Subunit Structure of Spinach Leaf Ribulose 1,5-Diphosphate Carboxylase*

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ABSTRACT: The presence of two nonidentical subunits in spinach leaf ribulose 1,5-diphosphate (RuDP) carboxylase has been shown by dissociating the carboxymethylated enzyme molecule in 8.0 M urea. The dissociated subunits were separated on columns of Sephadex G-75. The molecular weights, estimated by two different methods, (A) equilibrium analytical centrifugation and (B) sodium dodecyl sulfate–polyacrylamide gel disc electrophoresis, were approximately 60,000 and 20,000 for subunits A and B, respectively. The C-terminal amino acid of subunits A and B was found to be valine and tyrosine, respectively, based on (i) ^3H labeling of the C termini of the protein, and (ii) carboxypeptidase digestion. In agreement with these data, the native RuDP carboxylase molecule had both valine and tyrosine as C-terminal amino acids. The SH groups which are related to enzyme activity were mostly localized in the large subunit (A). The possible role of this large subunit in enzyme catalysis is discussed.

The elucidation of the subunit organization of RuDP[1] carboxylase is essential for the thorough understanding of the molecular mechanism of photosynthetic CO_2 fixation. The large molecular size of RuDP carboxylase has been known for many years (Weissbach *et al.*, 1956; Trown, 1965; Paulsen and Lane, 1966), and the results of recent investigations from several laboratories strongly indicate that the enzyme molecule consists of two different kinds of subunits. Rutner and Lane (1967) have demonstrated that spinach leaf RuDP carboxylase can be split by sodium dodecyl sulfate, after prior amino ethylation, into two different subunits on polyacrylamide gel disc electrophoresis. An essentially similar technique was employed by Kawashima (1969) for determining subunits from tobacco and spinach carboxylase enzymes. The latter author showed that the large subunits derived from both enzyme sources had similar amino acid compositions, whereas the small subunits were different. Moon and Thompson (1969) split spinach beet RuDP carboxylase into two subunits by treatment with 8.0 M urea after prior carboxymethylation of the enzyme protein. They estimated the molecular weight of each subunit to be 54,000 and 16,000, respectively.

Recently we have conducted experiments to examine the possible dissociation of spinach leaf RuDP carboxylase into an enzymically active component (Sugiyama *et al.*, 1970). After a prior incubation of the enzyme protein with either RuDP or $NaHCO_3$ plus $MgCl_2$, the subsequent addition of 4.0 M urea caused a splitting of the enzyme molecule into large and small components, separable by sucrose density gradient centrifugation. The enzyme activity was found to be associated only with the large component. More importantly, the banding patterns on gels of the subunits subsequently derived from each component after carboxymethylation in the presence of 8.0 M urea were clearly different, each closely resembling the subunits characterized by Rutner and Lane (1967) as well as by Moon and Thompson (1969). This finding suggested the possibility that each of our enzymically active and inactive components could be an oligomeric form of these large and small subunits, respectively, comprising the native RuDP carboxylase. We therefore pursued a more precise investigation of the subunit composition of this enzyme. In the experiments reported in this paper, we have undertaken the complete separation of two subunits of spinach leaf RuDP carboxylase, as well as the characterization of some of their biochemical and biophysical properties.

Experimental Procedures

Materials. [^3H]H_2O and [1-^{14}C]monoiodoacetamide were obtained from the Radiochemical Centre, Amersham, England. Diisopropyl fluorophosphate (DFP)-treated carboxypeptidase A (Worthington Biochemical Corporation, Freehold, N. J.) was a gift of Professor T. Murachi (Nagoya City University, Nagoya, Japan).

The following materials were purchased from the suppliers and used without further purification: Sephadex G-75 (Pharmacia), Tris (Boehringer), enzyme grade ammonium sulfate (Mann), protein markers (Mann), *N*-ethylmorpholine (Sigma), and sodium dodecyl sulfate (Sigma). All other chemicals used were of reagent grade.

