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Studies on Spinach Leaf Ribulosebisphosphate Carboxylase. Carboxylase and Oxygenase Reaction Examined by Immunochemical Methods†

Mikio Nishimura and Takashi Akazawa*

ABSTRACT: Rabbit antisera were developed against large and small subunits (A and B) of spinach leaf ribulosebisphosphate carboxylase. Specific cross-reactivity of anti-A and anti-B sera against respective antigenic subunits was proven by: (i) quantitative immunoprecipitation analysis using Sephadex G-200 column eluates, (ii) double immunodiffusion on agar plate, and (iii) sodium dodecyl sulfate polyacrylamide gel electrophoresis of antigen–antibody complexes. Specific inhibitory effect of anti-A sera on the ribulosebisphosphate carboxylase reaction provided an additional proof for the catalytic role of the larger subunit in the enzyme catalysis. Disappearance of the Mg^{2+}-induced optimum pH shift of the enzyme reaction by the anti-B-sera-treated enzyme preparation supported our previous notion of the regulatory role of the smaller subunit. A concomitant loss of the ribulosebisphosphate oxygenase activity shown by the anti-A-sera-treated carboxylase demonstrated that the larger subunit of the enzyme molecule shared the catalytic site for both the carboxylase and the oxygenase reactions. The anti-B-sera-treated enzyme preparation showed a marked shift of optimum pH in the oxygenase reaction to a neutral side, regardless of the presence of Mg^{2+} in the assay mixture. Experimental results altogether led to a definite conclusion that the catalytic function resided in the larger subunit (A) and the regulatory function in the smaller subunit (B) of the fraction 1 protein of chloroplasts.

Ribulosebisphosphate carboxylase[1] (EC 4.1.1.39) from spinach leaf comprises two different subunits (A and B) (Rutner and Lane, 1967; Sugiyama and Akazawa, 1970). The molecular weight is 5.4×10^4 for subunit A and 1.3×10^4 for subunit B (Nishimura *et al.*, 1973). An octameric

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[1] Abbreviations used are: RuP_2, ribulose bisphosphate; ammediol, 2-amino-2-ethyl-1,3-propanediol; SDS, sodium dodecyl sulfate; PMB, *p*-chloromercuribenzoate.

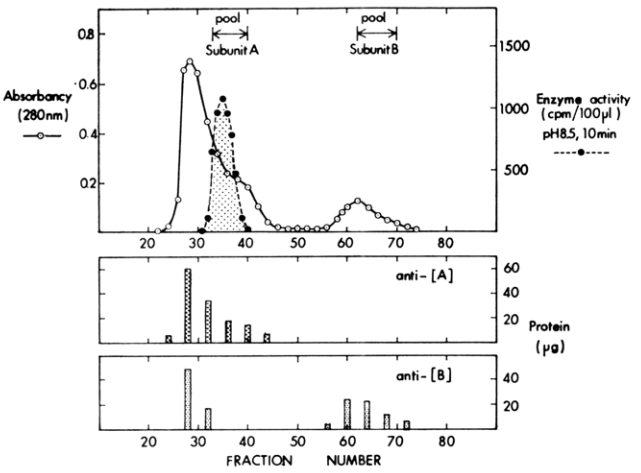

FIGURE 1: Quantitative immunoprecipitation analysis of Sephadex G-200 eluates. A PMB-treated enzyme protein sample (8.3 mg) prepared according to the method described in the text was applied to a column (1.5 × 90 cm) of Sephadex G-200, which had been equilibrated with 0.025 M Tris-HCl buffer (pH 9.0), and 1.5-ml fractions were collected. Pooled fractions of the larger (A) and small subunit (B) obtained from similar Sephadex G-200 column chromatography, as marked in the figure, were used for immunization of rabbits. Aliquots (0.1 ml) of the fractions (25–50) were incubated with excess β-mercaptoethanol for 3 hr at 25°, and 0.1-ml aliquots of the resulting samples were used for the enzyme activity measurements at pH 8.5. Separately aliquots (0.2 ml) of 12 fractions in every fourth tube were used for the immunoprecipitate analysis as explained in the text.

