Subunit Structure of Ribulose 1,5-Diphosphate Carboxylase from *Chlorella ellipsoidea**

T. Sugiyama, Tomoko Ito, and T. Akazawa

ABSTRACT: The ribulose 1,5-diphosphate carboxylase isolated from autotrophically grown cells of *Chlorella ellipsoidea* consisted of two distinct subunits, A and B. The two subunits, separated by gel filtration and by polyacrylamide gel disc electrophoresis, were found to contain different C-terminal amino acids, leucine for the large subunit A and valine for the small subunit B. The molecular weights of the subunits A and B were found to be 5.82×10^4 and 1.53×10^4, respectively, by the use of sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Properties and molecular structure of the ribulose 1,5-diphosphate carboxylase of *Chlorella* were compared with those of the spinach enzyme as reported previously.

In the previous papers of this series, we reported that the RuP_2-carboxylases[1] of the autotrophically grown cells of both *Chlorella ellipsoidea* and *Chromatium* strain D were similar to the spinach leaf enzyme in their large molecular sizes (18 S), kinetic properties, and immunochemical specificities (Sugiyama *et al.*, 1969; Matsumoto *et al.*, 1969; Akazawa *et al.*, 1970a). However, the RuP_2-carboxylases isolated from purple nonsulfur bacteria, such as *Rhodopseudomonas spheroides* and *Rhodospirillum rubrum*, were clearly distinguishable structurally and immunologically from those isolated from plant sources (Akazawa *et al.*, 1969, 1970b). The degree of structural and immunological resemblance of the RuP_2-carboxylase molecules in the phylogenetically distant organisms is of interest, for the enzymes play an important role in the carbon assimilation in the autotrophic organisms.

Investigations from several laboratories have previously established that a RuP_2-carboxylase from higher plant tissues consists of two nonidentical polypeptide chains (subunits) (Rutner and Lane, 1967; Moon and Thompson, 1969; Rutner, 1970). Kawashima (1969) reported that amino acid composition was remarkably similar in the larger subunits of RuP_2-carboxylases from various plant species, such as tobacco and spinach, but that it varied in the smaller subunits. He theorized that the large subunit, or subunit A, of a RuP_2-carboxylase was catalytically active and the smaller subunit, or subunit B, was enzymically inert and was responsible for the expression of the species specificity of the entire enzyme molecule. Recently we reported that the spinach leaf RuP_2-carboxylase consisted of two subunits, each containing a C-terminal amino acid different from the other. Our experimental data on the enzyme also supported the view that the larger subunit, or subunit A, was possibly engaged in the enzyme catalysis (Sugiyama *et al.*, 1970; Sugiyama and Akazawa, 1970). The work reported in the present paper deals with the subunit structures of *Chlorella* RuP_2-carboxylase and with the characterization of their biochemical properties, and is closely related to a study of the spinach leaf enzyme reported previously and mentioned above. Further investigation on the structural organization of RuP_2-carboxylases of different plant origins should provide the basis of comparing the quaternary structure of these enzymes. The comparison may eventually lead to better understanding of molecular evolution of photosynthetic carbon dioxide fixation from lower to higher autotrophic organisms.

Materials and Methods

Culture of C. ellipsoidea and Purification of RuP_2-Carboxylase. C. ellipsoidea was cultured in the Institute of Applied Microbiology, University of Tokyo, by the method described previously (Sugiyama *et al.*, 1969). The algal cells (about 40 g wet weight) at the exponential growth stage were harvested and washed twice with 2 mM K_2SO_4 and then brought to Nagoya University for the enzyme isolation. Extraction and purification of the *Chlorella* enzyme were carried out by the method reported in our previous papers (Sugiyama *et al.*, 1969; Matsumoto *et al.*, 1969). Spinach RuP_2-carboxylase used for this study was purified by the method reported previously (Sugiyama and Akazawa, 1970). The final enzyme preparations obtained from both sources were judged to be homogeneous from their electrophoretic patterns on polyacrylamide gel disc electrophoresis (Davis, 1964) and their sedimentation behavior in the analytical ultracentrifuge. A band pattern of such a preparation from *Chlorella* is presented in Figure 1.

