° for 3 hr was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Reference proteins of known molecular weight were also run at the constant current of 8 mA per tube.

Analytical Ultracentrifugation. The purified enzyme preparation was adjusted to a desired pH and was applied to a Hitachi analytical ultracentrifuge Model UCA-1-A equipped with schlieren optics.

Immunological Study. The procedure employed was similar to that reported previously for spinach and *Chlorella* RuP_2 carboxylases (Sugiyama *et al.*, 1969). The antibody was prepared by immunizing rabbits with subcutaneous injection of a purified preparation of spinach RuP_2 carboxylase. The method of Ouchterlony (1949) was used to carry out immunodiffusion tests on agar gel with approximately equal quantities of the enzyme proteins from spinach, *Chlorella*, and *Chromatium*.

Analytical Methods. The protein was determined by the Folin method modified by Lowry *et al.* (1951), with bovine serum albumin as standard.

Electron Microscopy. The diluted enzyme preparation was negatively stained with 1% sodium phosphotungstic acid (pH 6.9) (Sugiyama *et al.*, 1968b). An electron photomicrograph was kindly taken by Dr. K. Kojima at the Aichi Cancer Institute with a Hitachi high-resolution electron microscope Model 11D.

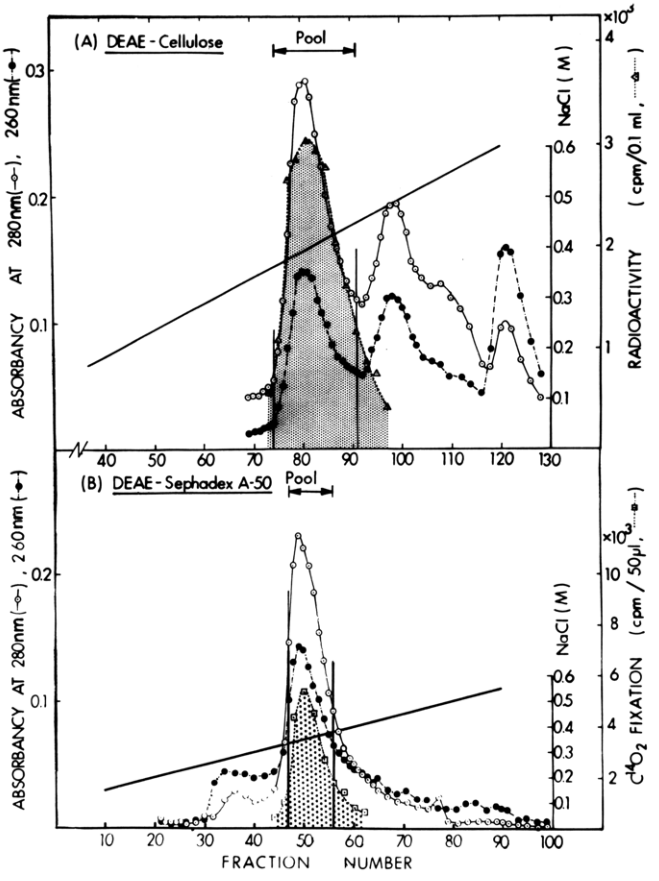

FIGURE 1: Purification of *Chromatium* RuP_2 carboxylase: (A) DEAE-cellulose column chromatography; (B) DEAE-Sephadex A-50 column chromatography. The experimental details are described in the text. The pooled fraction of the DEAE-cellulose (A) (tubes 74–91) were subjected to the DEAE-Sephadex A-50 column chromatography (B). The enzymatically active fractions (tubes 47–56) were combined and the precipitate formed at 80% ammonium sulfate saturation was dissolved in 0.05 M TM buffer and stored in an ice box after dialysis against the same buffer. In A, the radioactive labeling of the protein was shown by shadow, while the enzymic activities ($[^{14}C]CO_2$ fixation) were shown in B using different symbols by subtracting the radioactivity of each fraction.

