Localization and Characterization of Calvin Cycle Enzymes in *Chromatium* Strain D*

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ABSTRACT: The three enzymes, ribose 5-phosphate isomerase, phosphoribulokinase, and ribulose 1,5-diphosphate carboxylase, which function sequentially in CO_2 fixation *via* the Calvin cycle, are all released as soluble proteins from autotrophically grown cells of *Chromatium*. Breakage of cells by different methods, which resulted in differing degrees of membrane fragmentation, gave extracts in which the ratio of the activities was roughly constant. High-speed centrifugation of extracts showed no correlation between the sedimentation of the enzymes and of bacteriochlorophyll-containing material. The three enzymes were found to migrate separately in sorbitol density gradients; little overlap of activities occurred. Molecular weights of the enzymes were calculated to be approximately 500,000 for the carboxylase, 240,000 for the kinase, and 54,000 for the isomerase. Better than 90% recovery of carboxylase and of isomerase was obtained on the gradients, while recovery of the kinase was 45–55%. Complete separation of the isomerase from carboxylase and kinase was obtained on Sephadex G-200. These data indicate that the enzymes responsible for autotrophic CO_2 fixation in *Chromatium* exist as separate entities in cell extracts.

Functional association is not detectable by the methods employed.

The existence of soluble multienzyme systems has been demonstrated in only a few instances, such as the α-keto acid decarboxylases from animal and bacterial origins (Jagannathan and Schweet, 1952; Koike *et al.*, 1960) and the fatty acid synthetase complex in yeast (Lynen, 1961), although associations of other groups of enzymes catalyzing sequential reactions may also occur. In autotrophic carbon dioxide fixation by the Calvin cycle, the sequence of reactions between ribose 5-phosphate and 3-phosphoglyceric acid is catalyzed by three enzymes, ribose 5-phosphate isomerase (D-ribose 5-phosphate:ketol isomerase, EC 5.3.1.6), phosphoribulokinase (ATP:D-ribulose 5-phosphate 1-phosphotransferase, EC 2.7.1.19), and ribulose diphosphate carboxylase (3-phospho-D-glycerate carboxylase (dimerizing), EC 4.1.1.39). The last two serve no other function in the cell. Early findings that "fraction I" protein from plant sources contained R-5P[1] isomerase, as well as kinase (Ru-5P kinase) and carboxylase (RuDP carboxylase; Mendiola and Akazawa, 1964), suggested that these enzymes might be associated in a large unit. However, Trown (1965) subsequently demonstrated that fraction I protein from spinach could be purified by ammonium sulfate fractionation and passage through Sephadex G-200 to yield material having only RuDP carboxylase activity. If, therefore, specific rather than fortuitous association of the three enzymes occurs in plant material, it is relatively easily disrupted by mild procedures. Recently, functional association has been suggested for Ru-5P kinase and RuDP carboxylase in *Thiobacillus thioparus* and *Thiobacillus neapolitanus* (MacElroy *et al.*, 1968) since these two activities behave identically in density gradient centrifugation experiments.

During work on the purification of Ru-5P kinase from the purple sulfur bacterium *Chromatium*, we observed that R-5P isomerase accompanied Ru-5P kinase through a number of purification steps. This observation raised again the possibility of association between these enzymes, and we have therefore examined the behavior of extracted R-5P isomerase, Ru-5P kinase, and RuDP carboxylase in sorbitol density gradients and on columns of Sephadex G-200. Crude cell-free extracts have been prepared by several methods, calculated to produce differing degrees of disorganization of cellular contents. The results give no indication of any association of the three enzymes with each other or with the membranous "chromatophore" fraction of extracts prepared from this organism.