Separation of Subunits A and B. Both maleylation and carboxymethylation were found to be essential for preventing the formation of aggregates during the process of dissociating *Chlorella* RuP_2-carboxylase into subunits. The procedures were the same as those used for the spinach enzyme (Sugiyama and Akazawa, 1970). As in the case of the spinach enzyme preliminary maleylation of the enzyme molecule was also necessary to enhance solubility of the enzyme in aqueous media for subsequent steps of determining the C-terminal amino acids of subunits which will be described below. After these treatments, the enzyme solution containing about 30 mg of protein was applied to a column of Sephadex G-75 (2.5 × 80 cm), preequilibrated with 8.0 M urea–0.05 M borate KOH–0.1 M KCl (pH 8.0), and absorbancies of 5-ml fractions

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[1] Abbreviations used are: RuP_2, ribulose 1,5-diphosphate; SDS, sodium dodecyl sulfate.

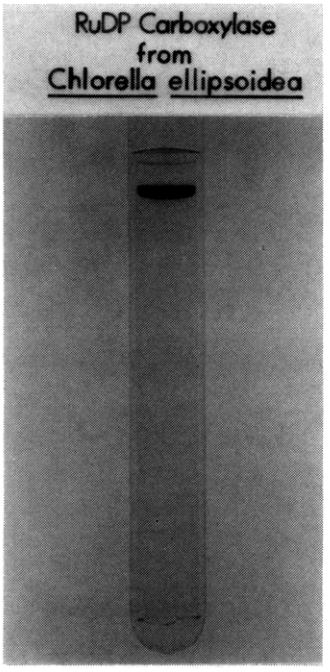

FIGURE 1: Polyacrylamide gel electrophoresis of *Chlorella* RuP_2-carboxylase. About 50 μg of a purified preparation of *Chlorella* RuP_2-carboxylase was subjected to polyacrylamide gel disc electrophoresis following the method of Davis (1970) using 15% of acrylamide and a discontinuous buffer system of Tris–glycine. Electrophoresis was carried out at 4 mA/tube. The gel was stained with Amido Schwarz 10B.

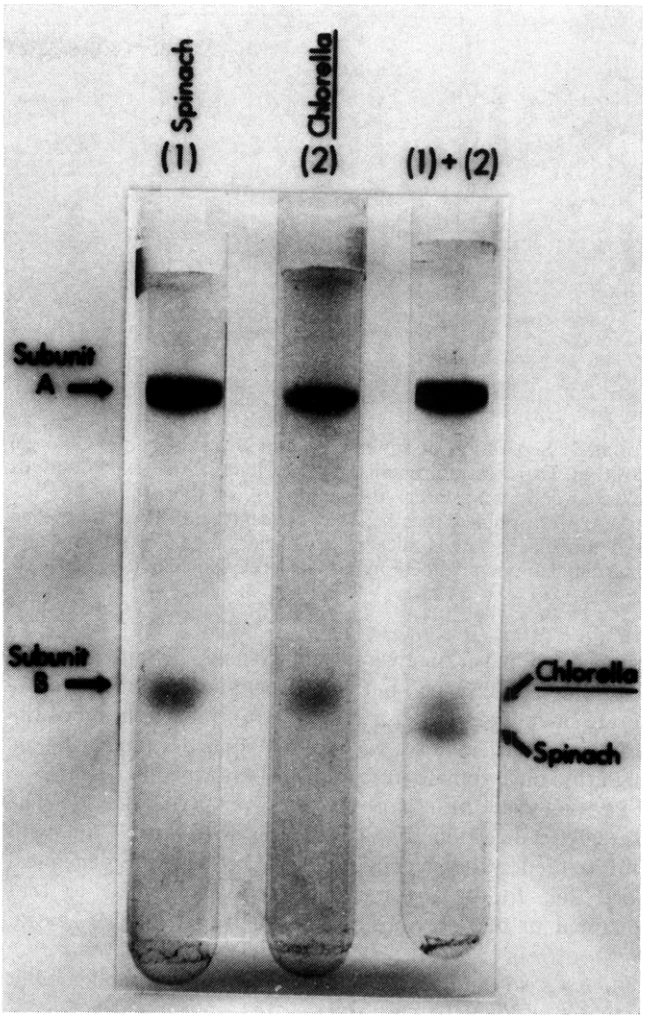

FIGURE 2: SDS–polyacrylamide gel disc electrophoresis of subunits A and B from *Chlorella* and spinach RuP_2-carboxylase. Experimental details are described in the text. Left (1): SDS-treated spinach enzyme. Middle (2): SDS-treated *Chlorella* enzyme. Right (1 + 2): equal amount of mixture of SDS-treated *Chlorella* and spinach enzymes.

collected were then read at 280 nm. Fractions containing either the subunit A or the subunit B were pooled separately and lyophilized after an extensive dialysis against water which was adjusted to pH 9.0 with NH_4OH.

