FURTHER PROOF FOR THE CATALYTIC ROLE OF THE LARGER SUBUNIT IN THE SPINACH LEAF RIBULOSE-1,5-DIPHOSPHATE CARBOXYLASE*

Mikio Nishimura and Takashi Akazawa

Research Institute for Biochemical Regulation
Nagoya University, School of Agriculture
Chikusa, Nagoya (464), Japan

Received August 7,1973

Summary:

The catalytically active oligomeric form of the larger subunit, A_m, obtained from spinach leaf ribulose-1,5-diphosphate carboxylase by pretreatment with p-mercuribenzoate at pH 7.5 followed by incubation at pH 9.0, was free of the smaller subunit based on C-terminal amino acid analyses. Valine was the predominant C-terminus of the A_m preparations, the release of tyrosine being negligibly small [cf. Sugiyama and Akazawa, Biochemistry 9 (1970) 4499]. The pH optimum of the ribulose-1,5-diphosphate carboxylase reaction by A_m was about 8.5, in comparison to the native enzyme which showed an alkaline pH optimum only in the absence of Mg^{2+}. The substrate saturation curve of the catalytic subunit with respect to bicarbonate followed the Michaelis-Menten equation, as contrasted to the anomalous reaction kinetics of the native ribulose-1,5-diphosphate carboxylase molecule reported previously. These overall results indicate that the allosteric properties of spinach ribulose-1,5-diphosphate carboxylase are possibly conveyed by a unique structural conformation that requires the presence of the smaller subunit in association with the larger catalytic subunit component of the enzyme molecule.

Our previous experiments demonstrated that ribulose-1,5-diphosphate (RuDP) carboxylase isolated from spinach leaf and Chromatium is composed of non-identical subunits, the intact molecule having the compositional formula, A_mB_n (1, 2, 3). Our current study showed that the spinach enzyme molecule can dissociate into eight multiple subunits designated as A_m, A_{m-1} $\cdots$ A_{m-7}, and mono- and dimeric forms of the small subunit (B_1 and B_2) upon initial treatment with p-mercuribenzoate (PMB) at pH 7.5 and subsequent incubation at pH 9.0 (4). It was found that the dissociated molecular species A_m retains the carboxylase activity in the absence of the smaller subunit upon incubation with excess β-mercaptoethanol which removes PMB, the activity was about 15%

* This is paper XXI in the series "Structure and Function of Chloroplast Proteins", and the work supported in part by research grants from the Toray Science Foundation (Tokyo) and the Ministry of Education of Japan (No. 758036).

of the native enzyme. The molecular weight determinations of the dissociated subunits revealed that probably eight pairs of the two kinds of subunits make up the symmetrical enzyme molecule represented as A_8B_8 (cf. 3, 5, 6). Evidence that the catalytically active larger subunit is not contaminated by the smaller subunit was based on the sodium dodecyl sulfate-polyacrylamide gel electrophoresis, by which the presence of contaminating small subunits was not detectable. The sensitivity limit of this particular technique, however, would not allow us to completely eliminate a possibility that the residual enzyme activity detected might be attributable to i) low levels of contaminated native enzyme or structurally altered native enzyme or ii) the presence of the smaller subunits as contaminants. That the A_m preparations are free of the smaller subunit was verified by C-terminal amino acids analysis, the results of which constitute this communication.

