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Isolation and Preliminary Characterization of Two Forms of Ribulose 1,5-Bisphosphate Carboxylase from *Rhodopseudomonas capsulata*

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The presence of two distinct forms of ribulose 1,5-bisphosphate carboxylase has been demonstrated in extracts of *Rhodopseudomonas capsulata*, similar to the form I (peak I) and form II (peak II) carboxylases previously described from *R. sphaeroides* (J. Gibson and F. R. Tabita, J. Biol. Chem. **252**:943-949, 1977). The two activities, separated by diethylaminoethyl-cellulose chromatography, were shown to be of different molecular size after assay on polyacrylamide gels. The higher-molecular-weight carboxylase from *R. capsulata* was designated form I-C, whereas the smaller enzyme was designated form II-C. Catalytic studies revealed significant differences between the two enzymes in response to pH and the effector 6-phosphogluconate. Immunological studies with antisera directed against the carboxylases from *R. sphaeroides* demonstrated antigenic differences between the two *R. capsulata* enzymes; cross-reactivity was observed only between *R. sphaeroides* anti-form II serum and the corresponding *R. capsulata* enzyme, form II-C.

Ribulose 1,5-bisphosphate (Rbu-P_2) carboxylase (3-phospho-D-glycerate carboxylase [dimerizing], EC 4.1.1.39) is the primary catalyst of CO_2 fixation in photosynthetic and chemosynthetic organisms. Distinct differences in the molecular weight of this enzyme from various sources led to its categorization by size into three classes: large ($M_r \geq 450{,}000$), intermediate ($M_r \simeq 360{,}000$), and small ($M_r \simeq 120{,}000$) (1, 9, 11). The large carboxylases are composed of eight large subunits ($M_r \simeq 55{,}000$) active in catalysis (11, 17, 23, 24) and, in many cases, eight small subunits (M_r = 12,000 to 20,000), polypeptides that may play a regulatory role in catalysis (25). This structurally complex two-subunit-type carboxylase, with a native molecular weight of approximately 550,000, has been found in all eucaryotic phototrophic organisms thus far examined, as well as in numerous procaryotes (2, 6, 9, 11, 16, 21, 26). Rbu-P_2 carboxylases of the intermediate and small type from certain procaryotes contain multiples of large subunits only (6, 10, 20, 22).

We previously reported the isolation of two structurally, catalytically, and immunologically distinct forms of Rbu-P_2 carboxylase from *Rhodopseudomonas sphaeroides* (6). Form I, similar to the carboxylase found in eucaryotes, has a molecular weight of 550,000 and contains the two types of subunits, catalytic and regulatory. Form II is a protein with a molecular weight of approximately 360,000 and is composed only of large subunits. The two forms of Rbu-P_2 carboxylase, isolated from extracts of the same organism, present an interesting and unusual opportunity to study how function is related to structure (6). It was thus of interest to examine the possibility that other members of the *Rhodospirillaceae*, in addition to *R. sphaeroides*, might also possess distinct forms of the enzyme. This investigation reports on the finding of two Rbu-P_2 carboxylase activities in extracts of *R. capsulata*.

MATERIALS AND METHODS

Materials. The following special reagents were commercial preparations: dithiothreitol, triethanolamine, tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid, and 6-phosphogluconate were from Sigma Chemical Co., St. Louis, Mo.; $Na_2{}^{14}CO_3$ (20 mCi/mmol) was from Amersham/Searle, Arlington Heights, Ill.; and diethylaminoethyl (DEAE)-cellulose was from Bio-Rad Laboratories, Richmond, Calif. Tetrasodium ribulose 1,5-bisphosphate was prepared by the procedure of Horecker et al. (7) as modified by Whitman and Tabita (28). Acrylamide (Eastman Kodak Co., Rochester, N.Y.) was recrystallized from chloroform.

Culture and growth conditions. A culture of *R. capsulata* B10, originally isolated by Barry Marrs, was kindly provided by Judy Wall. Cells were grown photoheterotrophically in the synthetic medium of Ormerod et al. (14) with 0.2% butyric acid instead of malate as the electron donor, essentially as previously described (6). The washed cell paste was frozen at −20°C.