Determination of Molecular Weights of Subunits A and B by SDS–Polyacrylamide Gel Electrophoresis. The molecular weights of the subunits A and B were determined by SDS–polyacrylamide gel disc electrophoresis of Weber and Osborn (1969) as described in our previous paper (Sugiyama and Akazawa, 1970). A sample of approximately 2 mg each of *Chlorella* and spinach leaf RuP_2-carboxylases and marker proteins was dissolved in 2 ml of 0.01 M phosphate buffer (pH 7.0) containing 1% SDS and 1% β-mercaptoethanol. Each solution was incubated for 3 hr at 37°. After dialysis of the solution against 0.01 M phosphate buffer (pH 7.0) containing 0.1% each of SDS and β-mercaptoethanol, a sample of 50–100 μl each of the protein solution was applied on gel containing 10% acrylamide. Electrophoresis was performed at a constant current of 8 mA/tube. After staining the gel with coomassie brilliant blue, the molecular weight of each subunit was estimated from a calibration curve made by the simultaneous running of the electrophoresis using the following protein markers (Mann) as reported previously: bovine serum albumin (6.7×10^4), human γ-globulin (H chain, 5.0×10^4; L chain, 2.0×10^4), 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). The numbers in parentheses are molecular weights.

Determination of C-Terminal Amino Acids. Two different techniques were applied to determine C-terminal amino acids of the subunits A and B. Experimental details were essentially the same as those for spinach leaf RuP_2-carboxylase reported previously (Sugiyama and Akazawa, 1970).

LABELING WITH TRITIUM. This was based on the method originally reported by Matsuo *et al.* (1966). A sample of approximately 1 mg each of lyophilized preparations of the subunits A and B was dissolved in a mixture of pyridine (0.2 ml) and tritiated water (0.1 ml, 50 mCi). To this solution in an ice bath 0.05 ml of acetic anhydride was added. The resulting mixture was allowed to stand at room temperature for 14 hr. To this mixture (slightly gelatinized in the case of the subunit A at this time) 0.2 ml of pyridine and 0.05 ml of acetic anhydride were added, and the reaction was allowed to proceed for another 2 hr at room temperature. Then the mixture was lyophilized. To remove the remaining ^3H completely, addition of water and the subsequent lyophilization were repeated five times. The tritiated protein sample was then hydrolyzed in 0.5 ml of glass-distilled 6 N HCl at 110° for 20 hr. The hydrolysate was evaporated to dryness and dissolved in water, and an aliquot was subjected to high-voltage paper electrophoresis in a solvent mixture containing pyridine–acetic acid–water (5:0.2:95, v/v, pH 6.5). After locating radioactivity on the paper in a Packard Tri-Carb liquid scintillation counter, the specifically ^3H-labeled neutral amino acid was extracted from the paper and chromato-

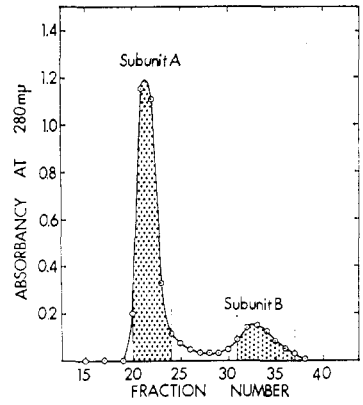

FIGURE 3: Separation of subunits A and B on Sephadex G-75 gel filtration. Experimental details are described in the text. Fractions collected containing either the subunit A or the subunit B (shadowed) were pooled together and concentrated. They were then used for subsequent studies of molecular weight estimation and C-terminal amino acid determinations as explained in the text.

graphed on paper in two solvent systems: 1-butanol–acetic acid–water (4:1:5, v/v, butanol layer) and phenol–*m*-cresol–borate buffer (pH 9.3) (25:25:7, w/w/v). Identification of the radioactive amino acid was carried out by comparing its migration on chromatogram to that of known amino acids.