Materials and Methods

The experimental details of purifying RuDP carboxylase from spinach leaf and the chemical modification of SH-groups of the enzyme molecule with PMB for the complete dissociation into two types of subunits were reported previously (4). The basic procedure of preparing the catalytic subunit, A_m, is as follows: sufficient PMB (2.5×10^{-3}M) in 0.05 M Tris-HCl buffer (pH 7.5) was added to an enzyme preparation so that the molar ratio of PMB to SH-residues in the enzyme protein was 2; the mixture was allowed to stand at 25° for 30 min. The preparation was then passed through a small column of Sephadex G-25 (fine) previously equilibrated with 0.025 M Tris-HCl buffer (pH 9.0) to remove the excess mercurial; the eluate was incubated for 3 hr at 25°. The modified enzyme protein was then applied to a column of Sephadex G-200 preequilibrated with 0.025 M Tris-HCl buffer (pH 9.0). Aliquots of the fractions were assayed for the RuDP carboxylase activities. The enzymically active fractions were concentrated in a collodion membrane bag to approximately 0.2 mg/ml. The preparation was then passed through a column of Sephadex G-25, preequilibrated with 0.025 M Tris-HCl buffer at pH 7.5, and

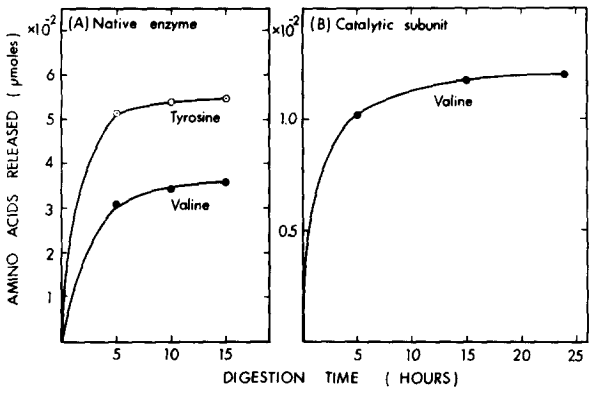

Fig. 1. Time course of carboxypeptidase hydrolysis of native RuDP carboxylase (A) and catalytic subunit A_m (B).

Basic experimental procedures were those previously described (1, 3). The reaction mixture of the proteolytic digestion contained 3.8 mg of native enzyme (about 0.06 μmole) or 0.8 mg of A_m preparations (about 0.02 μmole), 20 μmoles of N-ethylmorpholine acetate (pH 8.5), 3.6 μg (A) or 0.7 μg (B) of DFP-carboxypeptidase A dissolved in 10% (w/v) LiCl and 0.005 ml of toluene in a total volume of 1 ml. Incubation was carried out at 37°.

---

eluates incubated for 3 hr at 25° with a large excess of β-mercaptoethanol. The final preparation free of β-mercaptoethanol and PMB which was obtained by passage through a column of Sephadex G-25 (fine), previously equilibrated with 0.05 M Tris-HCl buffer at pH 8.5, was analysed for the C-terminal amino acids and its activity reassayed.

The methods of proteolytic digestion of intact native enzyme and purified A_m preparations using DFP-carboxypeptidase A (Worthington, Freehold, N. J.) were described previously (1, 3). The enzymic hydrolysis was stopped at various reaction intervals by heating the reaction mixture in a boiling water bath. After evaporating the hydrolysate to dryness, the sample was taken up with 0.2 M Na-citrate buffer (pH 2.2) and transferred to a Nihon Denshi Model JLC 5AH automatic amino acid analyzer equipped with a high sensitivity attachment and 10 mm light path cell.

The enzyme assay was carried out using the method reported by Murai and Akazawa (8). The compositions of the reaction mixture utilized are described in the respective figure legends. Following incubation the amount of

$^{14}CO_2$ fixed in aliquots of the reaction mixture was measured using a Packard Tri-Carb Model 3320 liquid scintillation counter.

Results and Discussion

The complete dissociation of the spinach leaf RuDP carboxylase molecule into two types of subunits was accomplished by the technique described in Materials and Methods. From the band patterns on the sodium dodecyl sulfate-polyacrylamide gel electrophoresis it was substantiated that each of large and small subunits comprise monomeric subunits A and B, respectively (4). The mean specific enzyme activity of the catalytic subunit A_m preparations was 36 mμmoles CO_2 fixed/mg protein/min (pH 8.5), which is about 12% of the intact native enzyme assayed at pH 7.0.