Materials and Methods

Chromatium strain D was grown in an autotrophic medium of the following composition: NaCl, 0.5%; KH_2PO_4, 0.1%; NH_4Cl, 0.1%; $MgCl_2 \cdot 6H_2O$, 0.05%; $CaCl_2$, 0.001%; trace element solution (Pfennig and Lippert, 1966), 1% v/v; $NaHCO_3$, 0.4%; $Na_2S_2O_3$, 0.3%; $Na_2S \cdot 9H_2O$, 0.05%; and final pH, 7.8. The cultures were grown in 10-l. bottles illuminated with 100-W incandescent bulbs at 30–34° and harvested when sulfur granules could no longer be seen in the cells. The cell pellet was washed once with medium lacking bicarbonate and sulfur compounds, and finally suspended in triethanolamine buffer (pH 7.8, 0.05 M), containing 10 mM $MgCl_2$.

Cell Breakage. LYSOZYME–FREEZE-THAW. The method of Kohler and Davis (1966) was modified as follows. NaCl and egg-white lysozyme were added to final concentrations of 0.4% and 1 mg/ml, respectively, and the suspension was frozen rapidly

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[1] Abbreviations used are: R-5P, ribose 5-phosphate; Ru-5P, ribulose 5-phosphate; RuDP, ribulose 1,5-diphosphate.

TABLE I: Effect of Cell Breakage Method on Enzyme Activity.[a]

	Protein (mg/ml)	Enzyme Activity					
		R-5P Isomerase		Ru-5P Kinase		RuDP Carboxylase	
		U/ml	Sp Act.	U/ml	Sp Act.	U/ml	Sp Act.
Lysozyme freeze-thaw	11.25	7,220	642	4,840	430	370	33.8
French pressure cell	60	19,300	322	17,800	297	1,145	23
Sonication	51	19,300	378	17,800	350	1,100	18.3

[a] Freshly harvested cells were suspended in two volumes of 0.05 M triethanolamine–10 mM $MgCl_2$ (pH 7.8). Activities are expressed in enzyme units as defined in the Materials and Methods section.

in a methanol–Dry-Ice bath; it was then allowed to thaw slowly in ice water. From three to six freeze-thaw cycles were required for cell breakage. Deoxyribonuclease and ribonuclease were added to a final concentration of 5 μg/ml each, and the preparation was incubated for 15 min at 30°.

FRENCH PRESSURE CELL. The suspension was passed once through the chilled cell at 9000 psi and treated with nucleases as above.

SONICATION. The suspension was sonicated for a total of 2 min, while the temperature was maintained at 4°. It was then treated with deoxyribonuclease and ribonuclease. The broken cell suspensions were centrifuged for 30 min at 36,700*g* in the cold to remove unbroken cells and large debris.

Enzyme Assays. RIBOSE 5-PHOSPHATE ISOMERASE. A modification of the cysteine–carbazole method (Axelrod and Jang, 1954) was used, in which the reaction mixture had the following composition: triethanolamine, pH 7.8, 50 mM; GSH, 5 mM; R-5P, 5 mM; 0.05 ml of enzyme solution; and total volume, 0.5 ml. After 10 min at 37°, the reaction was stopped by immersing the tubes in a boiling-water bath for 1.5 min. Ru-5P present in 0.3 ml of the reaction mixture was determined by adding 6 ml of H_2SO_4 (225 ml of concentrated H_2SO_4 mixed with 95 ml of water), 0.2 ml of 1.5% cysteine hydrochloride, and 0.2 ml of 0.12% carbazole in absolute ethanol. The color which developed in 30 min at 37° was measured in a Zeiss PMQ II spectrophotometer at 540 mμ. A standard curve was prepared using a solution of fructose, and the enzyme unit defined as the quantity of enzyme forming 1 nmole of ketose/min at 37°.