Enzyme purification. The partial purification of

Rbu-P_2 carboxylase from *R. capsulata* was based on the method described previously for *R. sphaeroides* (6). Frozen cells (15 g) of *R. capsulata* were suspended in an equal volume of buffer A [0.025 M tris(hydroxymethyl)aminomethane-chloride (pH 7.5) containing 1 mM ethylenediaminetetraacetate and 5 mM 2-mercaptoethanol] and passed twice through a French pressure cell at 15,000 lb/in^2. Unbroken cells and debris were removed by centrifugation at 16,300 $\times g$ for 15 min, followed by a high-speed centrifugation at 100,000 $\times g$ for 1 h. The high-speed supernatant fraction was subjected to a magnesium-heat treatment and streptomycin sulfate treatment (6, 19) to remove much of the contaminating protein and nucleic acids. After dialysis against buffer A, the extract was applied to a DEAE-cellulose column (2.5 by 20 cm) equilibrated with buffer A. After passage of 300 ml of this buffer through the column, followed by 300 ml of 0.1 M NaCl in buffer A, a linear gradient of 0.1 to 0.3 M NaCl (in buffer A) was started, resulting in the elution of two peaks of carboxylase activity (Fig. 1). Selected active fractions within each peak were pooled separately and concentrated by addition of solid ammonium sulfate to 75% saturation. After standing for 30 min in an ice bath, the suspensions were centrifuged at 27,000 $\times g$ for 15 min. The pellets were resuspended in a small amount of buffer A and dialyzed against the same buffer overnight. About 12% of the activity initially loaded on the column was recovered after elution and concentration by ammonium sulfate precipitation. The specific activity of the first activity peak (form II-C) was 0.21; the second activity peak (form I-C), after rechromatography on a small (2 by 7 cm) DEAE-cellulose column as before to remove residual form II-C, had a specific activity at this stage of 0.04. Both *R. capsulata* enzymes were unstable to prolonged storage and purification manipulation, in contrast to similar enzyme preparations obtained from *R. sphaeroides* (6). These *R. capsulata* preparations were used for further study.

Rbu-P_2 carboxylase assay. The assay for Rbu-P_2 carboxylase was as previously described (6, 12). Protein determinations were by the method of Lowry et al. (8), with crystalline bovine serum albumin used as the standard. Specific activity is given in units per milligram of protein, where 1 unit is defined as the amount of enzyme needed to carboxylate 1 μmol of ribulose 1,5-bisphosphate in 1 min at 30°C.

Polyacrylamide gel assay for Rbu-P_2 carboxylase. Samples to be analyzed were subjected to electrophoresis on triethanolamine-tris(hydroxymethyl) methyl-2-aminoethane sulfonic acid gels (15). After electrophoresis, the gels were cut into 2-mm slices. Each slice was placed in a tube containing the constituents of the carboxylase assay except Rbu-P_2, which was used to initiate the reaction at time zero. At 30 min after initiation, the assay was terminated by the addition of 99% propionic acid, and the acid-stable radioactivity was determined by liquid scintillation spectrometry.

Immunological studies. Antisera to form I and form II Rbu-P_2 carboxylases from *R. sphaeroides* were prepared as previously described (5, 6). Immunodiffusion was performed in 1% agarose containing 50 mM tris(hydroxymethyl)aminomethane-chloride (pH 7.5). Titration of enzyme activity was conducted by preincubating enzyme with portions of control (preimmune) serum or antiserum for 30 min at 30°C before assay.

RESULTS

Enzyme purification. Use of butyric acid as an electron donor resulted in growth of *R. capsulata* with higher levels of Rbu-P_2 carboxylase than with malate, the usual growth substrate, as previously demonstrated with *Rhodospiril-*