The native enzyme preparation was digested with DFP-carboxypeptidase A. The time-sequence analysis of C-terminal amino acids (Fig. 1, A) shows that both valine and tyrosine are released which is consistent with our previous experimental data based on the ^3H-labeling method and carboxypeptidase hydrolysis (1). The release of tyrosine, reaching approximately 0.9 equivalent per mol subunit B within 15 hour digestion, was faster than that of valine, reaching maximum at about 0.6 equivalent per mole subunit A. The reason for the incomplete release of the two C-termini is not clear, but it will be recalled that in our previous study the proteolytic digestion was carried out using the dissociated subunits prepared from the carboxymethylated enzyme samples. As less than a mg of the catalytic subunit preparation was available, the determination of the C-terminus of A_m was carried out using the amino acid analyzer with 10 mm light path cell and at the maximum sensitivity. The resolution was excellent and highly reproducible (Fig. 1, B), revealing that valine is the dominant amino acid released during a 24-hour digestion period. From the molecular weight of A_m, 4.0×10^5 (4), it was calculated that about 0.7 equivalent of valine is released. Under the experimental conditions presently employed, one can easily detect as little as 0.6 nmole tyrosine. However, a peak corres-

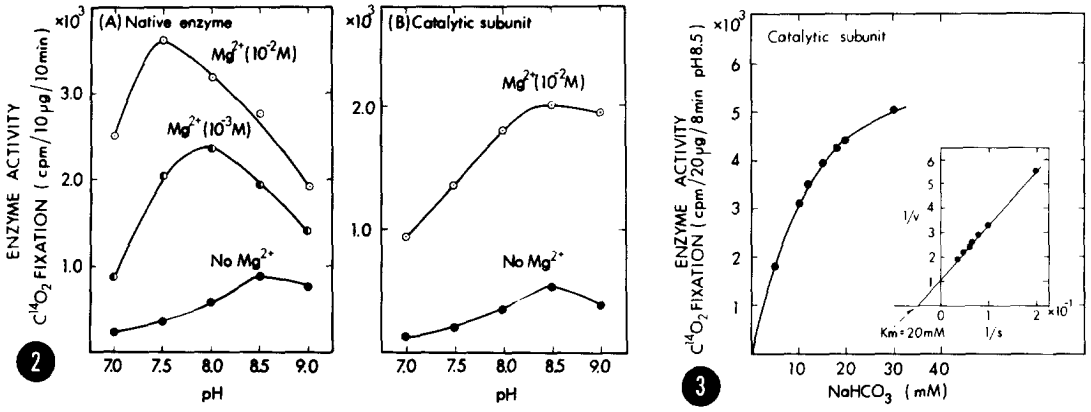

Fig. 2. Optimum pH of RuDP carboxylase reaction catalysed by native enzyme (A) and catalytic subunit A_m (B) with or without Mg^{2+}.

The optimal pH for native enzyme and enzymically active subunit fractions A_m separated by Sephadex G-200 gel filtration were determined in the presence or absence of Mg^{2+}. The reaction mixture contained the following components (μmoles): Tris-HCl of various pH, 100; RuDP, 0.7; $NaH^{14}CO_3$, 25 (2 μCi for A, and 20 μCi for B); $MgCl_2$, 5; and approximately 10-20 μg of the enzyme preparation in a total volume of 0.5 ml. Incubation was carried out at 25° for 10 min.

Fig. 3. Bicarbonate dependence of RuDP carboxylase reaction catalysed by catalytic subunit A_m.

The enzymically active subunit fractions A_m were separated by Sephadex G-200 gel filtration. The reaction mixture contained (μmoles): Tris-HCl (pH 8.5), 100; RuDP, 0.7; $NaH^{14}CO_3$ (25 mM, 20 μCi) at the indicated final concentrations; $MgCl_2$, 5; and 20 μg of enzyme protein in a total volume of 0.5 ml. The enzyme reaction was started by adding RuDP to the assay mixture which was preincubated at 25° for 10 min, and incubation continued for 8 min. The Km value of 20 mM was calculated from the double reciprocal plot analysis (inset).