PHOSPHORIBULOKINASE activity was measured using the coupled reaction described by Racker (1957). The reaction was followed in a Gilford 2000 spectrophotometer. The cuvets contained the following reaction mixture: triethanolamine, pH 7.8, 110 mM; KCl, 40 mM; $MgCl_2$, 10 mM; ATP, 2 mM; GSH, 5 mM; NADH, 0.128 mM; R-5P, 5.0 mM; and excess purified spinach R-5P isomerase, pyruvate kinase, and lactic dehydrogenase, in a final volume of 1 ml. After 5-min equilibration at 37°, the reaction was started by addition of the Ru-5P kinase solution to be tested. The enzyme unit was defined as the quantity giving an NADH oxidation rate of 1 nmole/min at 37°.

RIBULOSE 1,5-DIPHOSPHATE CARBOXYLASE was measured by the rate of incorporation of $^{14}CO_2$ into acid-stable form in the following reaction mixture: Tris buffer, pH 7.5, 60 mM; $MgCl_2$, 60 mM; GSH, 1.2 mM; RuDP, 0.6 mM; and $NaHCO_3$, containing 0.1–0.5 μCi of ^{14}C, 20 mM, in a total volume of 0.25 ml at 29°. The reaction was started by carboxylase addition, and 0.04-ml samples were withdrawn into scintillation vials containing 0.05 ml of 6 N acetic acid after 2, 4, and 6 min. A similar sample was also taken into 0.05 ml of 5 N NH_4OH. The vials were allowed to stand at room temperature for at least 2 hr before addition of scintillation fluid (Patterson and Green, 1965) and counting in a Packard Tri-Carb liquid scintillation counter. The rate of CO_2 fixation was linear over the 6-min period, and the inclusion of the alkaline sample allowed calculation of the proportion of CO_2 fixed. The enzyme unit was defined as the quantity catalyzing the fixation of 1 nmole of CO_2/min at 29°.

Catalase was assayed by the titrimetric method described by Herbert (1954).

Spinach R-5P isomerase was prepared as described by Hurwitz *et al.* (1956). Beef liver catalase, pyruvate kinase, and lactic dehydrogenase were obtained from Sigma Chemical Co.; $Na_2{}^{14}CO_3$ was obtained from New England Nuclear Co.; R-5P, ATP, NADH, dithioerythritol, Sephadex G-200, and one sample of RuDP were also obtained from Sigma; the latter compound was also prepared from R-5P using a partially purified enzyme preparation from spinach (Horecker *et al.*, 1956). Density gradient centrifugation was carried out essentially as described by Martin and Ames (1961) using the SW27 rotor in a Spinco Model L2-65 preparative ultracentrifuge. Linear 38-ml gradients were prepared from solutions containing 5 and 20% sorbitol, respectively, dissolved in 0.05 M triethanolamine (pH 7.8), 10 mM $MgCl_2$, and 0.1 mg/ml of dithioerythritol. The gradients were centrifuged for 21 hr at 27,000 rpm and 4°.

Protein was determined by the method of Kuno and Kihara (1967), and bacteriochlorophyll after extraction into acetone–methanol (7.2), taking $E^{1\,cm}_{mM}$ as 46 at 775 mμ.

Results

Methods of Cell Breakage. Of the three methods of cell breakage employed in this study, sonication was the most effective, as judged by the release of both protein and bacteriochlorophyll into the low-speed centrifugation supernatant. By comparison, the lysozyme–freeze-thaw method released only one-fifth the amount of protein found in the other supernatants. Although the total activities of the isomerase, kinase, and carboxylase in the extracts prepared by the

