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Pyridine Nucleotide Control and Subunit Structure of Phosphoribulokinase from Photosynthetic Bacteria

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With one exception, phosphoribulokinase from the *Rhodospirillaceae* requires reduced nicotinamide adenine dinucleotide for maximum activity. This mode of regulation is unique to the facultatively anaerobic photoorganotrophic photosynthetic bacteria, since the phosphoribulokinase from oxygen-evolving photosynthetic species is not subject to activation by reduced nicotinamide adenine dinucleotide. The enzyme was purified of fructose bisphosphatase activity from *Rhodopseudomonas capsulata* by means of affinity chromatography and was shown to have a native molecular weight of about 220,000. The homogeneous enzyme is composed of a single size polypeptide of 36,000 molecular weight. This study represents the first time the subunit structure of phosphoribulokinase has been determined from any source.

The two enzymes unique to the Calvin reductive pentose phosphate pathway are phosphoribulokinase (ATP:D-ribulose 5-phosphate 1-phosphotransferase; EC 2.7.1.19) and ribulose 1,5-bisphosphate (Rbu-P_2) carboxylase/oxygenase (3-phospho-D-glycerate carboxylase [dimerizing]; EC 4.1.1.39). The two enzymes catalyze sequential reactions with the subsequent production of two molecules of phosphoglycerate (Fig. 1).

Much interest has recently been directed towards the structure, function, and regulation of the carboxylase since it is a bifunctional enzyme which catalyzes both the carboxylation and oxygenolysis of ribulose bisphosphate, reactions which comprise the first steps in the two competing pathways of photosynthetic carbon dioxide reduction and photorespiratory carbon dioxide dissipation, respectively (8). Little, however, is known about the structure and function of phosphoribulokinase, the enzyme which catalyzes the synthesis of ribulose bisphosphate. Although there have been several studies which have examined certain of the kinetic and regulatory features of the kinase, all such studies suffer from the use of crude or only partially purified enzyme (2, 5, 13). In this investigation we describe the structure of homogeneous phosphoribulokinase isolated from the photosynthetic bacterium *Rhodopseudomonas capsulata* and we show that the phosphoribulokinase obtained from aerobic oxygen-evolving photosynthetic species is under a different mode of metabolic control than is the enzyme obtained from the facultatively anaerobic photosynthetic bacteria belonging to the *Rhodospirillaceae* (with one exception).

MATERIALS AND METHODS

Reagents. All biochemicals were purchased from Sigma Chemical Company, St. Louis, Mo. All common compounds were of reagent-grade quality. The affinity chromatography gel, Affi-Gel blue, was purchased from Bio-Rad Laboratories, Richmond, Calif., as was DEAE-Bio-Gel. Ribulose bisphosphate was prepared enzymatically from ribose 5-phosphate (6) as described earlier (26). $NaH^{14}CO_3$ (20 mCi/mmol) was purchased from Amersham Corp., Arlington Heights, Ill.

Organisms and growth. The photosynthetic bacteria used for these studies, *R. capsulata* B10, *Rhodopseudomonas sphaeroides* 2.4.1 Ga, *Rhodopseudomonas palustris*, *Rhodomicrobium vannielii*, *Rhodospirillum rubrum* S-1, *Rhodospirillum tenue*, and *Rhodospirillum molischianum*, were cultured in the medium of Ormerod et al. (16) with the carbon and nitrogen source specified. In addition, the following vitamin supplements were made to the basic media: 15 μg of biotin per liter for cultures of *R. rubrum*, *R. palustris*, *R. sphaeroides*, and *R. capsulata*; 1 mg each of nicotinic acid and thiamine hydrochloride per liter for *R. sphaeroides* and *R. capsulata*; and 0.1 mg of *p*-aminobenzoic acid per liter for *R. palustris*. Cultures of *R. palustris*, *R. vannielii*, *R. molischianum*, and *R. capsulata* B10 were kindly supplied by the laboratory of Howard Gest, Indiana University, Bloomington, Ind. A culture of *R. tenue* was kindly provided by Jurgen Oelze, of the University of Freiburg, W. Germany. Large batches of *R. capsulata* for the enzyme purification were obtained after inoculating 5-liter vessels of butyrate medium with a 5% inoculum of exponential-phase butyrate-grown cells. Small batches of photosynthetic bacteria were cultured in completely filled 850-ml Roux flasks, stoppered with paraffin wax to maintain anaerobiosis. The two species of cyanobacteria, *Anabaena* sp. CA and *Agmenellum quadruplicatum* strain PR-6, were cultured on Asp-2 medium at 39°C (24) as previously