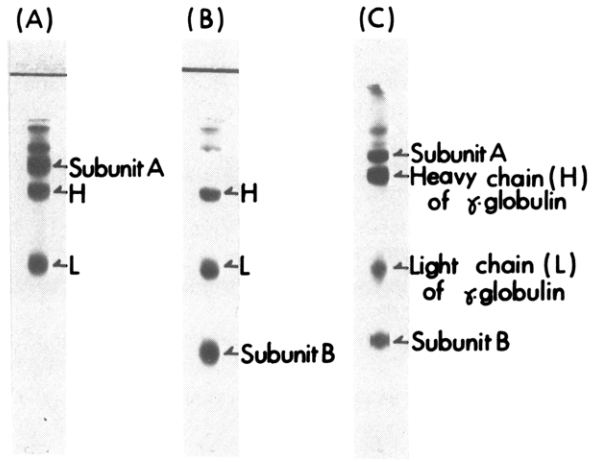

FIGURE 3: SDS polyacrylamide gel electrophoresis of antigen–antibody complexes. A mixture of 50 μg each of the large subunit A and small subunit B (*cf.* Figure 1) was added to 100–200 μg each of γ-globulin fraction of (A) anti-A, (B) anti-B, or (C) anti-native enzyme serum and the resulting mixture was left overnight at 4°. The antigen–antibody complex formed was collected by centrifugation, and washed repeatedly with 0.5 ml of 0.8% NaCl at 4°. An aliquot of the final precipitate dissolved in 0.1 ml of 0.02 M phosphate buffer (pH 7.0) containing 1% SDS and 1% β-mercaptoethanol was applied to SDS polyacrylamide gel electrophoresis.

oligomer of the larger subunit, A_s, produced upon an alkaline PMB treatment of the native enzyme and subsequent removal of the mercurial with excess β-mercaptoethanol, retains partial carboxylase activity in the absence of the smaller subunit (Nishimura *et al.*, 1973; Nishimura and Akazawa, 1974). The dissociation of the spinach enzyme into the catalytic oligomer, free of cross-contamination of the smaller subunit, is substantiated by our additional experimental results on the C-terminal amino acid analysis by the C-peptidase digestion (Nishimura and Akazawa, 1973). The enzymically active large subunit preparation does not exhibit the Mg^{2+}-induced shift of the optimal pH to an acidic side, indicating that the role of the smaller subunit in the enzyme reaction is closely related to the Mg^{2+} effect. This communication deals with the immunochemical investigation that would further clarify structure–function relationship of the two constituent subunits of the spinach enzyme.

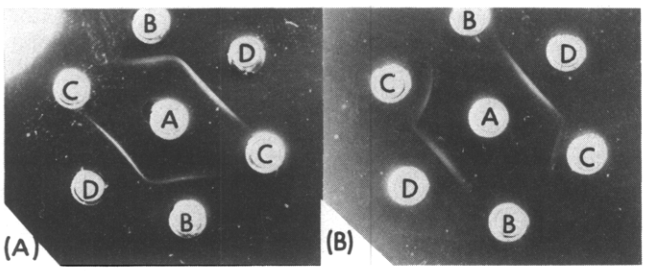

FIGURE 2: Ouchterlony double immunodiffusion experiment. Antibody wells contained 60 μg each of γ-globulin fraction of respective antiserum, and antigen wells contained *ca.* 10 μg of protein samples: (A) well A contained anti-A serum, and wells B, C, and D contained large (A) and small (B) subunits and native enzyme, respectively; (B) well A contained anti-B serum, and wells B, C, and D contained large (A) and small (B) subunits and native enzyme, respectively.

Materials and Methods

Enzyme Purification and Subunit Dissociation. A homogeneous preparation of spinach leaf RuP_2-carboxylase (0.6 unit/mg, pH 7.0) was used throughout this investigation. The outline of the enzyme dissociation by alkaline PMB treatment is as follows (*cf.* Nishimura *et al.*, 1973). The enzyme protein was initially treated by PMB (2 PMB/SH in the enzyme) at pH 7.5 for 30 min, and the resulting enzyme solution was applied to a small column of Sephadex G-25 (fine) equilibrated with 0.025 M Tris-HCl buffer (pH 9.0) to remove excess PMB. The eluate was applied to a column of Sephadex G-200, which had been equilibrated with 0.025 M Tris-HCl buffer at pH 9.0. Eluates were thus subjected to (i) ultraviolet (uv) absorption measurement at 280 nm, (ii) enzyme assay at pH 8.5, and (iii) immunoprecipitation analysis with the respective antiserum. A clear separation of the large and small subunit fractions was evidenced by the SDS polyacrylamide gel electrophoresis, although higher aggregates of the enzyme were eluted ahead of the catalytically active fractions (see Figures 1 and 3 of Nishimura *et al.*, 1973). Pooled fractions of large and small subunits were used for immunization of rabbits. The specific activity of the oligomeric catalytic subunit was 0.075 unit/mg, pH 8.5, 13% of that of native enzyme.