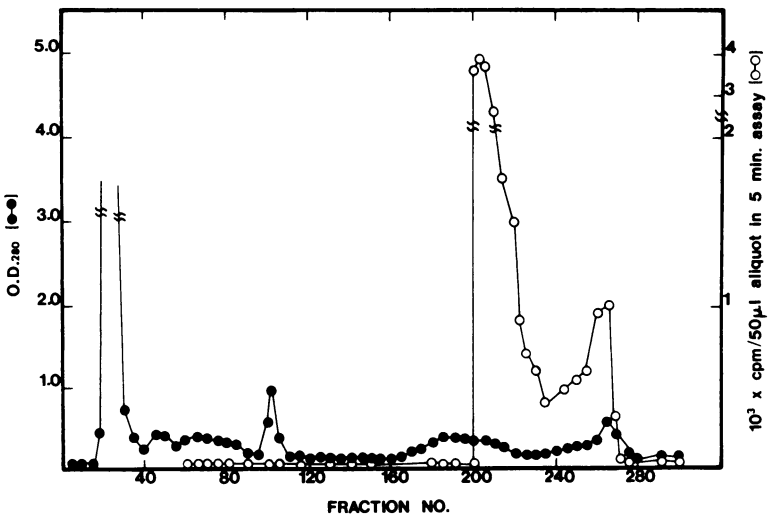

FIG. 1. *DEAE-cellulose chromatography of Rbu-P_2 carboxylase from R. capsulata.* (●) *Optical density at 280 nm (O.D. 280). Rbu-P_2 carboxylase activity* (○) *is expressed as counts per minute of [^{14}C]bicarbonate ($^{14}CO_2$) fixed during a 5-min assay per 50-μl portion from each fraction in the standard assay.*

lum rubrum (19) and *R. sphaeroides* (6). In this study, we used this observation to obtain relatively high yields of cells with workable levels of Rbu-P_2 carboxylase. The specific activity in crude 100,000 × *g* supernatants of *R. capsulata* was 0.07.

DEAE-cellulose chromatography resulted in the elution of two distinct peaks of Rbu-P_2 carboxylase activity from extracts of *R. capsulata* (Fig. 1). It was of interest to determine whether or not these two activities exhibited properties similar to those of form I and form II Rbu-P_2 carboxylases isolated from *R. sphaeroides*. A polyacrylamide gel carboxylase assay was employed to locate enzymatic activity on polyacrylamide gels. Figure 2 illustrates results obtained when crude extract and the two separated activities from the ion-exchange column were subjected to electrophoresis and subsequently sliced and assayed for carboxylase activity. The crude extract exhibited two activity peaks corresponding almost exactly to the relative migrations of form I and form II carboxylases of *R. sphaeroides*. However, the order of elution from the cellulose column was opposite to that obtained with extracts of *R. sphaeroides* (6). Thus, as shown by the polyacrylamide gel activity profiles of the DEAE-cellulose column fractions, a sample of the first carboxylase activity peak eluted from the cellulose column migrated further down the polyacrylamide gel than the second carboxylase activity peak (Fig. 2). For convenience of description, the higher-molecular-weight, slower-migrating carboxylase of *R. capsulata* was designated form I-C and the low-molecular-weight protein was designated form II-C, corresponding to the enzymes of *R. sphaeroides* (6). The verification of the relative molecular weights of the *R. capsulata* preparations was obtained after electrophoresis of each enzyme on gels of different acrylamide concentration. The migration of form I-C and form II-C was then compared with that of the corresponding *R. sphaeroides* enzymes (6). For each related enzyme, the slopes obtained after plotting the percent gel concentration against the relative electrophoretic migration were similar, corroborating the estimated molecular weights of the *R. capsulata* proteins.