PROTEOLYTIC DIGESTION WITH CARBOXYPEPTIDASE. The proteolytic digestion of each subunit was carried out with DFP-treated carboxypeptidase A (Worthington Biochemical Corp., N. J.), by the method essentially similar to that described in our previous paper (Sugiyama and Akazawa, 1970).

Amino Acid Analysis. A lyophilized sample of each subunit from *Chlorella* RuP_2-carboxylase was hydrolyzed in glass-distilled 6 N HCl *in vacuo* by the method of Moore and Stein (1963) at 110° for 20 hr. The amino acids were determined by a Nihon Denshi JLC-5AH automatic amino acid analyzer equipped with a high-sensitivity attachment.

Determination of Protein. Protein was determined by the colorimetric method of Lowry *et al.* (1951) and bovine serum albumin was employed as a standard.

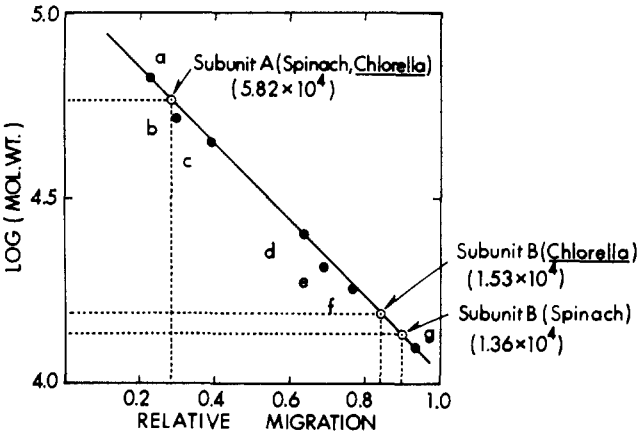

FIGURE 4: Estimation of molecular weight of subunits A and B by SDS–polyacrylamide gel disc electrophoresis. Experimental details for SDS–polyacrylamide gel disc electrophoresis are described in the text. After staining the gel with coomassie brilliant blue, relative mobility of each protein was plotted against its molecular weight on a semilogarithmic scale. From the calibration curve obtained with the standard proteins of known molecular weights, the molecular weights of the subunits A and B were determined. Standard samples used were (numbers in parentheses are molecular weights): (a) bovine serum albumin (6.7×10^4), (b) human γ-globulin (H chain) (5.0×10^4), (c) ovalbumin (4.5×10^4), (d) beef pancreas chymotrypsinogen (2.5×10^4), (e) human γ-globulin (L chain) (2.0×10^4), (f) sperm whale myoglobin (1.78×10^4), and (g) horse heart cytochrome *c* (1.3×10^4).