Both monoiodoacetamide and monoiodoacetic acid were recrystallized three times from hot chloroform. Urea was freshly recrystallized. Maleic anhydride was purified by sublimation from the highest grade reagent just before use. Fresh preparations of β-mercaptoethanol were obtained by redistillation under reduced pressure in the presence of nitrogen gas and stored in a deep freeze until used. Deionized water, redistilled from a glass pot still, was used throughout the experiments.

Purification of RuDP Carboxylase. Spinach leaf RuDP carboxylase was prepared following the methods reported in preceding papers (Sugiyama and Akazawa, 1967; Sugiyama

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[1] Abbreviation used is: RuDP, ribulose 1,5-diphosphate.

et al., 1968b). All the enzyme preparations used were ascertained to be homogeneous, as judged by their behavior on polyacrylamide gel disc electrophoresis as well as on analytical ultracentrifugation.

Maleylation and Carboxymethylation of RuDP Carboxylase. In some of the experiments dealing with the dissociated enzyme protein, specially for the determination of C-terminal amino acids, it was necessary to enhance the solubility of the carboxymethylated protein samples in aqueous media without urea and sodium dodecyl sulfate. Among several attempts, maleylation of the enzyme molecule following the method of Sia and Horecker (1968) prior to carboxymethylation proved to be most satisfactory for our purpose. The enzyme (2–6 mg/ml 0.05 M Tris-HCl buffer, pH 7.5) was dialyzed exhaustively against 0.05 M borate–NaOH buffer (pH 9.0) containing 10 mM β-mercaptoethanol, then maleic anhydride dissolved in acetone (0.5 g/ml) was added slowly to the enzyme solution, over a period of 15 min, in an ice bath. The amount of maleic anhydride added was about 10 times the lysine content of the protein. During this step, the pH was maintained between 8 and 9 by adding 5 N NaOH. At the end of the reaction time, the whole mixture was extensively dialyzed against H_2O which was adjusted to pH 9.0 with NH_4OH, and finally lyophilized. The maleylated protein samples were subjected to carboxymethylation following the method of Moon and Thompson (1969). The lyophilized protein (8–80 mg) dissolved in 10–15 ml of 8.0 M urea containing 0.02 M EDTA was deaerated by flushing the solution with O_2-free N_2 gas for 20 min. Redistilled β-mercaptoethanol was then added to the solution, to a final concentration of 0.14 M. After raising the pH to 10.5 by the addition of 5 N NaOH, the protein sample was again deaerated and left at room temperature for 5–12 hr. A solution of freshly recrystallized monoiodoacetic acid in 3.0 M Tris-HCl (pH 8.5) (0.33 g/ml) was added to the above protein sample at the ratio of 2.68 mg of iodoacetate per ml of protein solution and the alkylation reaction was allowed to proceed at 25° for 10–20 min. During this step the pH was maintained at 8.5 by adding 5 N NaOH.

Separation of Subunits A and B by Sephadex Gel Filtration. After carboxymethylation, excess reagents were removed by dialysis against 8.0 M urea. The pH of the dialysate was adjusted to 10.0 by adding concentrated NH_4OH and the dialysate applied to a column (2.5 × 80 cm) of Sephadex G-75, preequilibrated with 8.0 M urea–0.05 M Tris HCl–0.1 M KCl–0.2 M NH_4OH (pH 10.0). Elution was effected by the same buffer and 10-ml fractions were collected. Fractions corresponding to each subunit were pooled separately and lyophilized after extensive dialysis against H_2O.

Determination of Molecular Weights of Subunits A and B. For determining the molecular weights of subunits A and B, both (1) analytical ultracentrifugation following the short-column method of Yphantis (1960), as well as (2) sodium dodecyl sulfate–polyacrylamide gel disc electrophoresis by the method of Weber and Osborn (1969) were employed.

(1) A Spinco Model E analytical ultracentrifuge equipped with an 8-channel cell was used, at the Institute of Molecular Biology of this university. Each subunit was dissolved in 8.0 M urea in 0.05 M borate–NaOH buffer (pH 9.0). The apparent molecular weights were calculated from the equation

$$M_{app} = \frac{1}{\bar{r}c}\left(\frac{dc}{dr}\right)_{r=\bar{r}} \frac{RT}{\omega^2(1-\bar{V}\rho)}$$

making the necessary corrections for solvent density. The partial specific volume, $\bar{V}$, of the two subunits was calculated to be 0.730 ml/g (Schachman, 1957), based on the data of amino acid composition (*cf.* Table I).