Immunochemical Experiments. The appropriate protein samples, *i.e.*, native enzyme, and large and small subunits separated by Sephadex G-200 columns, were preincubated with excess β-mercaptoethanol for 3 hr at 25°, and subsequently dialyzed against 0.01 M phosphate buffer (pH 7.0) for 4 hr at 25°. During this step, higher aggregates of the native enzyme contaminated in the large subunit fraction became insolubilized, and could be removed by centrifugation. The specific immune response is thus guaranteed. Resulting protein samples (1 mg/ml) were emulsified with an equal volume of Freund's incomplete adjuvant, and 2-ml aliquots were injected intramuscularly into hip and subcutaneously along the back bone of the white rabbits (Kabat and Mayer, 1964). Forty days after the first injection, the booster was given in a 7-day interval over the period of 2 months. If necessary, antisera

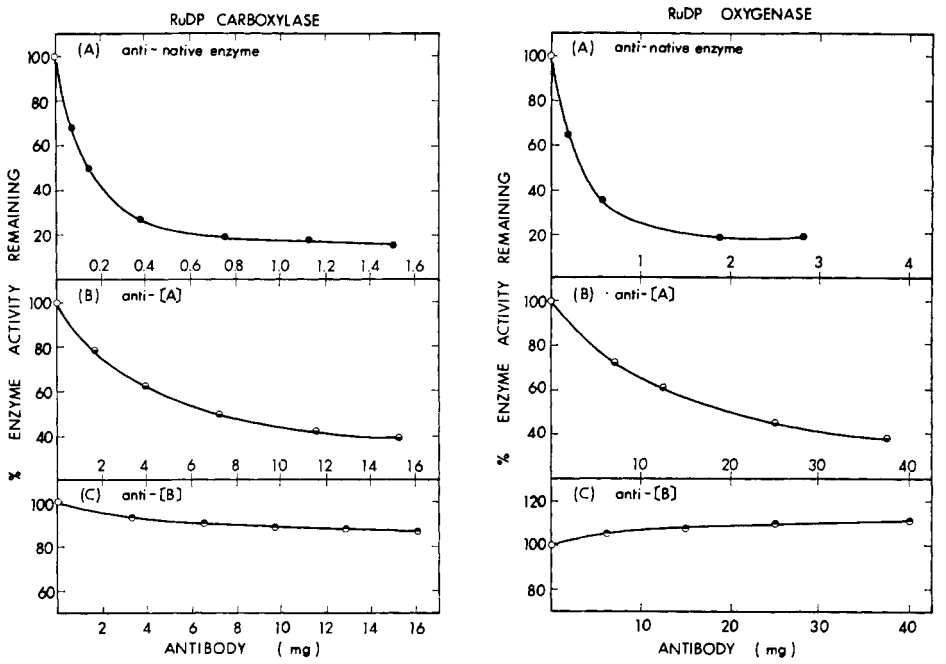

FIGURE 4: Effect of antiserum treatment on RuP_2-carboxylase (left) and RuP_2-oxygenase (right) activities. Each respective antiserum of various amounts was added to the intact native enzyme (20 μg with RuP_2-carboxylase and 50 μg with RuP_2-oxygenase) and incubated for 30 min at 25° before starting the enzyme assay. Determined enzyme activities in each system were calculated with respect to the per cent activity of the control system which received no antiserum treatment.