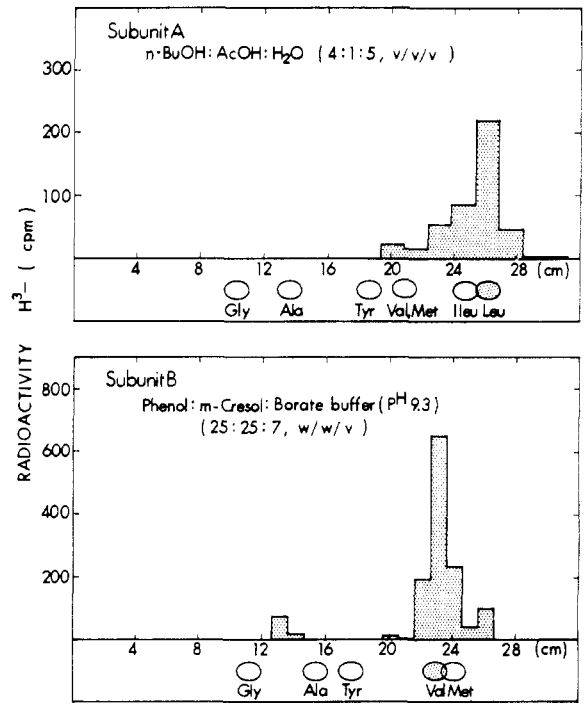

FIGURE 5: Radiochromatogram of acid hydrolysates of the ^{3}H-labeled subunits A and B (upper: subunit A; lower: subunit B). After the selective ^{3}H labeling of the C-terminal amino acid in the subunit A, followed by acid hydrolysis, the specifically labeled amino acid was separated by paper electrophoresis as described in the text. The spot containing the neutral ^{3}H-labeled amino acid was then separated by paper chromatography (24 hr at 25°) with a solvent system of 1-butanol–acetic acid–water (4:1:5, v/v, butanol layer). Experimental details for subunit B were the same as those for subunit A, except a solvent system of phenol–*m*-cresol–borate (pH 8.3) (25:25:7, w/w/v) was used for the final separation of the subunit B by paper chromatography.

Results

The results presented in Figure 2 showed the dissociation of *Chlorella* RuP_2-carboxylase into two distinct subunits, A and B, as demonstrated on SDS–polyacrylamide gel disc electrophoresis. The electrophoretic migration of the subunit A of the *Chlorella* enzyme was indistinguishable from that of spinach RuP_2-carboxylase, whereas the subunit B of the algal enzyme was clearly different from that of the spinach enzyme. This difference was further substantiated by applying a mixed protein sample to the electrophoresis.

The separation of the subunits A and B from the *Chlorella* enzyme was carried out by gel filtration on a Sephadex G-75 column. The elution profile of the two subunits shown in Figure 3 was similar to that obtained with the spinach enzyme (*cf.* Figure 1 of Sugiyama and Akazawa, 1970). Each subunit fraction as shadowed in the figure was ascertained to be homogeneous without mutual contamination, from its band pattern on polyacrylamide gel disc electrophoresis (not shown).

In order to determine the molecular weight of each subunit, relative migration of the subunit on SDS–polyacrylamide gel

TABLE I: Relative Amino Acid Composition of Ribulose 1,5-Diphosphate Carboxylases from *Chlorella ellipsoidea* and Spinach Leaf and Their Two Subunits (A and B).

	Subunit A		Subunit B		RuP_2-carboxylase			
					Chlorella			
	Chlorella[a]	Spinach[b]	*Chlorella*[a]	Spinach[b]	A[c]	B[d]	C[e]	Spinach[e]
Phenylalanine	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)
Tyrosine	0.98	0.94	0.91	1.74	0.96	0.97	1.33	1.09
Glycine	2.57	2.33	1.67	1.07	2.38	2.42	2.15	2.09
Lysine	1.01	1.13	0.74	1.09	0.95	0.97	2.10	1.12
Histidine	0.63	0.77	0.18	0.54	0.54	0.56		0.73
Arginine	1.74	1.48	1.82	1.10	1.76	1.75	1.54	1.41
Aspartic acid	2.37	2.20	2.61	2.20	2.42	2.41	2.11	2.20
Threonine	1.57	1.73	0.97	1.23	1.45	1.47	1.32	1.63
Serine	0.98	0.70	1.56	0.64	1.10	1.08	0.96	0.69
Glutamic acid	2.63	2.28	2.48	2.71	2.60	2.61	2.21	2.36
Proline	1.36	1.12	1.33	1.56	1.35	1.36	1.43	1.20
Alanine	2.72	2.18	2.00	0.79	2.57	2.60	2.35	1.92
Valine	1.24	1.67	1.78	1.12	1.35	1.33	1.44	1.57
Methionine	0.45	0.39	0.44	0.45	0.45	0.45	0.41	0.40
Isoleucine	0.84	0.92	0.61	0.66	0.79	0.80	0.82	0.87
Leucine	2.23	2.16	1.84	1.78	2.15	2.17	1.93	2.09

[a] Average of duplicate analyses. [b] Data taken from Table I of Sugiyama and Akazawa (1970). [c] Calculated from present data, by assuming eight each of subunits A (mol wt 5.82×10^4) and B (mol wt 1.53×10^4) (A_8B_8) make up RuP_2-carboxylase molecule. [d] Calculated from present data, by assuming eight subunits A (mol wt 5.82×10^4) and six subunits B (mol wt 1.53×10^4) (A_8B_6) make up RuP_2-carboxylase molecule. [e] Data taken from Figure 4 of Matsumoto *et al.* (1969). [f] Data are presented as relative content of individual amino acids in relation to phenylalanine taken as a unity (Rutner and Lane, 1967; Sugiyama and Akazawa, 1970).

