RECONSTITUTION OF SPINACH RIBULOSE-1,5-DIPHOSPHATE CARBOXYLASE FROM SEPARATED SUBUNITS*

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Summary: The addition of the smaller subunit (B) to the catalytic octamer of the larger subunit, A_8, of spinach ribulose-1,5-diphosphate carboxylase caused an appreciable stimulation of the enzymic activity, accompanied with the reconstitution of the original enzyme molecule. The reformation of the native enzyme was demonstrated by its immunochemical response to the rabbit antisera raised against larger (A) and smaller (B) subunits, whereas the catalytic oligomer only cross-reacted with the anti-[A] serum. The reconstituted enzyme exhibited the Mg^{2+}-dependent optimal pH-shift to a neutral side, supporting the notion concerning the regulatory role of the smaller subunit in the Mg^{2+}-effect in the enzyme catalysis.

Upon treatment with p-mercuribenzoate (PMB)** at alkaline pH (9.0), spinach leaf RuDP carboxylase (E.C. 4. 1. 1. 39) dissociates into two types of subunits, the multiple oligomeric forms of the large subunit A and the monomeric and dimeric forms of the small subunit B (1, 2). By the addition of excess thiol compounds, i.e. β-mercaptoethanol and DTT, octameric form of the larger subunit A preparation (A_8) was shown to retain the partial carboxylase activity in the absence of the smaller subunit (1, 3). Immunochemical evidence further supported the conclusion that the large subunit is the catalytic entity and the small subunit B is the regulatory subunit related to the Mg^{2+}-effect in the enzyme catalysis (4). Previously we reported a partial reconstitution of the original enzyme molecule from the separated constituent subunits, as evidenced by the polyacrylamide gel electrophoretic patterns

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** Abbreviations used: DTT, dithiothreitol; PMB, p-mercuribenzoate; RuDP, ribulose-1,5-diphosphate; SDS, sodium dodecyl sulfate.

(3). In this communication we report our further investigation seeking means for a clearer separation of subunits and stimulative effect of the small subunit on the enzyme activity conveyed by the catalytic oligomer. Results together with the immunochemical studies lend support the reconstitution of the enzyme molecule from two separated subunits.

Materials and Methods

Dissociation and reconstitution of RuDP carboxylase: Experimental procedures for separating two types of constituent subunits from the PMB-treated spinach RuDP carboxylase using Sephadex G-200 column chromatography were reported previously (1, 3). However, in these studies it was not possible to prevent the formation of higher aggregates of enzyme molecule, eluting ahead of the catalytic larger subunit [cf. Fig. 3 of (1); Fig. 5 of (2); Fig. 1 of (3)]. Therefore, in attempting to prevent structural deformation of separate subunits as much as possible, the stabilizing effect of several hydroxyl compounds was examined in the column chromatographic separation of subunits, and 10% glycerol was proved to be most effective. Total elution time of gel filtration on a column of Sepharose 4B, which was substituted for Sephadex G-200, preequilibrated with 0.025 M Tris-HCl buffer (pH 9.0) containing 10% glycerol, took about 5 hr. Aliquot of the column eluates were then subjected to (i) UV absorption at 280 nm, (ii) enzyme assay using standard assay mixture at pH 8.5 (10 min, 25°) (1), and (iii) SDS-polyacrylamide gel electrophoresis according to the method of Weber and Osborn (5). Excess of the enzymically inactive small subunit B fractions pooled in a separately run Sepharose 4B column was added to the catalytic subunit preparation and incubated for 3 hr at 25° in the presence of DTT (10 μmoles), and aliquots withdrawn were used for the determination of the carboxylase activity at pH 7.5 (10 min, 25°).

At the end of incubation, aliquots of the enzyme assay mixture, with or without subunit B treatment, were withdrawn and subjected to polyacrylamide gel electrophoresis at pH 8.9 following the method of Davis (6) and Hedrick