obtained were subjected to $(NH_4)_2SO_4$ fractionation to prepare γ-globulin fractions. Nonimmunized rabbit serum was used as a control antiserum. The antisera developed against large and small subunits are referred to as anti-A and anti-B, respectively, and their homogeneity was tested as follows. (i) For quantitative immunoprecipitation analysis, 0.2 ml aliquots of the Sephadex G-200 eluates (*cf.* Figure 1) were mixed with 0.2 ml of the respective antiserum preparation and the mixtures were left overnight at 4°. The precipitates collected by centrifugation were repeatedly washed with 0.5 ml of 0.8% NaCl at 4°. The protein content was analyzed by the colorimetric method of Lowry *et al.* (1951), using bovine serum albumin as a standard. (ii) For double immunodiffusion on agar plate, the method of Ouchterlony (1949) was employed, by dissolving 1 g of Difco special agar noble in 100 ml of 0.025 M Tris-HCl buffer (pH 9.0) containing 0.1% NaN_3. The precipitin line was observed after 24-hr incubation at 25°. (iii) For SDS polyacrylamide gel electrophoresis of antigen–antibody complexes, an antiserum–γ-globulin fraction (100–200 μg) was added to an equal mixture of subunits A and B and the resulting mixture was left at 4° overnight. The antigen–antibody complex formed was collected by centrifugation, and the precipitate was washed repeatedly with 0.5 ml of 0.8% NaCl at 4° (Platt *et al.*, 1972). After dissolving the precipitate in 0.1 ml of 0.02 M phosphate buffer (pH 7.0) containing 1% SDS and 1% β-mercaptoethanol at 37° for 3 hr, an aliquot was applied to the SDS polyacrylamide gel electrophoresis at 10% gel concentration (Weber and Osborn, 1969).

Enzyme Assay. (i) For RuP_2-carboxylase, unless otherwise stated, the enzyme assay method described in our previous report (Nishimura *et al.*, 1973) was followed exactly. In order to examine the effects of antibody treatment on RuP_2-carboxylase activity each antiserum preparation was added to an intact native enzyme preparation dissolved in 0.05 M Tris-HCl buffer (pH 7.5) and the mixture was incubated at 25° for 30 min. An aliquot withdrawn was then applied to the enzyme assay mixture. Incubation was carried out at 25° for 10 min at pH 7.5 and fixed $^{14}CO_2$ was determined in a Packard liquid scintillation spectrometer. (ii) For RuP_2-oxygenase, preliminary treatment of the enzyme samples with antiserum was the same as for the RuP_2-carboxylase reaction, and the standard reaction mixture of the RuP_2-oxygenase reaction in the air-saturated condition contained the following compositions (in μmol): ammediol-HCl buffer (pH 9.5), 100; $MgCl_2$, 5; RuP_2, 0.35; O_2, 0.12; and 50 μg of enzyme in a total volume of 0.5 ml. The reaction was started by adding RuP_2, and O_2 consumption was measured by a Rank Bros oxygen electrode (Bottisham, U. K.) at 25° (Akazawa and Osmond, 1974). The specific activity of the native enzyme was 0.03 unit/mg of protein (pH 9.5).

Results

Specific Cross-Reactivity of Antisera. The immunoprecipitation reaction of each antiserum (anti-A and anti-B sera) against the corresponding antigen was tested using the dissociated RuP_2-carboxylase separated on a column of Sephadex G-200. As shown in Figure 1, anti-A sera reacted only with the fast-eluting fractions containing higher aggregates of the native enzyme protein and catalytic oligomer, while anti-B sera cross-reacted with the later-eluting fractions. Of importance is that anti-A sera did not cross-react with the smaller subunit and that anti-B sera did not react with the catalytic subunit, comprising the larger subunit (shade). These results were perfectly aggreeable with the results of the double immunodiffusion precipitin lines on agar plate as presented in Figure 2. There was a continuous arc between anti-A sera and native enzyme–catalytic subunit and between anti-B sera and native enzyme–subunit B, indicating the immunochemical identities of each pair of molecules. An additional evidence supporting immunochemical specificity of each antiserum preparation was gained from the SDS polyacrylamide gel electrophoresis of antigen–antibody complexes. As presented