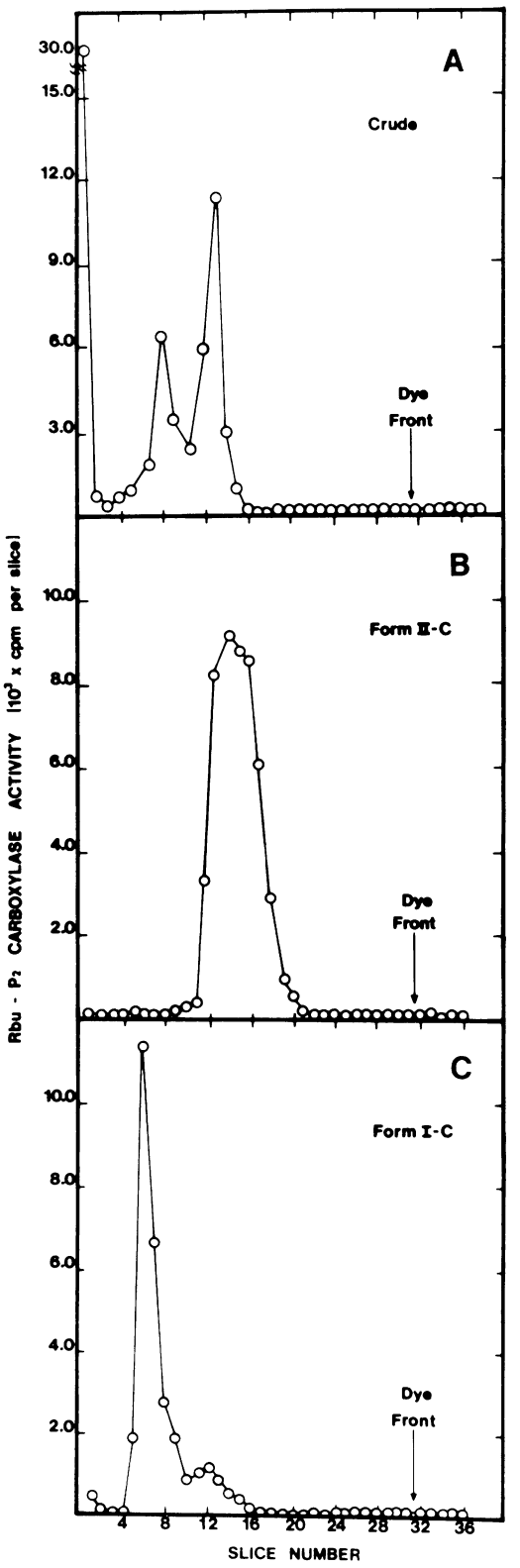

FIG. 2. *Assay of R. capsulata Rbu-P_2 carboxylase in polyacrylamide gels. Samples were applied to 7.5% acrylamide gels in a 10-μl volume containing 5% sucrose. (A) High-speed (100,000 × g) supernatant fraction (62 μg of protein); (B) 75% ammonium sulfate precipitate fraction (30 μg of protein) of first active DEAE-cellulose peak (form II-C); and (C) 75% ammonium sulfate precipitate fraction (40 μg of protein) of second DEAE-cellulose peak (form I-C).*

Catalytic studies. To further characterize the two enzymes from *R. capsulata*, various catalytic properties were investigated. Results obtained when carboxylase activity was determined at pH 7.2 and 8.0 for the enzymes from *R. capsulata* and *R. sphaeroides* showed form II and form II-C to be similar in their response to pH, exhibiting at pH 8.0 only 50 to 60% of the activity observed at pH 7.2. By contrast, the activities of form I and form I-C were not affected as dramatically as forms II and II-C, their activity being maximized at pH 8.0.

It has been shown that 6-phosphogluconate inhibits form I *R. sphaeroides* carboxylase activity; virtually no effect, however, is seen on form II (6). Titration of the activity of the two enzymes of *R. capsulata* with this metabolite yielded similar results (Fig. 3). Form I-C was inhibited significantly, to about the same extent as *R. sphaeroides* form I carboxylase (5), whereas Form II-C was relatively insensitive to this effector.

Immunological studies. Previous studies with antibodies directed against form I carboxylase from *R. sphaeroides* had shown no cross-reactivity with form II, nor did this antiserum inhibit form II enzymatic activity (6). More recently, antibodies directed against form II were obtained; again no cross-reactivity could be demonstrated with form I, nor did this antiserum inhibit form I activity (5). Since the form I enzyme appears to resemble form I-C of *R. capsulata*, and the form II enzyme resembles form II-C, the effect of the *R. sphaeroides* carboxylase antisera was investigated with regard to the enzymes from *R. capsulata*. The Ouchterlony double-diffusion technique revealed no precipitin band when anti-form I serum was reacted against crude extracts of *R. capsulata* or the partially purified form I-C and form II-C *R. capsulata* carboxylases (Fig. 4). A line of