disc electrophoresis was compared with that of marker proteins of the known molecular weights. As shown in Figure 4, the molecular weight of the subunit A was estimated to be 5.82×10^4 for both *Chlorella* and spinach enzymes. Molecular weight (1.53×10^4) of the subunit B from the *Chlorella* enzyme was slightly larger than that of the spinach enzyme (1.36×10^4) (*cf.* Figure 4 of Sugiyama and Akazawa, 1970).

Next the C-terminal amino acid of each subunit was identified by two different methods previously employed in the spinach enzyme studies. The results of the ^3H-labeling technique, summarized in Figure 5, demonstrated that the C-terminal amino acid of the subunit A was leucine and that of the subunit B was valine. In order to verify the finding further, each subunit was digested by carboxypeptidase A. The results of time-dependent release of amino acids analyzed by an automatic amino acid analyzer were presented in Figure 6. The results clearly supported the conclusion obtained by the ^3H-labeling method. Approximately 1 equiv of leucine per mole of the subunit A was released rapidly during the digestion, followed by the liberation of serine, valine, and alanine. One equivalent of valine was released from 1 mole of the subunit B, followed by the liberation of serine, which reached a maximum of 1 equiv/mole during the digestion. From the kinetic pattern of amino acid release during the proteolytic digestion, amino acid sequence in the C-terminal region of the subunits A and B was tentatively identified as $-Ala_4-(Val_3,Ser_2)-Leu_1$ and $-Ser_2-Val_1$, respectively.

Amino acid composition of the two subunits of *Chlorella* RuP_2-carboxylase was determined and compared to the data obtained for the spinach enzyme reported previously (Table I). Given in the table are contents of each amino acid in relation to that of phenylalanine taken as unity (Rutner and Lane, 1967; Sugiyama and Akazawa, 1970). The amino acid compositions of the subunit A from *Chlorella* are quite similar to that from the spinach enzyme. That of subunit B, however, is markedly different from that of the spinach enzyme. Amino acids which differed markedly were tyrosine, glycine, histidine, arginine, serine, alanine, and valine. The amino acid composition of the whole enzyme, *Chlorella* RuP_2-carboxylase, was calculated by assuming the enzyme was a *n* oligomer composed of either (i) eight monomers each of the subunit A and the subunit B (A_8B_8) or (ii) eight monomers of the subunit A and six monomers of the subunit B (A_8B_6) as in the case of the spinach enzyme (see Discussion). It can be seen in the table that the two models have nearly identical amino acid compositions within experimental errors and that the figures are in fairly good agreement with the data of our previous analysis except tyrosine, lysine, and a few other amino acids (Matsumoto *et al.*, 1970). A major factor of the discrepancy might probably be poor separation of lysine and histidine observed in the previous studies.

Discussion

Our previous studies showed that RuP_2-carboxylases from green alga *Chlorella* and spinach leaves have structural differences manifested by their amino acid composition, in spite of their similarities in large molecular size (Sugiyama *et al.*, 1969; Matsumoto *et al.*, 1969). Both immunodiffusion tests and quantitative precipitin tests carried out also showed that these two enzyme proteins were in fact quite different.