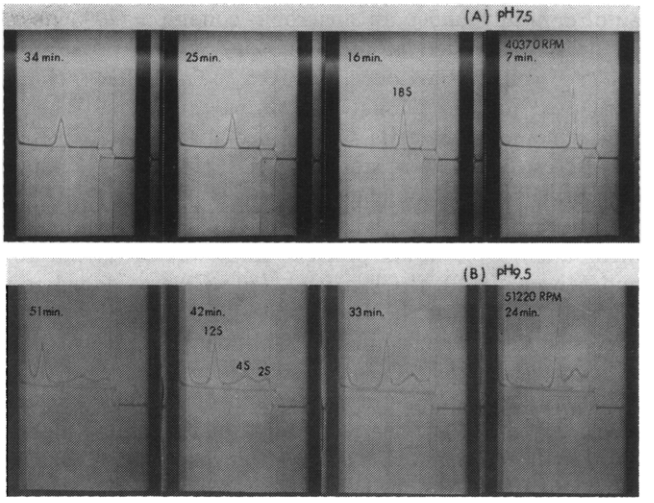

FIGURE 2: Sedimentation pattern of *Chromatium* RuP_2 carboxylase. (A) The enzyme dissolved in 0.05 M TM buffer (pH 7.5) (10 mg/ml) was placed in an analytical ultracentrifuge (40,370 rpm). (B) The enzyme solution (10 mg/ml) which was dialyzed against 0.05 M glycine–NaOH buffer (pH 9.5) for 4 hr at 4° was placed in an analytical ultracentrifuge (51,220 rpm). In both cases temperature was maintained at 20° and the bar angle of measurements was 60°.

Results

A typical example of the elution profile of RuP_2 carboxylase at the final two steps, DEAE-cellulose and DEAE-Sephadex A-50 column chromatography, is shown in Figure 1(A, B). Ready incorporation of ^{14}C into the carboxylase molecule occurred as evidenced by good correlation between the radioactive labeling of the enzyme and the uv absorption at 280 nm. The enzyme preparation obtained from column chromatography on DEAE-Sephadex was judged to be homogeneous on the basis of the sedimentation pattern after analytical ultracentrifugation (Figure 2A) as well as in polyacrylamide gel disc electrophoresis at pH 7.5 (Figure 3A). In the latter, formation of a minute amount of an aggregate was observed. In the gel electrophoresis experiment carried out at pH 8.9, appearance of an additional fast-moving band designated as B was seen, indicating dissociation of the protein molecule under the alkaline condition (Figure 3B). It will be noted that the mobility of the slow-moving band (A_m) is smaller than that of the native enzyme (A_mB_n) at pH 7.5, in spite of the fact that the charge/mass of the former is much higher than the latter. This was caused by the use of the same electrode buffer in two different electrophoresis experiments.

An electron photomicrograph of the purified *Chromatium* RuP_2 carboxylase is given in Figure 4. The molecular dimension of the enzyme protein at the ultrastructural level was analogous to that of the spinach (Sugiyama *et al.*, 1968b) and the *Chlorella* enzymes (Matsumoto *et al.*, 1969), *ca.* 100 Å in diameter. The presence of the central hole or depression strongly indicated oligomeric structural organization of the enzyme molecule.

By employing the Sephadex gel filtration method of Andrews (1964), the molecular weight of the bacterial RuP_2 carboxylase was estimated and calibrated with several reference proteins. The value of 5.5×10^5, which was quite similar to that of the plant enzymes, was obtained. In order to determine whether or not the enzyme consists of nonidentical subunits as in the case of the plant RuP_2 carboxylase, a *Chromatium* RuP_2 carboxylase preparation was subjected to sodium dodecyl sulfate–polyacrylamide gel disc electrophoresis as described by Weber and Osborn (1969). Formation of two bands designated as A and B in such an experiment was clearly seen (Figure 3G). The relative migrations of two bands were similar to those observed in either spinach or *Chlorella* enzyme (Sugiyama *et al.*, 1971). The approximate molecular weights were estimated to be 5.7×10^4 and 1.2×10^4 for monomeric subunits A and B, respectively. By combining these results, it can be proposed that the subunit composition of the *Chromatium* RuP_2 carboxylase is represented as A_mB_n (A_8B_8 or A_8B_6), signifying that the enzyme molecule consists of m molecules of subunit A and n molecules of subunit B.

In an attempt to understand the structure–activity relationship, sucrose density gradient centrifugation was applied to the enzyme, which dissociated under alkaline conditions. Representative results are shown in Figure 5(A–C). In agreement with our previous experiment (Akazawa *et al.*, 1970a), the carboxylase activity of the native enzyme sample exactly coincided with the fast-sedimenting 18S protein component (A). The reversible association of the alkali-dissociated