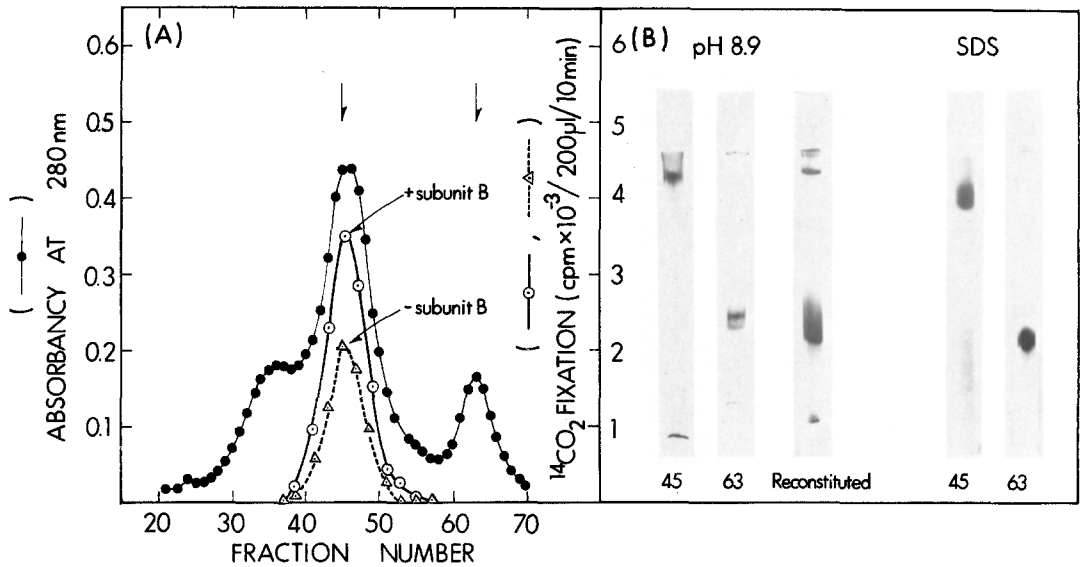

Fig. 1. Sepharose 4B gel filtration of subunits A and B of PMB-treated spinach RuDP carboxylase. Approximately 10 mg of a purified enzyme preparation which was treated by PMB as reported previously (1, 2) was placed on a column of Sepharose 4B (1.5 x 90 cm), preequilibrated with 0.025 M Tris-HCl buffer containing 10% glycerol (pH 9.0) and 2 ml fractions were collected. Two representative fractions as marked (#45,63) were subjected to polyacrylamide gel electrophoresis at pH 8.9 and SDS-polyacrylamide gel electrophoresis to characterize their molecular species. In order to examine the stimulative effect of the smaller subunit on the enzymic activity by the catalytic subunit, excess of subunit B (50 µg) collected in a separate Sepharose 4B column was added to 0.5 ml aliquots of fractions and incubated at 25° for 3 hr in the presence of RuDP (14 µmoles) and DTT (10 µmoles) in a total volume of 0.6 ml. 0.2 ml aliquots were withdrawn and used for enzyme assay in the standard assay system (pH 7.5 with subunit B and pH 8.5 without subunit B) and polyacryl amide gel electrophoresis at pH 8.9. The enzyme reaction was started by the addition of $NaH^{14}CO_3$ and incubation continued for 10 min.

and Smith (7) using different gel porosities. In the latter case one can estimate the molecular weight of the actual molecular species in the assay mixture (1).

Immunochemical methods: The experimental details for immunization of rabbits against the native enzyme molecule and constituent subunits were described in our preceding paper (4). The antisera raised against large and small subunits are referred as anti-[A] and anti-[B], respectively, and their specific cross-reactivity with respective antigenic molecules was confirmed.

The double immunodiffusion on an agar plate was carried out according to Ouchterlony (8), by burying into agar plates the electrophoresed gels of the assay mixture containing the catalytic subunit preparation with or without the small subunit B treatment. The precipitin lines produced with each of anti-[A] and anti-[B] after 48 hr incubation at 25° were stained with amido black 10B.

Results and Discussion

A clear separation of the catalytically active larger and inactive smaller subunits on a Sepharose 4B column chromatography of the PMB-treated enzyme sample as explained in Materials and Methods is presented in Fig. 1, A. It will be noted that the quantity of higher aggregates of the native enzyme eluting ahead of the catalytically active fraction was much smaller than that encountered in our previous investigations. Simultaneous examination of representative fractions by the gel electrophoresis show the absence of cross-contamination in two major fractions (Fig. 1, B). The curve of the residual enzyme activities assayed at pH 8.5 perfectly coincided with the fast-eluting protein fractions consisting of the larger subunit A; the specific enzyme activity (0.075 μmole CO_2/mg protein/10 min) being approximately 15% that of native enzyme determined at pH 7.0. This relative activity was consistent with our previous data (1, 3). The molecular weight of the catalytic subunit preparation was estimated to be 4.05×10^5 according to the method of Hedrick and Smith (7) (data not shown). As the molecular weight of the monomeric form of the large subunit A is 5.4×10^4 (1), we can reasonably conclude that the enzyme catalysis is conveyed by an octameric form of the large subunit, A_8. The addition of the excess subunit B preparation to the catalytic oligomer caused a significant increase (approximately 70%) of the enzyme activity, in a concomitant reformation of the native enzyme molecule, which was supported by the molecular weight determination (5.3×10^5) of the single banded molecular species in the assay mixture.