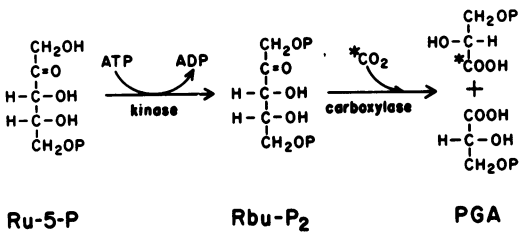

FIG. 1. *Enzymatic steps unique to the Calvin reductive pentose phosphate pathway.*

described (20, 23). *Chlorella sorokiniana* was cultured at 39°C in Cg-10 medium (25). In all cases, the cultures were bubbled with 1% CO_2 in air to maintain high growth rates at high cell densities. *A. quadruplicatum* strain PR-6 was obtained from Chase Van Baalen, University of Texas at Austin Port Aransas Marine Laboratory; the high-temperature *Chlorella* sp. strain was obtained from Warren Pulich of the same institution. All other strains were from our own collection.

Enzyme assay. Rbu-P_2 carboxylase was assayed exactly as previously described (27) by measuring the incorporation of $NaH^{14}CO_3$ into acid-stable 3-phosphoglycerate. In all cases enzyme was previously activated by a 5-min incubation with 20 mM $NaHCO_3$ and 10 mM magnesium acetate before initiating the reaction with Rbu-P_2. Phosphoribulokinase was assayed by coupling the formation of Rbu-P_2 to the subsequent carboxylation catalyzed by a large excess of *R. rubrum* Rbu-P_2 carboxylase, prepared as previously described (22). The phosphoribulokinase reaction mixture contained 64 mM Tris-chloride (pH 8.0), 20 mM $MgCl_2 \cdot 6H_2O$ or magnesium acetate, 2.6 mM ribulose 5-phosphate, 0.6 mM NADH where specified, and usually 10 or 20 μl of a suitable dilution of the enzyme preparation to be assayed (21). After a 5-min incubation at 30°C, 20 mM $NaH^{14}CO_3$ and 6 mM ATP were added to initiate the reaction and give a final volume of 250 μl. After the desired time, usually 5 min, the reaction was quenched by the addition of 100 μl of propionic acid. The vials were incubated in a hood for at least 1 h, and the reaction tubes were then subsequently centrifuged in a Sero-Fuge from Clay Adams, Parsippany, N.J. for 20 min under the hood to drive off all remaining gaseous $^{14}CO_2$ and to remove all precipitated protein. The resultant supernatant (200 μl) was then transferred to scintillation tubes, toluene-based scintillation cocktail was added (26), and the samples were counted for acid-stable radioactivity in a Beckman LS-100C liquid scintillation counter. Under these conditions, the activity was linear for up to 30 min and was directly dependent on protein concentration. Moreover, all substrates and cofactors were supplied in large excess to ensure saturation.

Protein was determined by the method of Lowry et al. (11), with crystalline bovine serum albumin as the protein standard.

Electrophoresis. Polyacrylamide gel electrophoresis was used to monitor the purity of the phosphoribulokinase preparations. A buffer system containing triethanolamine and *N*-tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid (TEA-TES system) as described by Orr et al. (17) was employed. Gels were stained with Coomassie brilliant blue G-250 (0.04%) in 3% perchloric acid. Sodium dodecyl sulfate-discontinuous gel electrophoresis was performed in 1.5 mM slab gels by the procedure of Lugtenberg et al. (12). Slab gels were stained with 0.1% Coomassie blue R in 25% isopropanol–10% acetic acid and destained, first with 10% acetic acid–10% isopropanol and then with 10% acetic acid.