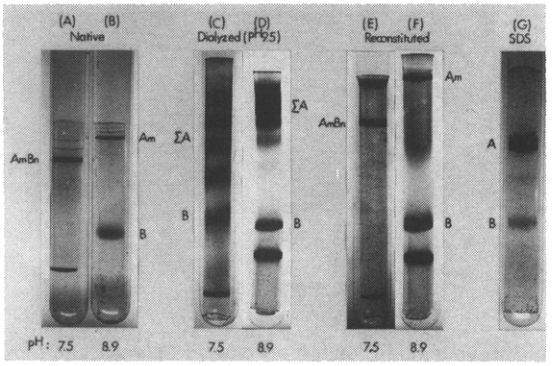

FIGURE 3: Polyacrylamide gel disc electrophoresis of *Chromatium* RuP_2 carboxylase. The experimental details for polyacrylamide gel disc electrophoresis at pH 7.5 (A, C, and E) and pH 8.9 (B, D, and F), and sodium dodecyl sulfate–polyacrylamide gel disc electrophoresis (G) are described in the text. The stock enzyme solution was directly applied to pH 7.5 and 8.9 gel electrophoresis, A and B, respectively. In each of C and D, the enzyme solution was preliminarily dialyzed against 0.05 M glycine–NaOH buffer (pH 9.5) for 10 hr at 4° and applied to gel electrophoresis at pH 7.5 and 8.9, respectively. The alkali-treated enzyme samples were thoroughly dialyzed against 0.05 M TM buffer (pH 7.5) for 4 hr and aliquots were applied to gel electrophoresis at pH 7.5 (E) and 8.9 (F), respectively. For the labels such as A_mB_n see the text. The same labels appear in Figure 5.

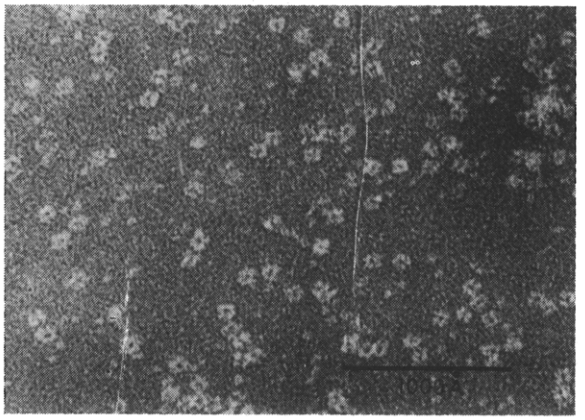

FIGURE 4: Electron micrograph of *Chromatium* RuP_2 carboxylase.

enzyme upon neutralization was demonstrated by the results given in B and C. In these experiments, the enzyme was first dialyzed against 0.05 M glycine–NaOH buffer (pH 9.5) for 4 hr, and an aliquot was directly loaded on the sucrose gradient at pH 9.5. The sedimentation profiles of B show formation of multicomponents due to the degradation of the 18S protein. The carboxylase activity of each sedimented fraction (tubes 6–14) was determined with a prolonged incubation time (60 min). The peak of the enzymatic activity fell at the leading edge of the first sedimenting peak and did not coincide with any of the sedimenting protein peaks. The fraction (tube 8) expected to be the peak containing 18S protein as well as the enzymatic activity showed little evidence of the presence of protein or activity. Instead the fraction (tube 10) expected to contain slower-moving proteins showed the enzymatic activity. The weak activity detected might have been ascribed to either nonsplit 18S protein or its re-formation from the dissociated enzyme molecules in the assay mixture which was maintained at pH 7.0. As can be seen from the electrophoretograms inserted, the resolution of dissociated components by density gradient centrifugation was not very good, and we cannot distinguish between these two alternatives. On the other hand, the reformation of the 18S protein by dialysis of the alkali-treated enzyme against the neutral buffer (pH 7.5) for 4 hr is clearly shown in C as well as its electrophoretogram at pH 7.5 and 8.9 (Figure 3E,F). All the RuP_2 carboxylase activity was found to reside in the 18S component. The maximum degree of re-formation attained was 70%, and specific activity of the re-formed enzyme was 64% of the native enzyme, partly due to the formation of the aggregated enzyme molecule. Gel electrophoretic behavior of the re-associated protein recovered (tube 8 of C) was identical with that of the native enzyme sample. However, it will be noted that the mode of enzyme splitting under a prolonged alkaline (pH 9.5) treatment (10-hr dialysis) was markedly different from that discernible from the electrophoretic pattern of the native enzyme on polyacrylamide gel at pH 8.9 without prior alkaline dialysis (Figure 3C,D). The longer the treatment the more the degradative dissociation of the large subunit, as marked ΣA (A_1, A_2 $\cdots$ A_m) in the figure. This was also supported by the gel electrophoretogram of the fractions as shown in the insert of Figure 5B. The sedimentation pattern after analytical ultracentrifugation of the enzyme preparation dialyzed against 0.05 M glycine–NaOH buffer (pH 9.5) for 4 hr shows dissociation into three components (12S, 4S, 1.5S), representing the transitory stage of the protein dissociation (Figure 2B).

