Purification of a Complex of Alkaline Fructose 1,6-Bisphosphatase and Phosphoribulokinase from *Rhodospirillum rubrum*

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IAN R. JOINT, † IAN MORRIS, AND R. C. FULLER‡

From the Department of Botany and Microbiology, University College London, Gower Street, London, WC1E 6BT, England, and The University of Tennessee—Oak Ridge Graduate School of Biomedical Science and Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830

SUMMARY

Two enzymes of the reductive pentose phosphate cycle, alkaline fructose 1,6-bisphosphatase and phosphoribulokinase, have been isolated and purified as a single complex from *Rhodospirillum rubrum*. During purification procedures involving ammonium sulfate precipitation and column chromatography with both DEAE-cellulose and Sephadex, the ratios of activity of one enzyme to that of the other remained approximately the same. The pure complex appeared as a single band on polyacrylamide gels, and the activities of the two enzymes showed the same profiles on an isoelectric focusing column. No other enzyme of the reductive pentose phosphate cycle could be detected in the complex. The complex was unstable, so that after 48 hours the activity of phosphoribulokinase was reduced by 80% and that of fructose 1,6-bisphosphatase by 50%. This loss of activity was accompanied by the appearance of two bands on the polyacrylamide gels.

These various studies have suggested that the important regulatory enzymes in the reductive pentose phosphate cycle are fructose 1,6-bisphosphatase (n-fructose 1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11), sedoheptulose 1,7-bisphosphatase (EC 3.1.3.11), ribulose 1,5-bisphosphate carboxylase (3-phospho-d-glycerate carboxylase (dimerizing), EC 4.1.1.39), and phosphoribulokinase (ATP:ribulose 5-phosphate 1-phosphotransferase, EC 2.7.1.19). However, the precise mechanism by which photosynthetic carbon dioxide assimilation is regulated remains unknown.

The present work began as an attempt to study the way in which the activity of fructose bisphosphatase in extracts from the photosynthetic bacterium *Rhodospirillum rubrum* might be modified by various effectors. However, during the purification of this enzyme the activity of another possible regulatory enzyme of the reductive pentose phosphate cycle, phosphoribulokinase, was found to parallel that of the bisphosphatases. The present paper describes the isolation and purification of a complex of these two enzymes.

EXPERIMENTAL PROCEDURE

Growth of Organism

*R. rubrum* strain S1 was obtained from the collection of one of us (R.C.F.). It was maintained as a stab in screw-cap test tubes filled with a complex medium of 0.3% (w/v) tryptone, 0.3% (w/v) yeast extract, and 2% (w/v) agar; the tubes being illuminated at 30°. The medium for growth of the bacterium was a modification of that described by Ormerod, Ormerod, and Gest (15) containing per liter of Pyrex-distilled water: 0.66 g of (NH₄)₂SO₄; 0.6 g of KH₂PO₄; 0.9 g of K₂HPO₄; 0.2 g of MgSO₄·7H₂O; 0.75 g of CaCl₂·2H₂O; 0.012 g of FeSO₄·7H₂O; 0.02 g of EDTA; 1.5×10⁻⁴ g of d-biotin; and 1 ml of a trace element solution containing per liter of Pyrex-distilled water: 2.8 g of H₂BO₃; 2.1 g of MnSO₄·4H₂O; 0.04 g of Cu(NO₃)₂·3H₂O; 0.24 g of ZnSO₄·7H₂O; and 0.79 g of Na₂MoO₄·2H₂O.

The organism was grown autotrophically by bubbling this basal medium continuously with hydrogen containing 2 to 5% (v/v) carbon dioxide at a rate of 2 liters per hour per liter of medium. The light intensity (provided by a series of 60-watt tungsten filament lamps) at the culture surface was about 800-foot candles, and the temperature was 30°. The bacteria were
harvested 48 hours (about three divisions) after inoculation with a 2.5% inoculum from the midexponential phase of a culture growing phototrophically on medium supplemented with 2.6 g of DL-malate per liter of medium. For phototrophic growth on acetate the basal medium was supplemented with 1.8 g of sodium acetate per liter of medium. Phototrophic growth occurred in bottles filled completely with medium and illuminated at 30°C for 36 hours.