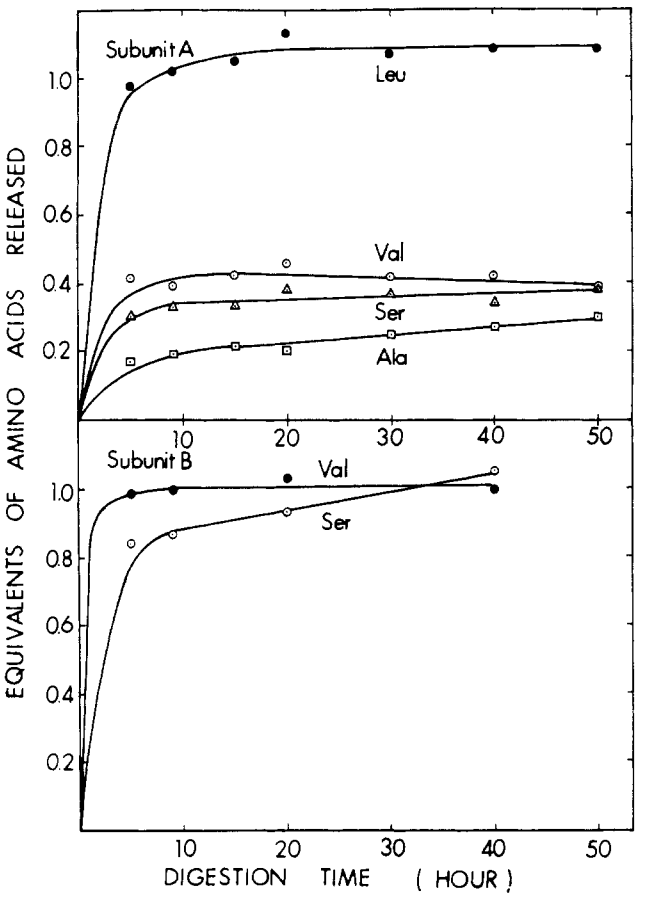

FIGURE 6: Carboxypeptidase digestion of subunits A and B. The reaction mixture contained about 0.1 μmole of either the subunit A or the B, 20 μmoles of *N*-ethylmorpholine–acetate buffer (pH 8.5), 3.5 μg of DFP–carboxypeptidase A dissolved in 10% (w/v) LiCl, and 0.005 ml of toluene in total volume of 0.2 ml. Proteolytic digestion of the proteins was carried out at 37°. The time-sequence analysis of the released amino acids was carried out by the use of a JLC-5AH automatic amino acid analyzer, as described in the text. Averaged values of duplicate analyses were then used to calculate the equivalents of each amino acid released per mole of subunit A (mol wt 5.82×10^4) and subunit B (mol wt 1.53×10^4), respectively.

The same studies made on the spinach RuP_2-carboxylase showed that amino acid sequence in the C-terminal regions of the subunit A and the subunit B determined by carboxypeptidase A was -Val_1 and -Phe_5-Leu_4-(Tyr_3,Thr_2)-Tyr_1, respectively (Sugiyama and Akazawa, 1970). Our present studies established the C-terminal amino acid sequence of the subunit A and the subunit B of *Chlorella* RuP_2-carboxylase to be -Ala_4-(Val_3,Ser_2)-Leu_1, and -Ser_2-Val_1, respectively. Despite the difference in the C-terminal amino acid sequence between the subunit A's of the *Chlorella* and spinach enzymes and despite the difference in the C-terminal amino acid sequence and in the amino acid composition between the subunit B's of these two enzymes, similarity in amino acid composition of the subunits A's and of the whole enzymes of these two organisms is clearly discernible. This perplexity is contrasted to the markedly dissimilar properties exhibited by the subunit B's of these two enzymes. It is certainly premature to speculate about the possible structural homology of the subunit A's of the two enzymes until the entire amino acid sequence is established.

Wishnick *et al.* (1970), using [^{14}C]RuP_2 and [^{14}C]2-carboxyribitol diphosphate, demonstrated that the spinach leaf RuP_2-carboxylase had eight binding sites for RuP_2 in its molecule. This view was further supported by Rutner (1970), who determined the molecular weight of the subunits A and B of this enzyme to be 55,800 ± 2300 and 12,100 ± 1600, respectively, and proposed a molecular organization containing eight catalytic subunits and eight to ten regulatory or structural subunits for this enzyme. On the other hand, Kawashima and Wildman (1970) reported the estimated molecular weight of the large and small subunits of tobacco leaf RuP_2-carboxylase (fraction I protein) to be 52,000 and 24,500, respectively, by Sephadex G-200 gel filtration technique. They thus proposed a model of the subunit structure of the protein consisting of eight large and six small subunits. Our data on the molecular weight of the spinach enzyme were in fairly good agreement with those of Rutner. From our present studies, it may be proposed that RuP_2-carboxylase of *Chlorella* is composed of eight each of the subunit A and the subunit B (A_8B_8) or eight subunit A's and six subunit B's (A_8B_6) (see Table I). The molecular weight of the entire enzyme molecule calculated from these assumptions (A_8B_8) (5.88×10^5) is 15–20% greater than that was obtained by gel filtration technique and was reported previously (Sugiyama *et al.*, 1969; Akazawa *et al.*, 1969). The discrepancy might have resulted from the different methods employed and the assumptions associated with these methods in the determination of the molecular weight.