(2) For polyacrylamide gel electrophoresis, approximately 1.0 mg each of carboxymethylated spinach RuDP carboxylase or marker protein was dissolved in 2 ml of 0.01 M phosphate buffer (pH 7.0) containing 0.1 % sodium dodecyl sulfate and 0.1% β-mercaptoethanol at room temperature. After a 4-hr incubation, 10–50 μl of protein solution was applied on gels with 10% cross-linkage. Electrophoresis was performed at a constant current of 8 mA per tube. After staining gels with coomasie brilliant blue, the mobilities of the proteins were determined. The marker proteins used for estimating the molecular weights were human γ-globulin (H chain, 5.0 × 10^4; L chain, 2.0 × 10^4), bovine serum albumin (6.7 × 10^4), ovalbumin (4.5 × 10^4), beef pancreas chymotrypsinogen A (2.5 × 10^4), sperm whale myoglobin (1.78 × 10^4), and horse heart cytochrome *c* (1.3 × 10^4).

Determination of C-Terminal Amino Acids. Two techniques were employed.

(1) Labeling with tritium (^3H). We applied the technique of selective labeling of C-terminal amino acids of proteins by ^3H developed recently by Matsuo *et al.* (1966, 1967), and the results were in perfect agreement with those obtained by the enzymic method. About 2 mg of the lyophilized protein (native enzyme or subunits A or B) was dissolved in a solution containing 0.1 ml of [^3H]H_2O (50 mCi) and 0.2 ml of redistilled pyridine. Then 0.05 ml of redistilled acetic acid anhydride was added slowly, at 0°. The reaction mixture was allowed to stand at room temperature for 12–16 hr. Afterward the mixture was evaporated *in vacuo* at 40°. Addition of H_2O followed by evaporation *in vacuo* was repeated 5 times to remove thoroughly the excess radioisotope. The residue of tritiated protein sample was then hydrolyzed in 0.5 ml of glass-distilled 6 N HCl at 110° for 20 hr. The hydrolysates were evaporated to dryness and aliquots were subjected to high-voltage paper electrophoresis, using as solvent, pyridine–acetic acid–H_2O (5:0.2:5, v/v, pH 6.5). After measurement of ^3H radioactivity on the paper in a Packard Tri-Carb liquid scintillation counter (Hayes, 1962), the ^3H-labeled amino acids (neutral amino acids) were extracted from the paper with H_2O, after thorough washing by toluene and acetone, and further characterized by a second paper chromatography using two solvent systems: 1-butanol–pyridine–acetic acid–H_2O (15:10:3:12, v/v) and 1-butanol–acetic acid–H_2O (4:1:2, v/v). The identities of the radioactive amino acids were determined by comparing the chromatograms to those of reference amino acids. Radioactivity of the spots on paper was measured in the scintillation counter using the nonaqueous scintillator system described above.

(2) Hydrolysis with carboxypeptidase. Proteolytic digestion of the protein samples was carried out at 37° in 0.2 M *N*-ethylmorpholine acetate using DFP-carboxypeptidase A (pH 8.5) (Ambler, 1967). The reaction mixture contained about 0.1 μmole of protein sample, 20 μmoles of *N*-ethylmorpholine acetate (pH 8.5), 3.6 μg of DFP-carboxypeptidase dissolved in 10% (w/v) LiCl, and 0.005 ml of toluene in a total volume of 0.2 ml. The reaction was stopped at various time intervals by immersing the reaction mixture into a boiling-water bath. The reaction mixture was then evaporated

in vacuo at 40° and the residue, dissolved in 0.2 M Na-citrate buffer (pH 2.2), was applied to a Hitachi KLA-3B automatic amino acid analyzer equipped with a high sensitivity attachment from the Central Research Laboratories of Ajinomoto Co. Ltd. (Tokyo).