Preparation of Cell-free Extracts

Up to 100 liters of organism were harvested in a continuous flow centrifuge, and the pellet was washed twice in ice-cold 0.02 M Tris-HCl buffer (pH 7.5) and resuspended in this buffer. The density was adjusted to about 0.3 g of cells, fresh weight, per ml of buffer, and the suspension was broken by a single passage through a French press at 10,000 p.s.i. After cooling in an ice bath (addition of commercial ribonuclease at this stage removed the coagulation caused by high concentrations of nucleic acids), the suspension was first centrifuged at 10,000 × g for 10 min, and then the suspension was centrifuged at 100,000 × g for 1 hour. All centrifugations were performed at 4°C. The pale red supernatant was removed carefully and used as the crude extract. Extracts were usually stored at −20°C for up to 4 days; no loss of activity was detected over a period of 2 weeks.

Assay of Fructose Bisphosphatase

The reaction mixture contained in 1 ml: 100 μmoles of Tris-HCl (pH 8.5), 5 μmoles of MgCl₂-6H₂O, 0.2 μmole of NADP⁺, excess (5 μg) phosphohexose isomerase (β-glucose 6-phosphate ketol isomerase, EC 5.3.1.9), excess (2 μg) glucose 6-phosphate dehydrogenase (β-glucose 6-phosphate:NAD⁺ oxidoreductase, EC 1.1.1.19), and 0.01 to 0.1 ml of extract. After 5 min at 30°C the reaction was started by adding 1.0 μmole of fructose 1,6-bisphosphate. The rate of reaction was measured continuously by following the increase in absorption at 340 nm. This continuous assay showed that the reaction was linear with time, and in some experiments the method was modified to a single point assay. The components of the reaction mixture omitting NADP⁺, phosphohexose isomerase, and glucose 6-phosphate dehydrogenase, were incubated for 5 min. The reaction was then stopped by placing the reaction mixture in a boiling water bath for 1 min and then cooling rapidly in an ice bath. The mixture was then incubated at 30°C with 1 μmole of reduced glutathione, 70 μmoles of NaHCO₃, 5 μmoles of NaH¹⁴CO₃ (1 Ci per mole), and excess purified ribulose bisphosphate carboxylase. After 10 min (preliminary experiments showed that all ribulose bisphosphate was converted to 3-phosphoglycerate in 2 to 3 min), 0.2 ml of the mixture was added to 1 ml of glacial acetic acid. After drying, scintillant (6 g of butyl-PBD, 750 ml of toluene, and 250 ml of methanol) was added, and the radioactivity was determined with a Packard Tri-Carb Scintillation Spectrometer, model 3320.

In the above assay absorption at 340 nm was measured with a Cary (model 14) or a Unicam S.P. 800 or S.P. 1800 spectrophotometer. Protein was measured by the method of Lowry et al. (18), and by absorbance at 280 nm.

Enzyme Purification Method

The final procedure presented in the results section involved the following procedures.

Dialysis—Adsorbed metal ions were removed by dialyzing the dialysis tubing in 0.02 M EDTA. EDTA was removed by boiling the tubing twice in Pyrex-distilled water. Extracts were dialyzed against 200 times their volume of buffer with at least one change of buffer.

Ammonium Sulfate Precipitation—The extract was stirred at 4°C and solid ammonium sulfate was added as one addition (recovery of the enzyme was not increased by using a saturated solution of ammonium sulfate). Stirring continued for 30 min after the ammonium sulfate had dissolved.

Column Chromatography on Sephadex and DEAE-cellulose—Sephadex G-25 and G-200 were prepared according to the manufacturer’s instructions. Sephadex G-200 columns (45 × 2.5 cm) were run with upward flow of buffer. Previously swollen micro-
granular DEAE-cellulose (DE 52) was prepared according to the manufacturer’s (Whatman) instructions. After degassing the cellulose and equilibrating it with the required buffer, the column (30 × 0.9 cm) was filled through an extension tube with a concentrated slurry of 1 wet settled volume of equilibrated DEAE-cellulose and 0.5 volume of buffer. Buffer was immediately pumped through the column at 75 ml per hour per cm² until the bed height reached a constant level.