Purification of phosphoribulokinase. Two volumes of buffer A (10 mM Tris-SO_4 [pH 8.0], 10 mM $MgSO_4$, and 0.5 mM 2-mercaptoethanol) were added to each gram (wet weight) of cells. The cell slurry was then passed two times through a chilled French pressure cell at 20 lb/in^2; all subsequent purification steps were done at refrigeration temperatures (0 to 4°C). Cell debris was removed from the French pressure cell eluant by centrifugation at 16,300 × *g* for 10 min. The resulting supernatant was clarified by high-speed centrifugation at 100,000 × *g* for 90 min. The supernatant after this centrifugation represents the crude extract in all cases, with all organisms, and was the starting material for the subsequent purification of the enzyme from *R. capsulata*.

To this high-speed-centrifuged supernatant fraction, enough 1 M $MgSO_4$ was added to bring the concentration to 0.22 M in $MgSO_4$. This was followed by the slow dropwise addition of 60% polyethylene glycol 4000 such that the final concentration of polyethylene glycol reached 37%. After standing on ice for 1 h, the cloudy solution was centrifuged at 16,300 × *g* for 20 min. The supernatant was carefully removed, and the tan pellet was suspended to 50 to 60 ml in buffer A. The suspended polyethylene glycol precipitate was then dialyzed overnight against a 100-fold excess of buffer A. This dialyzed fraction was then loaded onto a column (2.5 by 40 cm) of Affi-Gel blue previously equilibrated with buffer A. Buffer was passed through the column until the absorbance at 280 nm of the eluted fractions was negligible. At this time, the column was eluted with 200 ml (2 column volumes) of 10 mM ATP in buffer A. While this ATP-containing buffer removed much protein, virtually all the phosphoribulokinase remained bound to the column. The column was then washed with at least 2 column volumes of 10 mM Tris-SO_4 buffer containing 10 mM 2-mercaptoethanol. This was followed by elution with the same buffer containing 10 mM ATP. Alternatively, a gradient from 0 to 10 mM ATP in the same buffer was passed through the column. In each case, so long as the ATP-containing buffer lacked Mg^{2+}, phosphoribulokinase was quantitatively eluted from this column. Peak fractions were pooled and concentrated about four- to fivefold by ultrafiltration using an Aminco model 402 ultrafiltration cell with a Pellicon PTGC filter pad (Millipore Corp., Bedford, Mass.). This concentrated eluant was then dialyzed against a 100-fold excess of buffer A overnight. After dialysis, the concentrated eluate from the Affi-gel column was loaded onto a column (2.5 by 50 cm) of DEAE–Bio-Gel previously equilibrated with buffer A. The column was eluted with buffer A until approximately 3 column volumes passed through. At this time, a gradient of 0 to 0.5 M KCl in buffer A was

started, and the enzyme was eluted. Peak fractions were pooled (about 60 ml) and loaded onto a second Affi-Gel blue column (1.5 by 50 cm) equilibrated with buffer A. The column was washed with 100 ml of buffer A, then 100 ml of 10 mM ATP in buffer A was passed through followed by 100 ml of 10 mM Tris-SO_4 containing 10 mM 2-mercaptoethanol. Finally, a sharp peak of enzyme was eluted with 10 mM ATP in the same Tris-mercaptoethanol buffer. The fractions containing the highest specific activity were then pooled, concentrated by ultrafiltration as before, and dialyzed exhaustively. This preparation could be stored indefinitely at −70°C with no loss in activity.