Alkylation of RuDP Carboxylase with [^{14}C]Iodoacetamide. Labeling of the RuDP carboxylase molecule with [^{14}C]iodoacetamide was carried out according to the method reported in our previous paper (Sugiyama *et al.*, 1968a). The enzyme preparation was incubated with [^{14}C]iodoacetamide at a final concentration of 2.5×10^{-3} M; at this concentration enzyme activity is inhibited 100%. The subsequent steps of maleylation as well as carboxymethylation of the protein samples were essentially identical with those described above. The dissociated subunits were then separated on a column of Sephadex G-75 (2.5 × 40 cm), equilibrated with 8.0 M urea in 0.05 M borate–NaOH buffer (pH 9.0) containing 0.1 M KCl. Fractions (5 ml) were collected by elution with the same buffer. The absorbancy at 280 mμ as well as the radioactivity of the fractions were measured.

Amino Acid Analysis. The amino acid composition of subunits A and B were determined using a Beckman Spinco Model 120 automatic amino acid analyzer, at the Central Research Institute, Japan Monopoly Corporation, Tokyo, Japan. The lyophilized protein samples were hydrolyzed with glass-distilled 6 N HCl *in vacuo* by the method of Moore and Stein (1963) at 110° for 20 hr. The relative molar ratio of each individual amino acid was calculated in relation to phenylalanine.

Polyacrylamide Gel Disc Electrophoresis. Polyacrylamide gels containing 8 M urea were prepared, and the electrophoretic runs were performed using a discontinuous Tris–glycine buffer system prepared after Davis (1964). Electrophoresis was conducted at room temperature for 90 min at a constant current of 3 mA per tube. The gels were stained in a solution of 1% Amido Black 10B in 7% acetic acid.

Protein Determination. The protein concentration was measured by the colorimetric method of Lowry *et al.* (1951).

Results

The dissociation of RuDP carboxylase into two subunits by urea treatment can be seen from an elution pattern of a column of Sephadex G-75 (Figure 1). The banding patterns of the two subunits separated on polyacrylamide gels are shown in Figure 2. For dissociation, the carboxymethylation of the protein samples preceding urea treatment was found to be essential to obtain clear separation of the two subunits. When carboxymethylation was omitted, marked aggregation of both subunits occurred even in the presence of 10 mM β-mercaptoethanol, as indicated by the formation of many nondiscrete bands on polyacrylamide gel disc electrophoresis. This is understandable since many SH groups are present in the native enzyme molecule (Sugiyama *et al.*, 1968b).

The molecular weights of subunits A and B were calculated to be 60,000 and 20,800, respectively, from analytical ultracentrifugation (Figure 3). However, using six marker protein samples, the molecular weights of subunits A and B were estimated to be 60,000 and 14,000, respectively, from their mobilities on sodium dodecyl sulfate–polyacrylamide gel disc electrophoresis (Figure 4).

The amino acid compositions of subunits A and B are presented in Table I, along with the data of other investigators (Rutner and Lane, 1967; Moon and Thompson, 1969). It will be seen that our data are essentially similar to those reported by Rutner and Lane (1967), but differ slightly from those of Moon and Thompson (1969) on spinach beet enzyme protein, especially in their relative content of tyrosine, histidine, and arginine in subunit B.

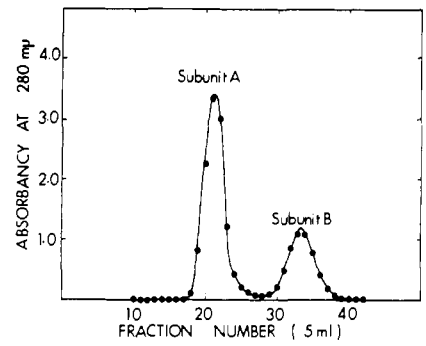

FIGURE 1: Sephadex G-75 gel filtration of subunits A and B. Approximately 80 mg of a purified preparation of RuDP carboxylase was subjected to maleylation, followed by carboxymethylation by the method described in the text. Samples were then placed on a column of Sephadex G-75 (2.5 × 80 cm), which was preequilibrated with 8.0 M urea–0.05 M Tris-HCl–0.01 M EDTA–0.1 M KCl–0.2 M NH_4OH (pH 10.0). Fractions (5 ml) were collected using the above buffer at a flow rate of 5 ml/hr, and the absorbancy at 280 mμ was measured.