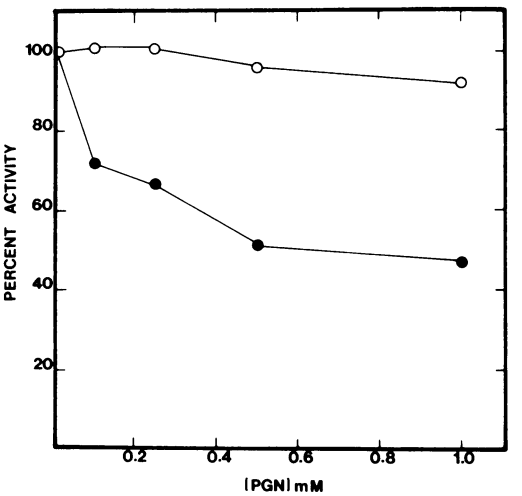

FIG. 3. *Effect of 6-phosphogluconate (PGN) on form I-C and form II-C Rbu-P_2 carboxylase from R. capsulata. Enzymes were preincubated for 5 min at 30°C in the presence of Mg^{2+}, $H^{14}CO_3^-$, and 6-phosphogluconate in 0.064 M tris(hydroxymethyl)aminomethane-chloride (pH 7.2) before initiation of the reaction with Rbu-P_2. The reaction was terminated after 5 min. Symbols: ●, form I-C; ○, form II-C.*

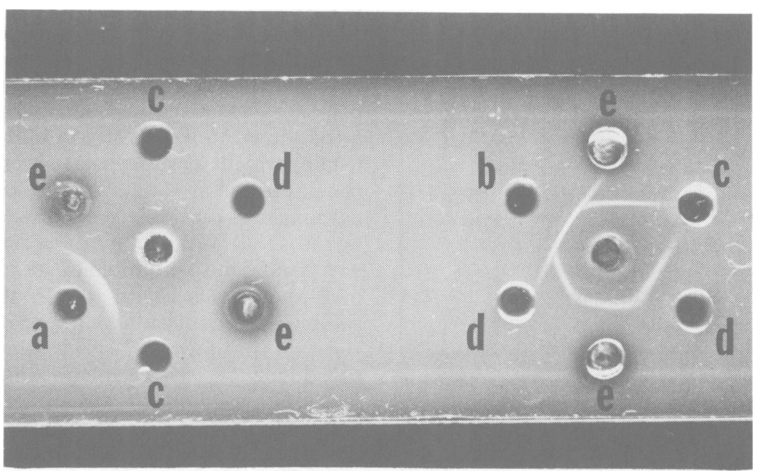

FIG. 4. *Ouchterlony double-diffusion analysis of form I-C and II-C R. capsulata Rbu-P_2 carboxylases. The left center well contained 10 μl of antiserum to R. sphaeroides form I carboxylase, and the right center well contained 10 μl of antiserum to R. sphaeroides form II carboxylase. Other wells contained: (a) R. sphaeroides form I carboxylase (30 μg); (b) R. sphaeroides form II carboxylase (7.5 μg); (c) R. capsulata form I-C carboxylase (25 μg); (d) R. capsulata form II-C carboxylase (30 μg); and (e) R. capsulata crude extract (200 μg).*

partial identity was observed when anti-form II serum was reacted against crude extract and the form II-C preparation, and a clear spur was observed when it was reacted against purified form II from *R. sphaeroides*. No reaction was evident towards the form I-C preparation (Fig. 4). The effect of both antisera on the activity of the two *R. capsulata* enzymes was also examined. Anti-form I serum inhibited neither enzyme (Fig. 5). Anti-form II serum inhibited the form II-C enzyme but had no effect on the form I-C enzyme (Fig. 5), consistent with results obtained in the double-diffusion experiment (Fig. 4).