Our previous experiments showed that prior treatment of spinach RuP_2 carboxylase with substrates, *i.e.*, RuP_2 or $NaHCO_3$ plus Mg^{2+}, gave rise to a marked protective effect against subsequent denaturation of the enzyme either by

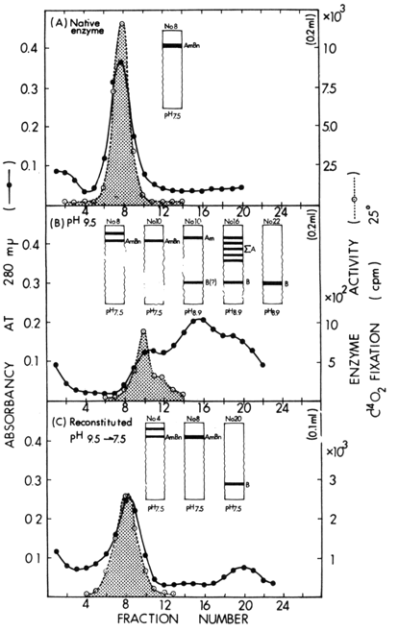

FIGURE 5. Sucrose density gradient centrifugation of *Chromatium* RuP_2 carboxylase: (A) native enzyme; (B) alkali-treated (pH 9.5) enzyme; (C) alkali-treated (pH 9.5), then neutralized by dialysis against 0.05 M TM buffer (pH 7.5). The experimental methods of alkali treatment and subsequent neutralization of the enzyme and the method of sucrose density gradient centrifugation are described in the text. The pH of the sucrose gradient was 7.5 in A and C and 9.5 in B. After the centrifugation, fractions were subjected to measurements of uv absorption at 280 nm and the RuP_2 carboxylase activities. Illustrations inserted are the electrophoretograms of the indicated fractions either at pH 7.5 or pH 8.9 obtained by the method described in Figure 3.

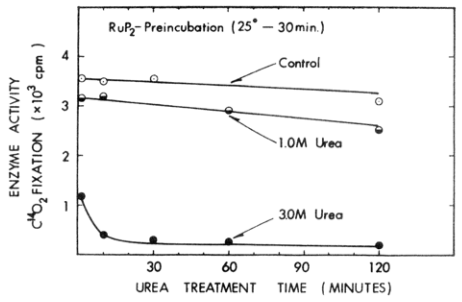

FIGURE 6: Effect of urea on the *Chromatium* RuP_2 carboxylase pretreated with RuP_2. The experimental procedures were the same as those employed in spinach (Sugiyama *et al.*, 1968c) of *Chlorella* enzymes (Matsumoto *et al.*, 1969). A 0.5-ml aliquot of the stock enzyme solution (1.0 mg/ml) was incubated with 0.4 ml of RuP_2 (14 μmoles/ml) at 25° for 30 min. A 0.3-ml aliquot of the substrate-treated enzyme was withdrawn and added to 0.3 ml of either (i) 0.05 M TM buffer (pH 7.0), (ii) 2.0 M urea, or (iii) 6.0 M urea. A 0.1-ml aliquot from each mixture was withdrawn at 1, 10, 30, 60, 90, and 120 min and added to the standard assay mixture described in the text. The reaction was carried out at 25° for 10 min.

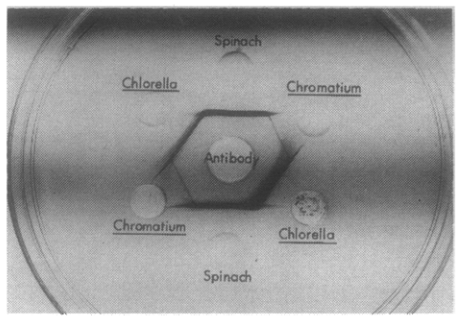

FIGURE 7: Immunodiffusion test of spinach, *Chlorella* and *Chromatium* RuP_2 carboxylase with rabbit antiserum against the spinach enzyme. The experimental procedures are described in the text.

urea or by sodium dodecyl sulfate and against proteolytic digestion (Sugiyama *et al.*, 1968b,c). A similar but lower protective effect occurred with RuP_2 carboxylase isolated from *Chlorella ellipsoidea* (Matsumoto *et al.*, 1969), indicating looser quaternary structure of the latter enzyme. Experimental results presented in Figure 6 showd a time-dependent effect of urea (1.0 and 3.0 M) treatment on the *Chromatium* carboxylase preincubated with RuP_2. A drastic decline of the enzyme activity was demonstrated even at 1 min after the addition of urea (3.0 M). Although it was difficult to compare the urea effect between the *Chlorella* and *Chromatium* enzymes since experiments were not conducted in parallel, the results appeared to indicate less rigid molecular architecture of the *Chromatium* RuP_2 carboxylase.