The results of ^3H labeling of the native RuDP carboxylase summarized in Figure 5 clearly demonstrate that only valine and tyrosine are specifically labeled in the native protein, the ratio of radioactivity ([^3H]valine/[^3H]tyrosine) is approximately 2. When subunits A and B isolated by Sephadex G-75 gel filtration (*cf.* Figure 1) were labeled separately, the C-terminal amino acid proved to be valine for subunit A and tyrosine for subunit B, as clearly shown in Figures 6 and 7. Samples of the isolated subunits A and B were digested with

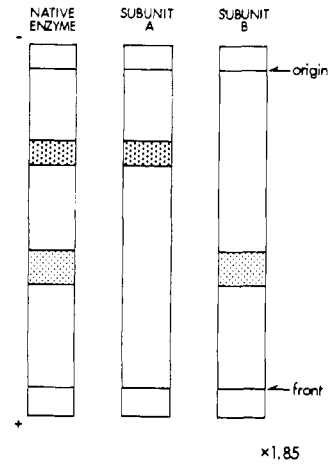

FIGURE 2: Polyacrylamide gel disc electrophoregram of RuDP carboxylase and its two subunits. Experimental details are described in the text: (a) 8.0 M urea-gel loaded with carboxymethylated enzyme preparation; (b) 8.0 M urea-gel loaded with subunit A; (c) 8.0 M urea-gel loaded with subunit B. In both a and c staining property of subunit B is very weak.

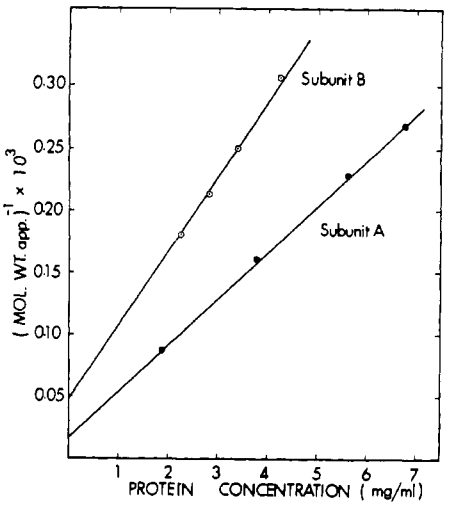

FIGURE 3: Estimation of molecular weight of subunits A and B by analytical ultracentrifugation. Experimenta details of analytical ultracentrifugation are described in the text. At each of the concentrations indicated, subunits A and B were centrifuged at the rotor speed of 29,500 rpm and 42,040 rpm, respectively. In each case, data were obtained 150 min after reaching the maximum speed.

carboxypeptidase A and the results are illustrated in Figure 8. Only valine was released from subunit A, reaching a maximum of 1 equiv. On the other hand, about 2 equiv of tyrosine was released from subunit B, followed by the liberation of 1 equiv of threonine. These calculations were based on the mol wt 14,000 of subunit B determined by sodium dodecyl sulfate–polyacrylamide gel disc electrophoresis. Subsequently leucine and phenylalanine were slowly released. These data indicate the C-terminal sequence of subunit B to be -Phe_5-Leu_4-(Tyr_3, Thr_2)-Tyr_1. It can be thus seen that the results of the time-sequence analyses of C-terminal amino acids based on enzymic method are in good agreement with the data obtained by ^{3}H-labeling experiments.

For further characterization of subunits A and B the specific localization of functional SH groups was determined. The enzyme protein was preliminarily labeled with [^{14}C]iodoacetamide at a concentration which inhibited enzyme activity 100%. Under these conditions approximately 8–10 SH groups per enzyme molecule were blocked. The enzyme protein was subsequently dissociated as outlined above, and the subunits produced were separated on Sephadex G-75. The results presented in Figure 9 show that most of the SH groups blocked by [^{14}C]iodoacetamide are localized in the large subunit A, indicating a possible role for this subunit in enzyme catalysis.