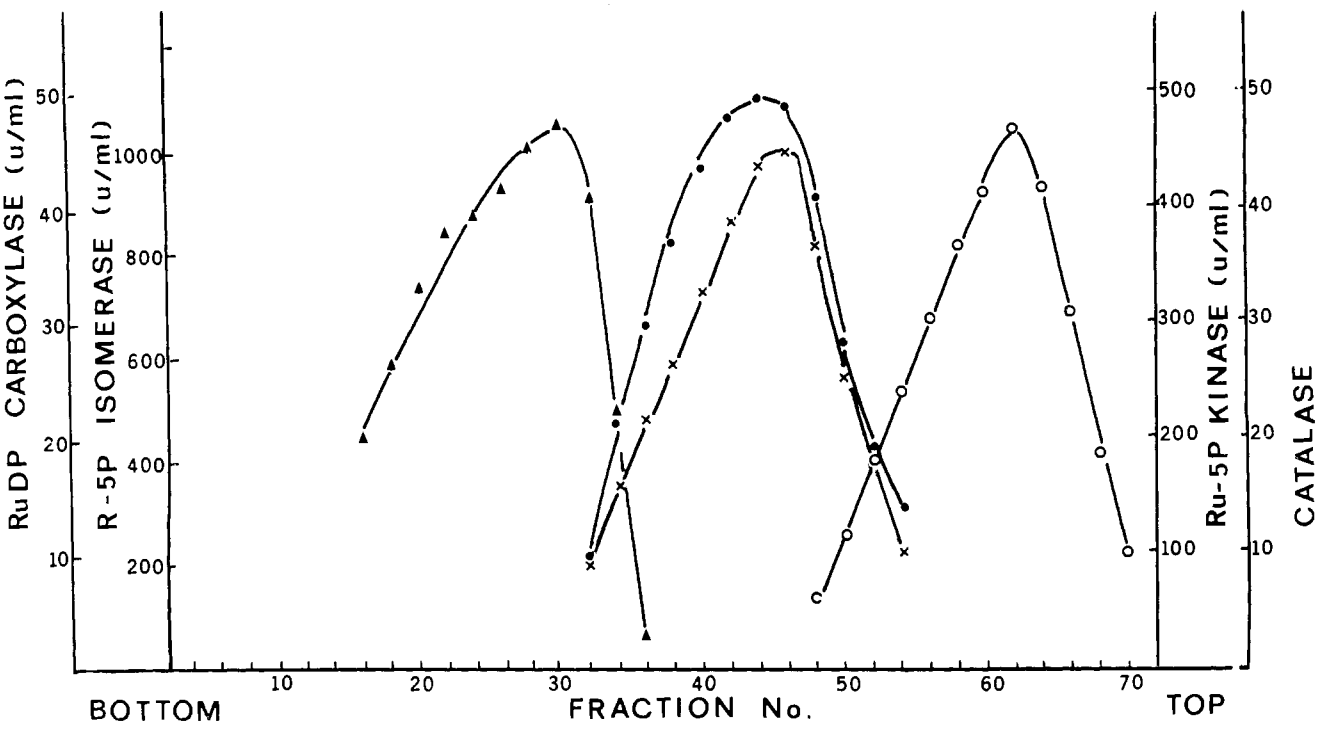

FIGURE 1: Profile of RuDP carboxylase, Ru-5P kinase, and R-5P isomerase after centrifugation through a sorbitol density gradient. Lysozyme freeze-thaw extract (0.5 ml) containing 13.5 mg of protein, 425 units of RuDP carboxylase, and 5500 units of Ru-5P kinase; 7222 units of R-5P isomerase was mixed with 27 μg of crystalline beef liver catalase and layered on a linear gradient of 5–20% sorbitol in 50 mM triethanolamine–10 mM $MgCl_2$–0.1 mg/ml of dithioerythritol. Gradients were centrifuged at 27,000 rpm in the SW27 rotor for 21 hr at 4°. The gradients were tapped and 20 drop fractions were collected. (▲) RuDP carboxylase, (×) Ru-5P kinase, (○) R-5P isomerase, and (●) beef liver catalase. Enzyme units are defined in Materials and Methods except for catalase, which is expressed in arbitrary units.

TABLE II: Effect of High-Speed Centrifugation on Enzyme Activity and Pigment Content.