Analytical Polyacrylamide Gel Electrophoresis—Polyacrylamide gels were prepared by the method of Davis (19). Protein samples (containing 20 to 50 μg of protein per gel) in 10% sucrose were layered onto the surface of the gels. A constant current of 2 ma per gel was applied from a direct current power supply until the tracking dye was within 0.5 cm of the base of the gel. Protein was stained with 1% (v/v) naphthalene black in 7% (v/v) acetic acid and destained with 7% acetic acid.

Preparative Polyacrylamide Gel Electrophoresis—The gels were prepared by the method of Davis (19) and the apparatus used was the Buchler apparatus (Poly Prep). The buffer in the upper chamber was 0.01 M Tris-base and 0.525 M glycine (the same as the elution buffer); in the lower chamber the buffer was 0.5 M Tris-HCl (pH 9.0). The protein sample in 5% sucrose was pumped onto the surface of the gel, and a constant current of 50 ma was applied. The voltage increased from 200 to 400 volts during the run, fractions being collected every 30 min.

Isoelectric Focusing—An LKB 8101 column was used. Carrier ampholyte (LKB Ampholine) of pH range 3.0 to 6.0 (constant voltage, increased stepwise from 300 to 700 volts during the first 2 to 3 hours) were used. After 3 days of isoelectric focusing the column was drained and 2-ml fractions were collected each minute.

RESULTS

Studies with Crude Cell-free Extracts—Most photosynthetic organisms have two fructose bisphosphatases with different pH optima. The enzyme with the alkaline pH optimum appears to be that of the reductive pentose phosphate cycle, and that with the more neutral pH optimum is associated with glucogenesis (20). However, no data were available for R. rubrum and it therefore seemed desirable to determine whether this organism also possessed two fructose bisphosphatases, and whether the enzyme associated with photosynthetic carbon dioxide assimilation was, in fact, the one we were studying.

Two pieces of evidence suggested that R. rubrum did contain both enzymes and that the one with the more alkaline pH optimum was the photosynthetic one. Firstly, the pH profile of fructose bisphosphatase activity in crude cell-free extracts became modified after dialysis (Fig. 1). The activity of the dialyzed preparation showed a more rapid loss of activity with decreasing pH below 8.5, suggesting a reduction in the activity of an enzyme with a pH optimum between 7.5 and 8.5. This loss of activity at neutral pH values was also observed by Smillie (20) in extracts of Euglena gracilis. A second piece of evidence came from an examination of the effects of photobacteriophage growth on the activity of fructose bisphosphatase at various pH values. Anderson and Fuller (21) showed that photobacteriophage growth on malate or acetate reduced the activity of the reductive pentose phosphate cycle in photosynthetic carbon dioxide assimilation by R. rubrum. Table I shows that photobacteriophage growth had a most marked effect on the bisphosphatase activity at the more alkaline pH values, indicating that the enzyme with the alkaline pH optimum was associated with the photoassimilation of carbon dioxide in this organism. Therefore, pH 8.5 was used for all further assays of fructose bisphosphatase activity described in this paper.

Purification Procedure for Fructose Bisphosphatase—Photobacteriokinase Complex—After the pH of the crude extract was adjusted (where necessary) to pH 6.0 with lactic acid, solid ammonium sulfate was added, and the fraction precipitated between 25 and 40% saturation was retained (no activity was detected in the other ammonium sulfate fractions). The precipitate was dissolved in 0.02 M Tris-HCl buffer (pH 8.0) and the ammonium sulfate was removed by passage through Sephadex G-25.