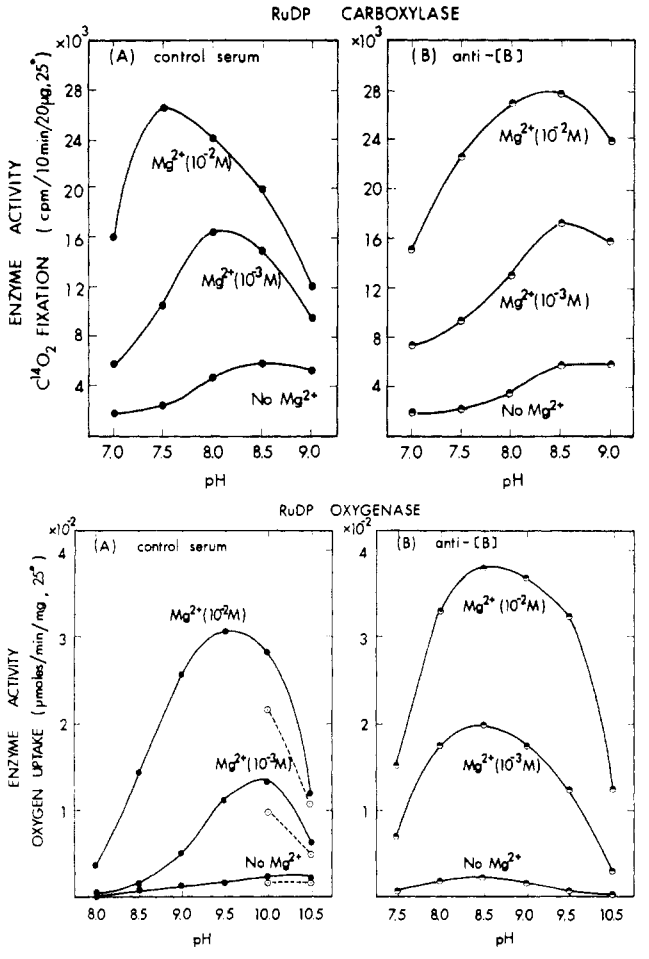

FIGURE 5: Effect of anti-B treatment on the optimum pH of RuP_2-carboxylase (top) and RuP_2-oxygenase (bottom) reactions. Native enzyme (20 μg, RuP_2-carboxylase; 50 μg, RuP_2-oxygenase) was treated either with 15 mg of nonimmunized control serum (A) or 15 mg of anti-B sera (B) for 30 min at 25°. Aliquots were used for measuring the RuP_2-carboxylase and oxygenase activities at different pH values in the presence (10^{-3} and 10^{-2} M) or absence of Mg^{2+}. In the RuP_2-carboxylase assay 1 M Tris-HCl buffer was used, and in the RuP_2-oxygenase assay both 1 M ammediol buffer (solid line) and 1 M glycine–NaOH buffer (pH 10.0 and 10.5) (dotted line) were used.

in Figure 3, each complex produced only a single band of the corresponding monomeric subunit species, free of cross-contamination of other subunit species, in addition to the heavy and light chains of γ-globulin.

Effects of Antisera on RuP_2-carboxylase Reaction. The results showing effects of antisera on the enzymic reaction catalyzed by the intact native RuP_2-carboxylase are summarized in Figure 4A–C (left). A strong inhibitory effect was exerted on the carboxylase activity by the treatment with anti-native enzyme (A). The addition of anti-A serum caused a marked inhibitory effect, although a much larger amount of serum was required (B). On the other hand, there was practically no inhibitory effect exhibited by adding anti-B serum (C). The results clearly showed that the larger subunit comprising subunit A was indeed the catalytic entity. It should be emphasized that the inhibitory effect of anti-A serum was not due to the alteration of pH–activity curve by the antibody treatment. By the addition of the nonimmunized control serum, the optimum pH shifted to acidic side (pH 7.5) in the presence of Mg^{2+} like the case of the intact native enzyme without the serum (Figure 5A, top). Exactly the same relationship was observed by the enzyme preparation treated with either anti-native enzyme or anti-A sera, although the actual enzyme activities were much lower (data not shown). However, by the treatment with anti-B serum, characteristic Mg^{2+}-dependent shift of optimal pH of the carboxylation reaction disappeared, and the optimum pH remained at an alkaline side at about pH 8.5 (Figure 5B, top).