Breakage Method	Time at 90,000g (min)	Bacteriochlorophyll in Supernatant (mM)	Relative Enzyme Recovery in Supernatant		
			R-5P Isomerase	Ru-5P Kinase	RuDP Carboxylase
Lysozyme freeze-thaw	0	0.37	100	100	100
	30	0	117	96	85
	60	0	121	91	82
	120	0	125	91	51
French pressure cell	0	3.13	100	100	100
	30	3.01	117	91	74
	60	0.59	123	91	88
	120	0.04	124	91	81
Sonication	0		100	100	100
	30	5.53	120	100	87
	60	1.63	124	100	117
	120	0.02	127	111	117

[a] The same extract used in Table I was employed.

lysozyme freeze-thaw method were lower than in the other extracts, the specific activities of these enzymes were appreciably higher (Table I). The behavior of the extracts on centrifugation indicated that much less comminution of membranous material had occurred in the enzymatic lysis, since bacteriochlorophyll was no longer detectable after 30-min ultracentrifugation of extracts prepared with lysozyme, while the other extracts still contained traces after as long as 120 min at 90,000g (Table II).

Density Gradient Centrifugation. When extracts of *Chro-*

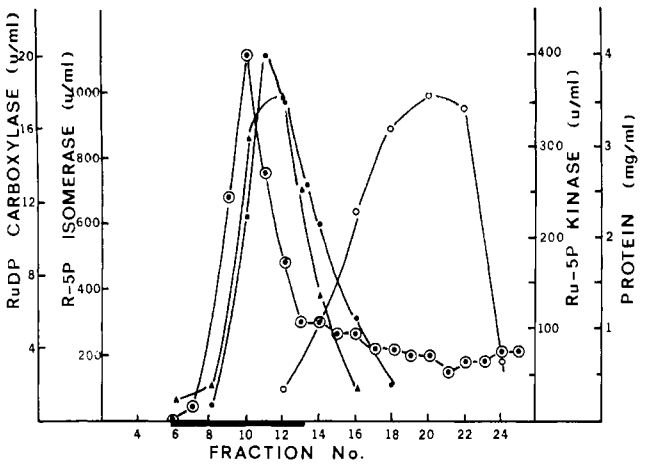

FIGURE 2: Elution of RuDP carboxylase, Ru-5P kinase, and R-5P isomerase from Sephadex G-200. Lysozyme freeze-thaw extract (1 ml) containing 27.7 mg of protein, 850 units of RuDP carboxylase, 11,000 units of Ru-5P kinase, and 14,444 units of R-5P isomerase was applied to a Sephadex G-200 column (1.4 × 26 cm, void volume 19.5 ml), equilibrated with Tris buffer (0.05 M, pH 7.4) containing 0.1 mg/ml of dithioerythritol, and eluted with the same buffer at a flow rate of 30 ml/hr. Fraction volume, 1.5 ml. All operations were carried out at 4°. (▲) RuDP carboxylase, (●) Ru-5P kinase, (○) R-5P isomerase, and (⊙) protein. Enzyme units as defined in Materials and Methods.

matium were centrifuged through 5–20% sorbitol gradients, R-5P isomerase, Ru-5P kinase, and RuDP carboxylase were found in different parts of the gradient, with little overlap of activities. More than 90% of isomerase and carboxylase was recovered in gradient fractions, while 40–60% of kinase activity was found. In agreement with the results of Anderson *et al.* (1968a) and of Kieras and Hazelkorn (1968), RuDP carboxylase migrated at a rate appropriate for a protein of molecular weight of about 500,000, calculated from the position of beef liver catalase added to the extract to serve as marker (Martin and Ames, 1961). All the Ru-5P kinase appeared in a peak close to the marker, corresponding to a molecular weight of about 240,000. R-5P isomerase is associated with a much smaller molecular weight of about 54,000 (Figure 1). Similar distributions of activities were observed in extracts prepared by different methods of cell breakage. The behavior of Ru-5P kinase in crude extracts was exactly like that of preparations which had been purified 100-fold.