The protein solution (about 23 ml, still highly pigmented) was pumped on to the DEAE-cellulose column (pH 8.0) at the rate of 50 ml per hour. Most of the pigment was not absorbed and
was washed through with 30 ml of buffer. No bisphosphatase or phosphoribulokinase activity was detected in this eluate. Protein was eluted from the column with 400 ml of a linear gradient of 0.2 to 0.3 M KCl in 0.02 M Tris (pH 8.0). The activities of both fructose bisphosphatase and phosphoribulokinase appeared in the same fractions.

The fractions of peak activity were pooled, and ammonium sulfate was added. The precipitate from the 25 to 40% fraction was dissolved in 0.02 M malonate buffer (pH 6.0) and the solution was desalted by passage through Sephadex G-25. The protein solution (about 15 ml, still slightly pigmented) was pumped on to a second DEAE-cellulose column (pH 6.0) at a rate of 30 ml per hour. After washing with 30 ml of buffer (removing some of the pigment) the protein was eluted with 300 ml of a 0.1 to 0.25 M linear gradient of KCl in 0.02 M malonate buffer (pH 6.0). Again the peaks of activities of both enzymes coincided.

The protein from the pooled peak fractions was precipitated by adding a saturated solution of ammonium sulfate to a final value of 50% saturation. After dissolving the precipitate in 1 ml of 0.02 M malonate buffer (pH 6.0), the solution was applied to a Sephadex G-200 column and eluted with the same buffer. Again the peaks of activity of the two enzymes coincided (Fig. 2). Also, in the eluate from this column the protein peak was well defined and coincided with that of the two enzyme activities (Fig. 2).

The two enzymes could not be separated on an isoelectric focusing column (Fig. 3). Furthermore, Fraction 10 from the G-200 column (Fig. 2) appeared as a single protein band on analytical polyacrylamide gels (Fig. 4).

The purification procedure is summarized in Table II, which also shows that the ratio of the bisphosphatase activity to the
Table II

Purification of fructose bisphosphatase-phosphoribulokinase complex

| Fraction | Volume | Protein | Fructose 1,6-bisphosphatase | Phosphoribulokinase | Biphosphatase recovered | Kinase to biphosphatase ratio |
|----------|--------|---------|-----------------------------|---------------------|-------------------------|-----------------------------|
|          | ml     | mg/mg   | total units | specific activity | total units | specific activity | % | % |
| Crude extract | 100 | 21 | 102 | 0.048 | 280 | 0.135 | 100 | 2.8 |
| (NH₄)₂SO₄ and Sephadex G-25 | 25 | 8 | 33 | 0.162 | 66 | 0.328 | 33 | 2.0 |
| DEAE-cellulose (pH 8.0) | 33 | 1.4 | 25.4 | 0.544 | 58 | 1.27 | 25 | 2.3 |
| (NH₄)₂SO₄ and Sephadex G-25 | 15 | 2 | 29.2 | 0.98 | 58 | 1.97 | 28.5 | 2.0 |
| DEAE-cellulose (pH 6.0) | 40 | 0.12 | 13.8 | 2.68 | 28.3 | 5.6 | 13 | 2.2 |
| (NH₄)₂SO₄ and Sephadex G-200 | 15 | 0.018 | 6 | 22.5 | 13.3 | 49.1 | 6 | 2.2 |

a Micromoles of substrate consumed per min per ml.
b Units per mg of protein.

phosphoribulokinase activity remained constant throughout the various stages in the procedure.

Stability of Enzyme Complex—The pure complex eluted from Sephadex G-200 column was unstable, 50% of the biphosphatase activity and 80% of the phosphoribulokinase activity being lost during 48 hours at 4°C. Also, after this period the original single band on polyacrylamide gels was largely replaced by two others (Fig. 4).

Partial separation of the two enzymes was also achieved by preparative polyacrylamide gel electrophoresis (Fig. 5). It seemed likely that the original complex had broken down to its component parts. One piece of evidence in favor of this idea was the rapid movement of the two enzymes through this preparative gel; it appears unlikely that an enzyme that was excluded from Sephadex G-100 would move through a 7.5% gel so rapidly. Also, the activity of the phosphoribulokinase was much less than that of the fructose bisphosphatase, again suggesting that the complex had broken down to its two components.