TABLE I: Relative Amino Acid Composition of Two Subunits of Ribulose 1,5-Diphosphate Carboxylase.[a]

	Subunit A			Subunit B		
Amino acids	*b*	*c*	*d*	*b*	*c*	*d*
Phenylalanine	1.00	1.00	1.00	1.00	1.00	1.00
Tyrosine	0.94	0.92	0.76	1.74	1.57	0.99
Glycine	2.33	2.10	2.23	1.07	1.15	1.58
Lysine	1.13	1.18	1.15	1.09	1.21	1.44
Histidine	0.77	0.67	0.62	0.54	0.45	0.21
Arginine	1.48	1.44	1.26	1.10	0.99	0.58
Aspartic acid	2.20	2.18	1.95	2.20	2.14	1.44
Threonine	1.73	1.75	1.40	1.23	1.19	0.77
Serine	0.70	0.81	0.88	0.64	0.77	0.92
Glutamic acid	2.28	2.20	2.30	2.71	2.26	2.17
Proline	1.12	1.13	1.06	1.56	1.56	1.43
Alanine	2.18	2.16	2.13	0.79	0.86	1.09
Valine	1.67		1.57	1.12		1.41
Methionine	0.39	0.42	0.33	0.45	0.46	0.28
Isoleucine	0.92	0.88	0.88	0.66	0.56	0.70
Leucine	2.16	2.09	1.91	1.78	1.63	1.54
1/2-Cystine			0.35			0.35

[a] Data are presented as relative content of individual amino acids in relation to phenylalanine taken as a unity (Rutner and Lane, 1967). [b] Present: average of duplicate analyses. Details are described in the text. [c] Spinach leaf RuDP carboxylase, Rutner and Lane (1967). [d] Spinach beet RuDP carboxylase, Moon and Thompson (1969).

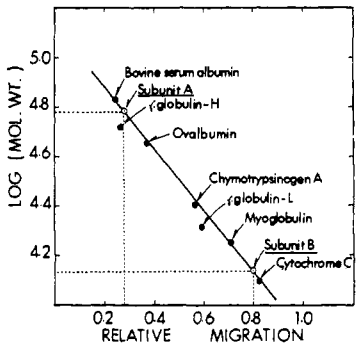

FIGURE 4: Estimation of molecular weights of subunits A and B by sodium dodecyl sulfate–polyacrylamide gel disc electrophoresis. Experimental details for polyacrylamide gel disc electrophoresis are described in the text. Relative mobilities of each sample are plotted against its reported molecular weight on semilogarithmic scale.

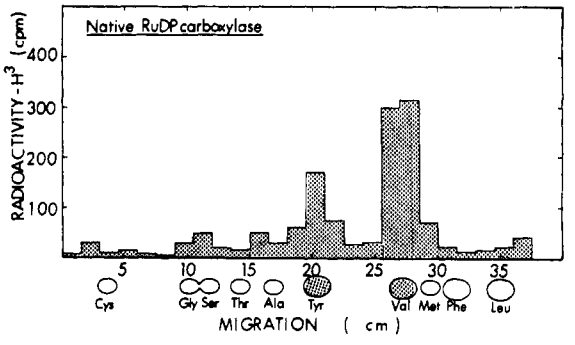

FIGURE 5: Radiochromatogram of hydrolysate of ^{3}H-labeled native RuDP carboxylase. The selective labeling of C-terminal amino acids in the enzyme protein by ^{3}H and the subsequent procedures of acid hydrolysis as well as separation by paper electrophoresis and paper chromatography are described in the text. Result represents the radioactivity distribution of neutral amino acids separated by rechromatography (23 hr/25°) using solvent system of 1-butanol–acetic acid–H_2O (4:1:2, v/v).

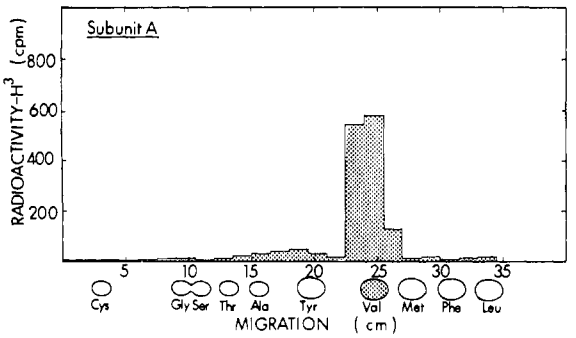

FIGURE 6: Radiochromatogram of hydrolysate of ^{3}H-labeled subunit A. Experimental details were essentially the same as those given in Figure 5.