The results of the agar plate immunodiffusion test are shown in Figure 7. The formation of spur in the precipitation line between spinach/*Chlorella*, spinach/*Chromatium*, and *Chlorella*/*Chromatium* enzymes indicated a partial immunological resemblance of each pair, although the RuP_2 carboxylase from spinach and *Chlorella* more closely resemble each other than the enzyme from *Chromatium*, which is phylogenetically distant from the enzymes from the former two organisms (*cf.* Fuller and Gibbs, 1959; Anderson *et al.*, 1968; Akazawa *et al.*, 1970b).

Discussion

Several lines of experimental evidence presented in this paper support our previous notion concerning the structural similarity of *Chromatium* RuP_2 carboxylase to green plant enzymes (Akazawa *et al.*, 1970a). It is certainly interesting that the *Chromatium* enzyme also consists of two nonidentical subunits. However, ready dissociation into subunit components under an alkaline condition is a unique property of the bacterial enzyme. A number of enzymes or proteins are known to be reversibly dissociated under environmental ionic changes; they are, for example, aldolase (Stellwagen and Schachman, 1962; Deal *et al.*, 1963), *Neurospora* malate dehydrogenase (Munkres, 1965), isocitrate dehydrogenase (LeJohn *et al.*, 1969), rat liver mitochondrial malate dehydrogenase (Mann and Vestling, 1969), squid hemocyanin (Van Holde and Cohen, 1964), and mouse nerve growth factor (Varon *et al.*, 1967, 1968). It is assumed that the dissociation of these proteins occurs due to an increase or decrease in the net surface charge of oligomeric structure of the proteins, depending on environmental ionic changes. It is thus of particular interest to see that such a reversible dissociation occurred only in the RuP_2 carboxylase molecule of a photosynthetic bacterium, *Chromatium*, but not in that from plant sources. It is fascinating to imagine that the comparatively less rigid quaternary structural organization of the *Chromatium* carboxylase signifies the initial evolutional step toward further molecular development of the enzyme into a more rigid structural assembly. Although more detailed analytical studies are needed to support the view that the structure of the large subunit (A) of *Chromatium* carboxylase is indeed similar to that of plant enzymes, it can be speculated that the catalytic subunit, present as an ancestory form, of the RuP_2 carboxylase in procaryotic organisms is evolutionaly left intact in eucaryotes, *e.g.*, spinach and *Chlorella*. One may note that the primitive sulfur bacterium, *Chromatium*, lacking the lamellar chloroplast structure as photosynthetic apparatus, carries a molecular assembly of RuP_2 carboxylase apparently similar to that of plant enzymes. One may also note, as reported by Kuehn and McFadden (1969) that RuP_2 carboxylase from the heterotrophically grown cells of *Hydrogenomonas facilis* is structurally akin to plant enzymes in its large molecular size but consists of identical subunits. As shown already by us RuP_2 carboxylase from both autotrophically and heterotrophically grown cells of purple nonsulfur bacteria, *Rhodopseudomonas spheroides* and *Rhodospirillum rubrum*, is in many respects distinguishable from plant enzymes (Akazawa *et al.*, 1969; Akazawa *et al.*, 1970b).

Our previous experiments indicate that the spinach RuP_2 carboxylase molecule dissociates into two subfragments upon treatment with 6.0 M urea after a prior incubation with substrates, RuP_2 or $NaHCO_3$ plus Mg^{2+}, and reassociates by the subsequent dilution with Tris-buffer (Sugiyama *et al.*, 1970). However, the strucutral nature of the dissociated molecules is not well characterized, partly because urea splits the enzyme nonspecifically. As reported by Kawashima and Wildman (1970), and Criddle *et al.* (1970) tobacco leaf fraction-I protein and barley leaf RuP_2 carboxylase dissociate into two subunits at alkaline pH, although reversibility of the process is not established yet.

It is difficult to conclude at the present stage of the study what the degraded forms of the large subunit capable of reassembling into the enzymically active molecule are. As shown in Figure 5C, when alkali-treated protein samples were thoroughly dialyzed against pH 7.5 buffer and then applied to the sucrose density gradient centrifugation, the recovery of the 18S protein became greater, and extraneous protein bands

of smaller molecular sizes diminished, although a certain amount of aggregates was produced. However, there remains a possibility that A_m (or another oligomeric form) retains a catalytic activity without the aid of the small subunit. A more refined technique of separating two subunits is needed to resolve some of the problems discussed above, and experiments along this line are now in progress.

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