RESULTS

Previous results show that photosynthetic bacteria cultured with reduced fatty acids, such as butyric acid as the electron donor, in the presence of exogenous bicarbonate synthesize high levels of Rbu-P_2 carboxylase (3, 4, 22). Results obtained in this laboratory with several species of photosynthetic bacteria including *R. sphaeroides*, *R. capsulata* and *R. rubrum* have shown that high levels of both Rbu-P_2 carboxylase and phosphoribulokinase can be obtained under these growth conditions. Thus, photosynthetic bacteria were cultured in a butyrate-bicarbonate medium to obtain extracts with high phosphoribulokinase activity for the subsequent experiments.

Activation of phosphoribulokinase by NADH. Crude and partially purified preparations of phosphoribulokinase from a number of microbial sources have been shown to be activated by NADH (2, 10, 13, 18). The activation by NADH, although specific for this reduced nucleotide, does not follow any specific pattern since the enzyme from several chemosynthetic and photosynthetic bacteria is not activated by NADH (5, 14). Among photosynthetic species, phosphoribulokinase from species of *Rhodospirillaceae* appears unique in its requirement for NADH (Table 1). In fact, with the exception of the enzyme from *R. vannielii*, the kinase from all the *Rhodospirillaceae* tested is regulated by NADH. Interestingly, there appeared to be some variation in the amount of activity obtained in the absence of NADH since the *R. palustris* and *R. sphaeroides* enzymes showed nearly an absolute requirement, whereas the others showed significant activity in its absence. This effect was obtained with both dialyzed and undialyzed preparations. Control experiments have also shown that there is no significant enzymatic oxidation of NADH during the time course of the assay, and the purified *R. capsulata* enzyme behaves exactly like the crude enzyme. Phosphoribulokinase from oxygen-evolving photosynthetic organisms, whether procaryotic (*Agmenellum* sp. and *Anabaena* sp.) or eucaryotic (*Chlorella* sp.), was unaffected by this ligand (Table 1). Indeed, spinach leaf phosphoribulokinase is also not regulated by NADH (10). No other pyridine nucleotide (NADPH, NAD^+, or $NADP^+$) affected the *Agmenellum* sp., *Anabaena* sp., or *Chlorella* sp. enzyme. It thus appears that phosphoribulokinase from oxygen-evolving photosynthetic species is under a different mode of metabolic control.

TABLE 1. *NADH regulation of phosphoribulokinase from photosynthetic organisms*

Organism	Phosphoribulokinase activity (nmol of CO_2 fixed/min)	
	+NADH[a]	−NADH
Rhodopseudomonas capsulata	21.5	4.2
Rhodopseudomonas sphaeroides	17.7	0.8
Rhodopseudomonas palustris	15.5	0.3
Rhodospirillum rubrum	8.9	2.8
Rhodospirillum tenue	15.7	5.6
Rhodospirillum molischianum	23.0	9.2
Rhodomicrobium vannielii	12.9	12.1
Agmenellum quadruplicatum[b]	17.5	17.5
Anabaena sp. CA[b]	5.7	5.7
Chlorella sorokiniana[b]	17.8	17.3

[a] NADH added to either dialyzed or undialyzed extracts at 0.6 mM.

[b] NADH also added at 3.0 mM.

Purification of phosphoribulokinase from *R. capsulata*. For several years, *R. capsulata* was the only photosynthetic procaryote for which a well-defined genetic system was available (15); studies were thus initiated to learn more of the structure of the NADH-regulated enzyme obtained from this organism. Preliminary experiments showed that up to 65% of the total phosphoribulokinase units released from the cell were associated with the intracytoplasmic membrane fraction obtained after centrifugation at 200,000 × *g*. The enzyme did not appear to be tightly associated with these membranes since gel filtration of this preparation on a Bio-Gel A 1.5 M column nicely separated enzyme activity from the pigmented membrane material.