---

ponding to tyrosine was found to be negligibly small, unable to estimate its content. We thus conclude that the A_m preparation free of the smaller subunit can exhibit the RuDP carboxylase activity in the absence of the smaller subunit.

It is known that RuDP carboxylase from spinach leaf (9, 10) as well as from Chlorella ellipsoidea (11) and Chromatium (7, 12) exhibits a shift in the optimum pH toward a neutral or acidic from an alkaline side upon the addition of Mg^{2+} to the assay mixture. As shown in Fig. 2, the pH optimum of the enzymically active fractions separated by Sephadex G-200 gel column in the presence (10^{-2}M) or absence of Mg^{2+} was about 8.5 (B), in comparison to the native enzyme which showed an alkaline pH optimum only in the absence

of Mg^{2+} (A). The absence of the shift in the optimum pH of the enzyme reaction by the catalytic subunit suggests a role for the smaller subunit is to modulate the Mg^{2+}-effect of the enzyme reaction. A similar phenomenon was observed in the alkaline dissociated RuDP carboxylase from Chromatium (7). The bicarbonate saturation curve of the A_m preparation was found to follow the Michaelis-Menten equation, K_m value calculated by the double reciprocal plot analysis being 20 mM (Fig. 3). This is sharply contrasted to the anomalous reaction kinetics of the intact RuDP carboxylase from either spinach or maize leaves with respect to bicarbonate (or CO_2) concentrations (8, 9, 10, 13, 14).

Our allied investigation (7) concerning the catalytic nature of the larger subunit, A_m, prepared from the photosynthetic bacterium, Chromatium, together with results here indicate that the allosteric properties of RuDP carboxylase are conveyed by a unique structural conformation that requires the presence of the smaller subunit.

Acknowledgment

The authors thank Tatsuo Sugiyama and Tetsuko Takabe for invaluable discussions in connection with this work and Dr. Kay Ryugo for his kind help during the preparation of the manuscript.

References

1. Sugiyama, T., and T. Akazawa Biochemistry, 9, 4499 (1970).
2. Akazawa, T., H. Kondo, T. Shimazue, M. Nishimura, and T. Sugiyama Biochemistry, 11, 1298 (1972).
3. Sugiyama, T., T. Ito, and T. Akazawa Biochemistry, 10, 3406 (1970).
4. Nishimura, M., T. Takabe, T. Sugiyama, and T. Akazawa J. Biochem. (Tokyo) in press (1973).
5. Kawashima, N., and S. G. Wildman Ann. Rev. Plant Physiol., 21, 325 (1970).
6. Siegel, M. I., M. Wishnick, and M. D. Lane In The Enzymes. Edited by P. D. Boyer, Academic Press, New York. Vol. 6, p. 169 (1972).
7. Takabe, T., and T. Akazawa Arch. Biochem. Biophys., 157, 303 (1973).
8. Murai, T., and T. Akazawa Biochem. Biophys. Res. Commun. 46, 2121 (1972).
9. Sugiyama, T., N. Nakayama, and T. Akazawa Biochem. Biophys. Res. Commun. 30, 118 (1968).
10. Sugiyama, T., N. Nakayama, and T. Akazawa Arch. Biochem. Biophys., 126 737 (1968).

11. Sugiyama, T., C. Matsumoto, T. Akazawa, and S. Miyachi Arch. Biochem. Biophys., 129, 597 (1969).
12. Akazawa, T., K. Sato, and T. Sugiyama Plant Cell Physiol., 11, 39 (1970).
13. Andrews, T. J., and M. D. Hatch Phytochemistry, 10, 9 (1971).
14. Buchanan, B. B., and P. Schurman Fed. Proc., 31, 461 Abs. (1972).