The second proof for the reconstitution of the native enzyme molecule

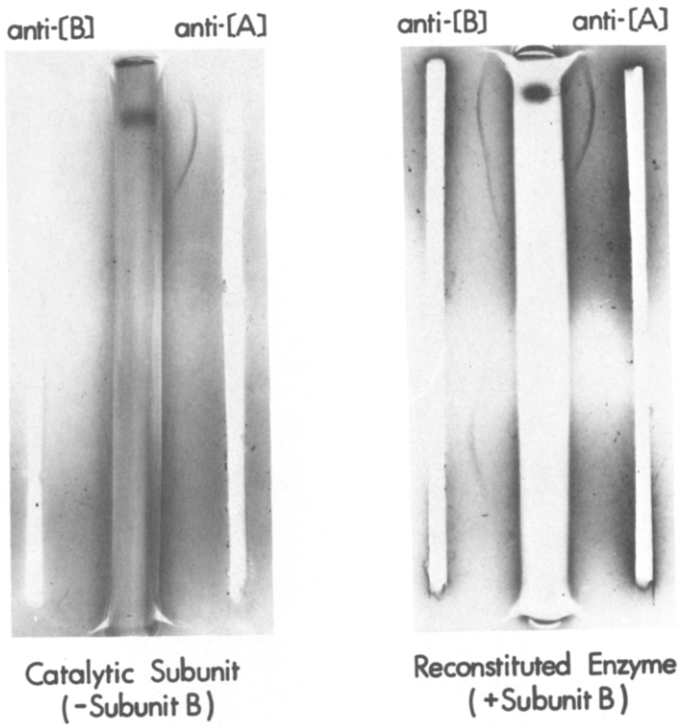

Fig. 2. Double immunodiffusion of catalytic subunit and reconstituted enzyme molecule against rabbit antisera to larger and smaller subunits. Catalytic subunit without subunit B treatment and reconstituted preparation after subunit B treatment (#45) were subjected to polyacrylamide gel electrophoresis at pH 8.9 as shown in Fig. 1, B. At the end of the electrophoretic run, the gels were buried into agar plate (1% Difco special agar noble containing 0.1% NaN_3). Afterwards, γ-globulin fraction of the respective rabbit-antiserum against each subunit was placed in each well (ca. 0.3 mg anti-[A] and ca. 0.4 mg anti-[B]) and immunoprecipitin lines were observed after 48 hr incubation at 25°.

came from results of the immunochemical studies. After the electrophoresis gel samples of the reaction mixture containing the catalytic oligomer, with or without subunit B treatment, were subjected to immunodiffusion experiment. As shown in Fig. 2, the catalytic subunit preparation only cross-reacted with anti-[A], whereas the enzyme molecules after reconstitution cross-reacted with both anti-[A] and anti[B] sera.

Our current studies have shown that in both spinach leaf (2) and Chromatium (9) RuDP carboxylases, the enzyme reaction catalyzed by the catalytic subunit does not exhibit the optimal pH-shift in response to the addition

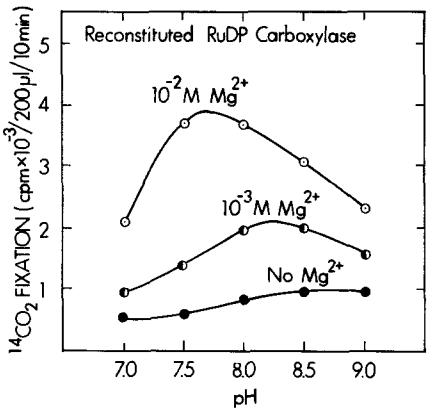

Fig. 3. Mg^{2+}-dependent optimal pH-shift of the reconstituted enzyme molecule. The reconstituted enzyme sample (#45) prepared as described in Fig. 1, was subjected for RuDP carboxylase activity measurement at different pH's using 1.0 M Tris-HCl buffer, in the presence (10^{-3}M and 10^{-2}M) and absence of Mg^{2+} (1).

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of Mg^{2+}. The regulatory role of the small subunit in relation to the Mg^{2+}-effect was further supported by the immunochemical studies (3), showing the absence of Mg^{2+}-induced optimal pH-shift in the anti-[B]-treated spinach enzyme. We tested the effect of Mg^{2+} on the optimum pH of the carboxylase reaction catalyzed by the reconstituted enzyme sample. Results of Fig. 3 clearly show that in the presence of 10^{-2} M Mg^{2+} the optimum pH was at pH 7.5, whereas it resided in an alkaline side in the absence of Mg^{2+}. This behavior is identical with that observed by the native enzyme molecule (10).

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