Eventually, a procedure for the purification of the enzyme was developed using a combination of polyethylene glycol precipitation and column chromatography with DEAE-Bio-Gel and the generalized affinity matrix, Affi-Gel blue. Virtually all the enzyme was separated from substantial amounts of extraneous protein in the polyethylene glycol precipitation step. Indeed, a reproducible slight (10 to 20%) increase in the total amount of enzyme units was always obtained (Table 2). Subsequently, it was found

that the enzyme would tightly bind to Affi-Gel blue and only high concentrations of salt (0.5 M KCl) would elute the enzyme from such a column. Much more specific elution was obtained by eluting the enzyme from such a column with low concentrations of ATP. However, only small or negligible amounts of phosphoribulokinase were eluted when the ATP was applied in the usual buffer A. Only if the column was re-equilibrated with buffer A lacking Mg^{2+} would the subsequent application of ATP elute the large bulk of the enzyme originally applied (Fig. 2). Two such columns were employed, along with anion exchange chromatography, to isolate homogeneous enzyme. There was no fructose 1,6-bisphosphatase activity associated with the pure protein. A summary of the purification procedure shows that over a 25% recovery may be obtained, although only peak fractions were pooled from the three columns (Table 2).

TABLE 2. *Purification summary of phosphoribulokinase from R. capsulata*[a]

Fraction	Total protein (mg)	Total units	Sp act	Percent recovery	Fold purification
100,000 × *g* supernatant	1,122	1,375	1.2		
Polyethyleneglycol precipitate	708	1,580	2.2	115	1.8
First Affi-Gel blue column eluates (pooled)	130	726	5.6	53	4.7
DEAE-Bio-Gel eluates (pooled)	26	579	22.5	42	18.8
Second Affi-Gel blue column eluates (pooled)	7.6	370	48.7	27	40.6

[a] In this purification scheme, only the soluble enzyme was processed.

Molecular weight and subunit structure. The molecular weight was determined by both sucrose density gradient centrifugation (Fig. 3) and gel filtration by using a calibrated Bio-Gel A 1.5 M column. By the two procedures, the molecular weight was determined to be between 200,000 and 220,000. Moreover, the phosphoribulokinase from *R. capsulata* migrated just ahead of bovine liver catalase in native polyacrylamide gels of several concentrations, suggesting that the kinase is slightly smaller than catalase, which has a molecular weight of 232,000 (19).

Upon dissociation and electrophoresis of the enzyme in the presence of sodium dodecyl sulfate, a single polypeptide species was found corresponding to a subunit molecular weight of 36,000 (Fig. 4). Thus, phosphoribulokinase from *R. capsulata* appears to be a hexamer of subunits of equal molecular size, although further studies of the aggregation properties of the native enzyme are warranted before this assumption can be verified. No heterogeneity of subunits was found.

DISCUSSION

It is interesting that phosphoribulokinase from the facultatively anaerobic photosynthetic bacteria is activated by NADH (18) as is the enzyme from certain chemosynthetic bacteria (1, 2, 13). Of course, NADH is produced as a result of bacterial photosynthesis and there

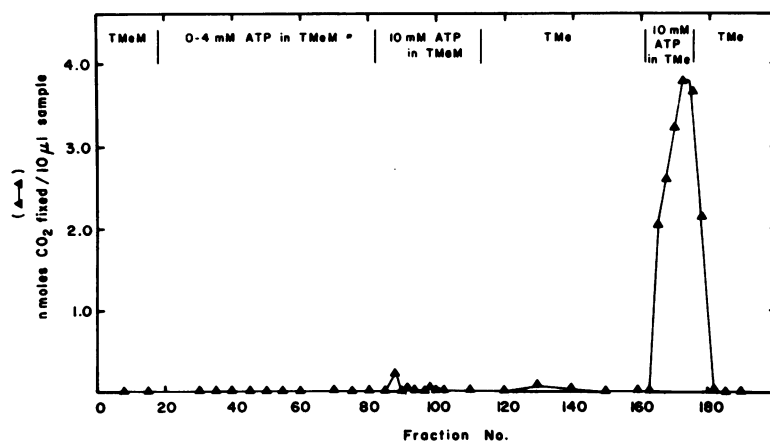

FIG. 2. *Affinity chromatography purification of R. capsulata phosphoribulokinase. A column (1.5 by 50 cm) of Affi-Gel blue was equilibrated with buffer A. After applying the enzyme sample, the column was washed extensively with the equilibration buffer. Subsequently a gradient of 0 to 4 mM ATP, and eventually 10 mM ATP in buffer A, was passed through the column. At this time, buffer A lacking Mg^{2+} was washed through the column, followed by buffer A lacking Mg^{2+} and containing 10 mM ATP.*