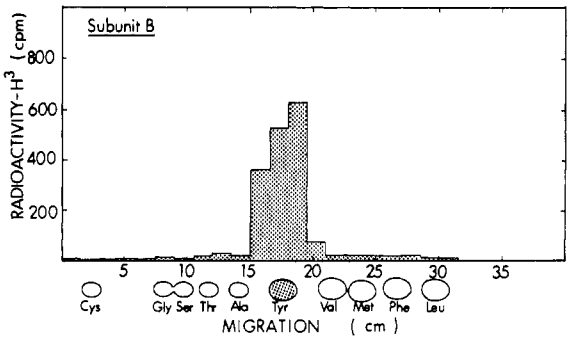

FIGURE 7: Radiochromatogram of hydrolysate of ^{3}H-labeled subunit B. Experimental details were essentially the same as those given in Figure 5.

Discussion

The result reported in the present paper furnish further strong evidence for the nonidentical subunit organization of RuDP carboxylase which has been reported previously by several workers (Rutner and Lane, 1967; Sugiyama and Akazawa, 1967; Kawashima, 1969; Moon and Thompson, 1969). The two subunits, separable by gel filtration or polyacrylamide gel electrophoresis contain different C-terminal amino acids, valine for the large subunit A and tyrosine for the small subunit B. Furthermore, most of the SH groups blocked by monoiodoacetic acid and resulting in complete inactivation of the enzyme are located in the large subunit A.

The molecular weights for subunits A and B were estimated to be approximately 60,000 and 20,000, respectively. These values differ slightly from those of spinach beet enzyme (54,000 and 16,000), as reported by Moon and Thompson (1969). While the values for the molecular weight of subunit A as determined by two independent methods agreed perfectly, there was a discrepancy in the values similarly obtained for subunit B. At present, we cannot explain this discrepancy. Although quite reproducible results have been obtained for determining the molecular weights of many types of proteins by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, Weber and Osborn (1969) have pointed out that the accuracy of the latter method should not be overemphasized, in comparison to other physicochemical methods, such as equilibrium centrifugation.

Kawashima (1969) observed the similarity in amino acid composition of the large subunits obtained from tobacco and spinach leaf RuDP carboxylases, and the differences between the respective small subunits. He therefore postulated that the large subunit A may be related to enzyme activity, whereas the small subunit B may be related to species specificity. Although more rigorous data are needed to substantiate this thesis, it is interesting to recall that there are immunological resemblances between the RuDP carboxylase of different plants and those of microbial origin (Dorner *et al.*, 1957; Kawashima, 1969; Sugiyama *et al.*, 1969; Matsumoto *et al.*, 1969; Akazawa *et al.*, 1969). We are also tempted to speculate on the catalytic role of subunit A from the following observations: (i) localization of most SH groups closely related to enzyme activity in the large subunit A (*cf.* Sugiyama *et al.*, 1970), and (ii) the banding pattern of subunit A on polyacrylamide gels containing 8.0 M urea is the same as that of an enzymically active component obtained by treatment of the native enzyme with 4.0 M urea (*cf.* Sugiyama *et al.*, 1970). In fact, our previous experiments demonstrated the localization of the "catalytic" SH groups in the enzymically active component which was separated in sucrose density gradient. Although our experiments do not provide definitive proof for the role of SH groups in enzyme catalysis, it is evident that they are localized unevenly in the two subunits (A and B) now isolated (*cf.* Sugiyama *et al.*, 1968a). However, in view of the fact that subunit B is also carboxymethylated, though to a much lesser extent, we reserve our final conclusion that only subunit A is engaged in enzyme catalysis.