The belief that the subunit A plays a catalytic role in the RuP_2-carboxylase was derived from our previous experiments showing that eight to ten SH groups closely related to the enzyme reaction are mostly localized in the large subunit (Sugiyama *et al.*, 1970; Sugiyama and Akazawa, 1970). Since the *Chlorella* RuP_2-carboxylase was found to exhibit analogous kinetic properties as those observed by the spinach enzyme, it is an intriguing problem of future investigation to elucidate the intrinsic role of each subunit of the enzyme molecule in the mechanism of RuP_2-carboxylase reaction.

Criddle *et al.* (1970) recently reported a differential synthesis of RuP_2-carboxylase in barley leaf using specific inhibitors of protein synthesis. They found that the subunits A and B of the RuP_2-carboxylase were synthesized in chloroplasts and in cytoplasm, respectively. However, it is premature to speculate freely that synthesis of subunit A, with a composition that is relatively invariable among plant species, is genetically controlled under chloroplast DNA, while that of the subunit B, whose species specificity was manifested by its immunochemical specificity, is under the control of cytoplasmic DNA. In fact, a study of Armstrong *et al.* (1971) using synchronously growing cells of green alga *Chlamydomonas reinhardi* indicates that the synthesis of RuP_2-carboxylase does not require the transcription on chloroplast DNA. Their data suggest the complex picture in the control on the translational level; both 70S chloroplast ribosomes and 80S cytoplasmic ribosomes are engaged in the synthesis of RuP_2-carboxylase. Further work is needed to understand roles of subunit B in the photosynthetic carbon dioxide fixation reaction. A preliminary discussion on the relation between the structure and function of oligomeric RuP_2-carboxylase was previously presented by Moon and Thompson (1969) and Akazawa (1970).

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Stabilization of Allosteric Adenosine Monophosphate Nucleosidase by Inorganic Salts, Substrate, and Essential Activator*

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ABSTRACT: Adenosine monophosphate nucleosidase (AMP nucleosidase, EC 3.2.2.4) has been purified over 80-fold from *Azotobacter vinelandii* OP and used to determine the mechanism of inactivation which occurs when the enzyme is placed in a low ionic strength environment. The kinetics of inactivation, together with results of gel filtration studies, indicate that the native enzyme exists in a polymeric form with an approximate molecular weight of 360,000. The enzyme is maintained in this form by a variety of inorganic salts as well as by the substrate (AMP) or essential activator ($MgATP^{2-}$). Removal of these protective agents results in a rapid loss of activity accompanied by the appearance of an inactive form of the enzyme having an apparent molecular weight of about 180,000. The inactive enzyme may be converted to the native form by the addition of salts, substrate, or activator. It is proposed that inorganic salts stabilize the native enzyme by neutralizing excess electrostatic charges in the molecule, which, when unshielded, disrupt the quaternary structure of the protein. Substrate and essential activator protect not by ionic shielding, but by combination at specific binding sites on the enzyme. Such combinations cause a conformational or charge distribution change which is sufficient to stabilize the quaternary structure in the absence of protection by inorganic salts. Comparison of initial velocity and stability experiments to both AMP and $MgATP^{2-}$ indicate that the substrate combines well with the enzyme either in the presence or absence of essential activator. Thus the function of $MgATP^{2-}$ must be to provide a catalytically active conformation rather than to form a binding site for the substrate.

In recent years a wide variety of allosteric enzymes have been studied kinetically with the results usually being interpreted in terms of the hypotheses proposed by either Monod *et al.* (1965) or Koshland *et al.* (1966). Because these hypotheses have been based only on the binding of ligands, with no consideration given to kinetic effects (rate changes as a consequence of multiple ligand bindings), such interpretations are, at best, only approximations to the actual mechanism of allo-

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