should thus be a significant intracellular pool of this reduced nucleotide during growth; the intracellular level found in *Anacystis* sp. (7) (60 μM) is more than enough to activate phosphoribulokinase in photosynthetic bacteria. With the isolation of homogeneous phosphoribulokinase from *R. capsulata*, the structural basis of this regulation and the nature of this regulatory site might now be delineated. Certainly it will be of deep interest to elucidate the structure of a phosphoribulokinase that is not activated by NADH.

In *R. rubrum*, phosphoribulokinase was isolated as a complex associated with fructose bisphosphatase (9). The complex appeared as a single band in polyacrylamide gels and both activities comigrated upon isoelectric focusing. This complex, however, was unstable, losing 50% of the fructose bisphosphatase activity and 80% of the phosphoribulokinase activity after 48 h at 4°C. Interestingly, after this time, the single band on polyacrylamide gels was replaced by two faster migrating species, perhaps indicating that the complex had broken down to its component parts (9). The homogeneous phosphoribulokinase isolated in this investigation from *R. capsulata* does not possess any fructose bisphosphatase activity; moreover, the enzyme is comprised of only a single size polypeptide species, making it unlikely that the enzyme isolated here is complexed with any other protein. The partially purified *Alcaligenes eutrophus* enzyme was also devoid of fructose bisphosphatase activity and has a molecular weight similar to the *R. capsulata* enzyme (2). The molecular weight of the *Chromatium* sp. enzyme is also about 200,000 (5). Perhaps the purification procedure used in this investigation, employing affinity chromatography on Affi-Gel blue, is harsh enough to destroy any such association in facultatively anaerobic photosynthetic bacteria, or these enzymes are more tightly associated in *R. rubrum*. It will, indeed, be fascinating to probe for associations of enzymes of the Calvin cycle, and current investigations in this laboratory are directed toward this end. A necessary first correlate, however, is to isolate and determine the structure of individual components of any putative complex. The present investigation represents the first time that the subunit structure of phosphoribulokinase has been described from any source.

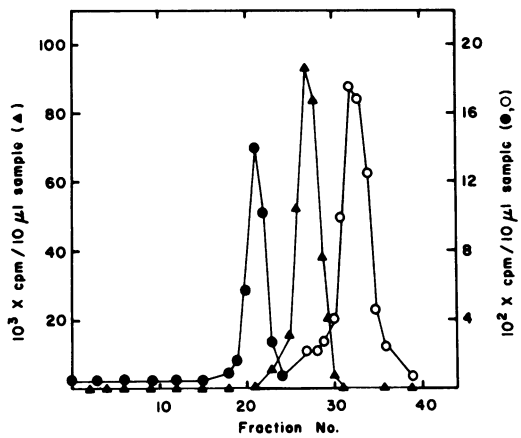

FIG. 3. *Sucrose density gradient (0.2 to 0.8 M sucrose in buffer A) molecular-weight determination of R. capsulata phosphoribulokinase. Purified phosphoribulokinase (▲) and R. rubrum (○) and spinach leaf (●) Rbu-P_2 carboxylases were applied to a 38-ml gradient and the tubes were centrifuged for 24 h at 27,000 rpm in a Beckman SW27 rotor. Fractions of 1.0 ml were collected from the bottom of the tube, and each fraction was assayed as described in the text. The direction of sedimentation is from left to right (bottom of tube).*

FIG. 4. *Sodium dodecyl sulfate slab gel electrophoresis of homogeneous R. capsulata phosphoribulokinase. The enzyme was electrophoresed in 11% gels (12).*

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