An alternative, though equally interesting possibility, for the role of subunit B may be in relation to a structural or

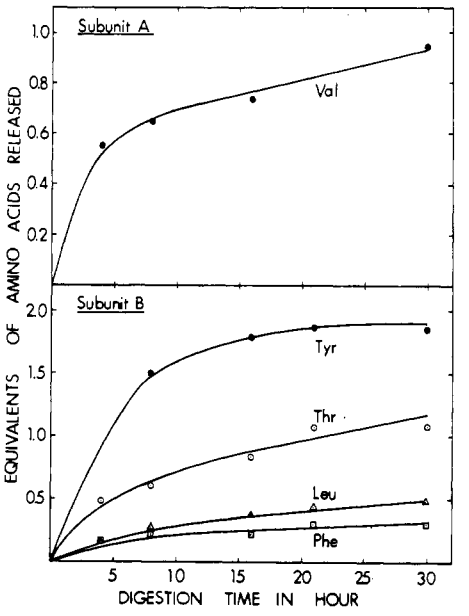

FIGURE 8: Carboxypeptidase hydrolysis of subunits A and B. Experimental details are described in the text. DFP-carboxypeptidase was added to each of subunits A (mol wt 60,000) and B (mol wt 14,000) at the molar ratio of approximately 1:1000 and the enzymic hydrolysis was allowed to proceed at 37°. Released amino acids were determined using a Hitachi KLA-3B automatic amino acid analyzer. Averaged values of duplicate analyses are presented in the figure.

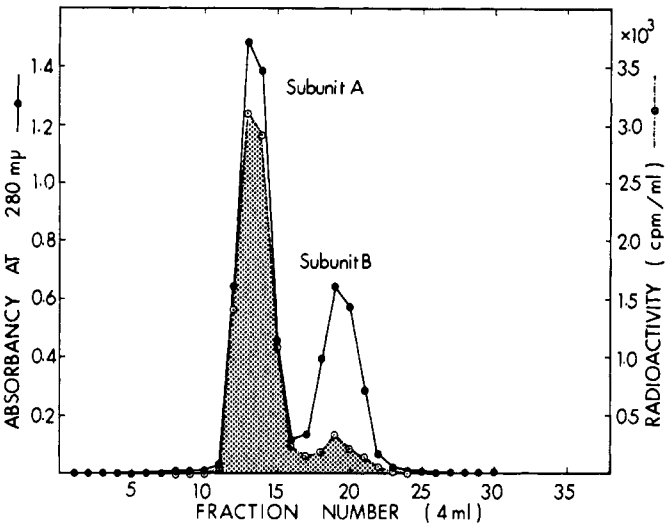

FIGURE 9: ^{14}C-Labeling of SH groups in subunits A and B. Principles of experimental procedures are described in the text. Enzyme preparation (2.70 ml ≙ 24.6 mg of protein) was treated with 0.30 ml of 2.5×10^{-3} M [^{14}C]iodoacetamide (100 μCi) at 25° for 30 min. At the end of incubation, 5 μl of 10 mM β-mercaptoethanol was added to stop the alkylation reaction. The resulting mixture was extensively dialyzed against 0.05 M borate–NaOH buffer containing 10 mM β-mercaptoethanol (pH 9.0). The subsequent steps of maleylation as well as carboxymethylation of the protein molecule were described in the text. The dissociated subunits were then separated on a column of Sephadex G-75, and the absorbancy at 280 mμ (–●–) as well as radioactivities (–⊙–) were measured.

regulatory function, as has been discussed by Moon and Thompson (1969) and Akazawa (1970). Our previous experiment has demonstrated the unique properties of spinach RuDP carboxylase with respect to (i) the deviation of the $NaHCO_3$ saturation curve from Michaelis–Menten kinetics and (ii) the characteristic activation by Mg^{2+} (Sugiyama *et al.*, 1968b). We have thus proposed the cooperative interaction between CO_2 molecules in the enzyme reaction, RuDP carboxylase being under allosteric control. However, in view of the recent findings indicating CO_2 to be the active molecular species reacting with the enzyme, our proposal has been questioned by Cooper *et al.* (1969). Regardless of the validity of the allosteric nature of RuDP carboxylase, it would certainly be interesting to study whether or not this enzyme consists of nonidentical subunits, differing in their function, as in the case of ATCase studied by Gerhart and Schachman (1965), Meighen *et al.* (1970), and Weber (1968).

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