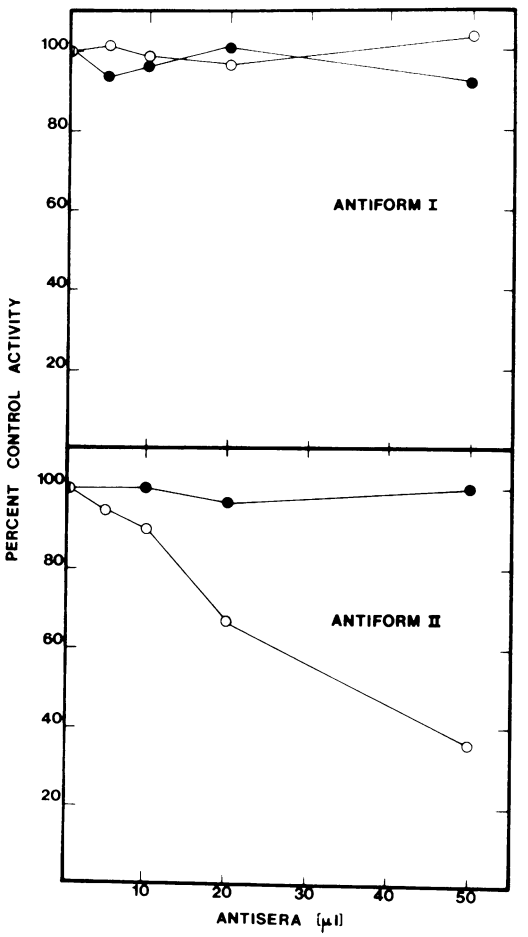

FIG. 5. *Antibody titration of form I-C and form II-C R. capsulata Rbu-P_2 carboxylase activity. Samples of antisera directed against form I and form II carboxylases of R. sphaeroides were incubated with form I-C (●) and form II-C (○) for 30 min at 30°C (pH 7.2) and subsequently assayed as described in the text. Percent activity was based on the activity obtained with equivalent amounts of control (preimmune) sera.*

Thus, from the electrophoretic, catalytic, and immunological studies, it is apparent that form I *R. sphaeroides* Rbu-P_2 carboxylase is similar to the form I-C *R. capsulata* enzyme, whereas the form II *R. sphaeroides* carboxylase resembles the form II-C enzyme from *R. capsulata*.

DISCUSSION

Our previous study demonstrated the presence of two forms of Rbu-P_2 carboxylase in extracts of *R. sphaeroides* (6). The same procedure developed for separating the two enzyme species proved effective in showing the presence of two forms of carboxylase in extracts of *R. capsulata*. By the polyacrylamide gel Rbu-P_2 carboxylase assay developed in this laboratory, we found that the two major peaks of activity from DEAE-cellulose columns comigrated with form I and form II Rbu-P_2 carboxylases from *R. sphaeroides*. The first peak of activity eluted from the cellulose column proved to be the intermediate-size carboxylase (form II-C), whereas the large-molecular-weight, slower-migrating carboxylase (form I-C) eluted shortly thereafter. This order of elution is opposite to that found with *R. sphaeroides* (6), and probably indicates different exposed charged groups on the respective proteins of the two organisms.

A comparison of the catalytic properties of the Rbu-P_2 carboxylases of *R. capsulata* and *R. sphaeroides* revealed marked similarities. The intermediate-size enzymes from both organisms exhibited significantly higher activity at pH 7.2 than at pH 8.0, the activity being almost twofold greater in both cases. The response to pH by the large enzymes was not so dramatic, similar to previous results (6); the activity of form I-C was essentially the same at both pH's, whereas that of form I was slightly greater at pH 8.0. The effector, 6-phosphogluconate, has been shown to selectively inhibit the large-molecular-weight carboxylases (3, 4, 18), whereas the intermediate- and small-molecular-weight carboxylases are relatively insensitive to this ligand (18). With *R. sphaeroides*, only form I was significantly inhibited by low concentrations of 6-phosphogluconate (6). In this study, our findings are consistent with previous observations, in that form I-C was inhibited by 6-phosphogluconate, whereas the intermediate-size form II-C was not affected.

Immunological studies revealed cross-reactivity between anti-form II serum and form II-C only. This could be demonstrated by double-diffusion experiments and the more sensitive enzymatic assay. Of interest is the fact that no cross-reactivity was observed between anti-form I serum and form I-C. Certainly, the altered order of elution of the *R. capsulata* form I-C

protein upon DEAE-cellulose chromatography may be indicative of differences in charge, a result consistent with the lack of immunological cross-reactivity towards antiserum to *R. sphaeroides* form I carboxylase.

The demonstration of two forms of Rbu-P_2 carboxylase in extracts of *R. capsulata* has important implications. First, the previous demonstration of two molecular forms of carboxylase in *R. sphaeroides* has an obvious parallel in *R. capsulata*, although there are significant immunological and chemical differences between the form I and form I-C proteins. Secondly, a functional genetic system in *R. capsulata* now exists (13, 27); such a development may be useful in determining whether or not the two Rbu-P_2 carboxylases (i.e., the catalytic subunits of these proteins) produced by both *R. sphaeroides* and *R. capsulata* are actually distinct gene products.

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