Fractionation on Sephadex G-200. A portion of the same extract as was used for the sorbitol density gradient represented in Figure 1 was passed through a column of Sephadex G-200, previously equilibrated with Tris buffer (0.05 M, pH 7.4) containing 0.1 mg/ml of dithioerythritol and maintained at 5°. The distribution of protein and of enzyme activities in fractions of the eluate is shown in Figure 2. Both Ru-5P kinase and RuDP carboxylase emerged from the column in the void volume and were well separated from the isomerase, as would be expected if the molecular sizes were as indicated by the gradient centrifugation data.

Discussion

The three enzymes concerned with the conversion of ribose 5-phosphate into 3-phosphoglyceric acid are readily extracted from *Chromatium* when the cells are broken by methods causing differing degrees of comminution of cell contents. The gentlest of these, lysis by lysozyme and several freeze-thaw cycles, which has been successfully used to release intact polysomes from *E. coli* (Kohler and Davis, 1966), liberates very much less protein and pigment into the supernatant obtained after low-speed centrifugation, than do the others. The specific activities of these lysozyme extracts are, however, actually higher than sonicated or pressure cell preparations, suggesting that the enzymes are more easily extracted from the large cell debris than are some other proteins. The results of high-speed centrifugation showed conclusively that there is no correlation between the sedimentation of bacteriochlorophyll-containing material and enzyme activity. Significant decrease in activity was observed only in lysozyme extracts after 2 hr at 100,000*g*, in which the carboxylase activity only decreased to about half the original, while the extract was effectively freed of membrane fragments carrying bacteriochlorophyll after 30 min at the same speed. Although the smaller fragments produced by the other extraction methods sedimented only after longer centrifugations, these experiments indicate that R-5P isomerase, Ru-5P kinase, and RuDP carboxylase were released in all cases as soluble proteins which were not significantly associated with membranous material.

The behavior of the three enzymes in sorbitol density gradients as well as on columns of Sephadex G-200 gives no suggestion of association. Consistently with the results of others, RuDP carboxylase behaves as a large protein of molecular weight about 500,000, and similar in this property to the fraction I protein from plant sources. Gradient analysis suggested that the Ru-5P kinase has a molecular weight of about 240,000, which is about half the size of the carboxylase; R-5P isomerase is smaller still, with a calculated molecular weight of about 54,000, which is approximately the same size as the R-5P isomerase from *Rhodospirillum rubrum* studied by Anderson *et al.* (1968b). The conclusion that the enzymes are not associated with each other in extracts is reinforced by the excellent recovery of R-5P isomerase and RuDP carboxylase in fractions from the gradient. Although only about 50% of the Ru-5P kinase was accounted for, the absence of activity in any part of the gradient other than the main peak suggests strongly that the losses were due to enzyme inactivation during the long centrifugation.

Density gradient centrifugation data are supported by results of column chromatography on Sephadex G-200. The position at which R-5P isomerase was eluted from the column indicated that this enzyme has a molecular weight smaller than 200,000 and that it is not bound to either the kinase or the carboxylase. Since the molecular weights of RuDP carboxylase and Ru-5P kinase are greater than 200,000, both enzymes are excluded from the gel.

Although the evidence presented here indicates that the proteins are not linked physically in extracts, the possibility of a close association between these sequentially functioning enzymes *in vivo* cannot be excluded. MacElroy *et al.* (1968) have concluded that Ru-5P kinase and RuDP carboxylase in differentially centrifuged extracts of *Thiobacillus* species sediment together in sucrose gradients. The assay procedures used for localizing the enzymes were, as pointed out by the authors, able to detect Ru-5P kinase only in the presence of RuDP carboxylase, and it is therefore not possible to compute total enzyme recoveries in their experiments. Although the

peak activities of RuDP carboxylase and combined Ru-5P kinase–RuDP carboxylase occur in the same fraction from both gradient and column, this could represent only a small part of the total Ru-5P kinase originally present. What appears at first sight to be a major difference in enzyme organization in *Chromatium* and in *Thiobacillus* may therefore be in part or whole the result of differences in analytical methods used in the investigations.

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