Effects of Antisera on RuP_2-oxygenase Reaction. Recently Tolbert and his colleagues (Andrews *et al.*, 1973) reported that soybean and spinach leaf fraction 1 proteins catalyzed the RuP_2-oxygenase reaction, producing oxidatively phosphoglycolate and 3-phosphoglycerate from RuP_2. In a recent experiment, Takabe and Akazawa (1973b) demonstrated that the RuP_2-carboxylase preparation from the photosynthetic bacterium, *Chromatium*, catalyzed the oxidative formation of phosphoglycolate from RuP_2, and that the RuP_2-binding site in both of carboxylase and oxygenase reactions existed in the larger subunit of the bacterial enzyme. We herein examined effects of antisera on the RuP_2-oxygenase reaction catalyzed by the spinach carboxylase. Results summarized in Figure 4A–C (right) are essentially comparable to those observed with the RuP_2-carboxylase reaction. The potent inhibitory effect of anti-A sera on the oxygenase reaction supports the fact that the larger subunit of the carboxylase molecule also contains the catalytic site of the oxygenase reaction. Note that a slight but significant enhancement of the oxygenase activity was observed by the anti-B-sera-treated enzyme preparations (C). This is reminiscent of our previous investigation concerning the catalytic subunit of the *Chromatium* RuP_2-carboxylase exhibiting the greater oxygenase activity than the native enzyme. Another notable finding was an apparent shift of the optimum pH to neutral side in the oxygenase reaction catalyzed by the anti-B-sera-treated enzyme preparation. As can be seen in Figure 5 (bottom), the native enzyme treated by the control serum exhibited a slight shift of the optimum pH as a function of Mg^{2+} concentrations. The behavior of the anti-A-sera-treated enzyme was essentially comparable to this, optimum pH at 9.5 with 10^{-2} M Mg^{2+} (data not shown). On the other hand, the addition of anti-B sera caused a significant shift of the optimum pH to pH 8.5, regardless of the presence of Mg^{2+} in the assay mixture (Figure 5B, bottom).

Discussion

A sizable amount of evidence has accumulated showing that the oligomer of the larger subunit A is catalytic entity in both spinach and *Chromatium* RuP_2-carboxylases (Nishimura *et al.*, 1973; Nishimura and Akazawa, 1973, 1974; Siegel *et al.*, 1972; Sugiyama and Akazawa, 1970; Takabe and Akazawa, 1973a; Wishnick *et al.*, 1970). In the work reported herein we applied immunochemical techniques to demonstrate both the structural homogeneity of each constituent subunit of the spinach carboxylase and its functional role, and the results were conclusive in that the oligomeric larger subunit A is the catalytic entity of both RuP_2-carboxylase and RuP_2-oxygenase reactions in the absence of the smaller subunit B.

Our separate experiment reported elsewhere showed on the basis of molecular weight determination that the catalytic entity of the spinach RuP_2-carboxylase was an octameric oligomer of subunit A, A_8 (Nishimura *et al.*, 1973; Nishimura and Akazawa, 1974). We further showed that the catalytic subunit, A_m, of the *Chromatium* RuP_2-carboxylase was active as a RuP_2-oxygenase in the absence of the smaller subunit (Takabe and Akazawa, 1973b). Thus, the specific inhibition by the anti-A serum on both RuP_2-carboxylase and -oxygenase

reactions shown in this report indicate that the larger polypeptide chain (subunit A) contains the RuP_2 binding site for the two different enzyme reactions. Furthermore the fact that the addition of anti-B serum has little effect on the carboxylase reaction, yet causing the disappearance of the Mg^{2+}-induced shift of optimum pH from alkali to neutral side, appears to strengthen our view that the small subunit modulates the role of Mg^{2+} in the enzyme catalysis (Nishimura and Akazawa, 1973; Takabe and Akazawa, 1973a). A newly discovered function of the small subunit in controlling the activity of RuP_2-oxygenase, as manifested in an increase of the enzyme activity as well as an apparent shift of optimum pH of the reaction shown by the anti-B-sera-treated enzyme preparation, is of special interest. We cannot dismiss, however, a possibility that the manifested results are artifacts caused by the antibody binding and may have no bearing on the role of the subunit B in the RuP_2-carboxylase and -oxygenase reactions. In this connection it may be noted that the optimum pH measurement was carried out at low oxygen concentrations in comparison with the K_m (0.75 mM for O_2) of RuP_2-oxygenase reported by Andrews *et al.* (1973). Nonetheless, it may be reasonably speculated that the ratio of RuP_2-carboxylase:oxygenase activities by fraction 1 protein in chloroplasts is modulated by the mutual interaction of the constituent subunits making up the whole enzyme molecule. The reports by Andrews *et al.* (1973) and Ryan *et al.* (1973) showed that the ratio of the two enzyme activities exhibited by spinach and bean leaf enzymes at each respective optimum pH was variable with several factors. In our present experiment, the ratio was shown to be 30:1 with the native enzyme and 24:1 with the anti-B-sera-treated enzyme. Future studies on the enzyme structure at quaternary level will certainly further our understanding on this challenging problem of the functional role of RuP_2-carboxylase–oxygenase in photosynthetic CO_2 fixation and photorespiration in chloroplasts.

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