Further evidence for the instability of the complex came from a comparison of the elution profile from Sephadex G-200 of the freshly prepared pure complex (Fig. 6A) with that of the same protein solution after 48 hours of storage at 4°C (Fig. 6B). Again, this experiment showed a loss of activity of both enzymes (this being particularly marked for phosphoribulokinase) and a change in the position of the peaks in the eluate.

The pure complex did not show any activity of the following enzymes of the reductive pentose phosphate cycle: glycerate 3-phosphate kinase, glyceraldehyde 3-phosphate dehydrogenase, triose phosphate isomerase, aldolase, and pentose phosphate iso-
Possible Regulatory Characteristics of Enzyme Complex—Both phosphoribulokinase and fructose bisphosphatase have been implicated as possible control points in the regulation of photosynthetic carbon dioxide assimilation. We have examined various aspects of the isolated complex in an effort to understand the way in which the activities of the component enzymes might be regulated. Phosphoribulokinase activity was inhibited by AMP, with \( K_i \) values between 1.2 and 1.8 mM (depending on whether the concentration of ribulose 5-phosphate or of ATP is varied) and was activated by 0.05 mM NADH. These results agree with those obtained with other autotrophic bacteria \( (9, 10, 12) \). The most potent inhibitors of fructose bisphosphatase were MgATP\(^{2+}\), 2.6 mM giving 60% inhibition, and magnesium pyrophosphate (Mg\(\text{PO}_4\))\(^{2-}\), 3.3 mM giving 50% inhibition. These results agree with those of Morris \( (8) \) with the fructose bisphosphatase from spinach chloroplasts. However, our results do not agree with those of Morris \( (8) \) and Preiss, Biggs, and Greenberg \( (7) \) in that the relationship between reaction rate and fructose bisphosphatase concentration is hyperbolic and not sigmoidal as observed with the enzyme from spinach chloroplasts. Also, the pH optimum of the chloroplast fructose bisphosphatase is altered by increasing Mg\(^{2+}\) concentration \( (7) \) but there is no such change with the enzyme from \( R. \) rubrum.

**DISCUSSION**

Two enzymes of the reductive pentose phosphate cycle in the photosynthetic bacterium \( R. \) rubrum have been isolated and purified as a complex. The ultraviolet absorption spectrum of the pure complex shows a peak at 250 nm and is similar to bovine serum albumin. Also, it appears as a single band on analytical polyacrylamide gels. Furthermore, two enzyme activities remain associated (and show a constant ratio of activities) during several different fractionation techniques. These various observations suggest that the complex is a single protein.

However, we have not made physicochemical measurements of this protein. Thus, although it appears likely that the complex consists of two subunits, each of which is associated with the activity of one of the component enzymes, the size of the complex and the precise nature of the association between the subunits remains unknown. Indeed, its instability would make analysis with (for example) the analytical ultracentrifuge difficult.

Whatever the precise nature of this link between the two enzyme activities, an association between two enzymes not catalyzing consecutive reactions would appear to be of interest. A "multienzyme complex" has been defined as "an orderly association (not involving peptide linkages) of various enzymes that catalyze successive steps in a reaction sequence" \( (\text{definition of Henning, quoted by Gaertner and De Moss}) \).

The complex described here does not fit in with this concept since the two enzymes do not catalyze successive reactions in the reductive pentose phosphate cycle. Rather, the association reported here more closely resembles that between aspartokinase and homoserine dehydrogenase in *Escherichia coli* K-12 \( (23, 24) \). These two enzymes catalyze the first and third steps in the sequences leading to the synthesis of methionine and threonine. The activities of both aspartokinase and homoserine dehydrogenase are regulated by the end products of the biosynthetic pathways, and it seems probable, therefore, that an association between them has some regulatory function. Possibly, the association between fructose bisphosphatase and phosphoribulokinase reported here also has some kind of regulatory function. Certainly, both enzymes have been implicated in the control of the reductive pentose phosphate cycle \( (1-14) \).

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