Subcellular Segregation of Phosphoribulokinase and Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase in the Cyanobacterium *Chlorogloeopsis fritschii*

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The Calvin cycle enzyme phosphoribulokinase has been localized in terms of catalytic activity and enzyme protein in the cyanobacterium *Chlorogloeopsis fritschii*. In contrast to the CO₂-fixing enzyme of the Calvin cycle, D-ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), which occurs in the cytoplasm and in the carboxysomes, phosphoribulokinase is essentially (95%) cytoplasmic in extracts from cells grown photoautotrophically to late exponential phase. Total phosphoribulokinase accounted for 0.6% of total cell protein in these cells. Immunochemical identity was found between the phosphoribulokinase located in the cytoplasm and the remaining enzyme (5%) associated with the particulate fraction of the cell obtained following differential centrifugation. On density gradient centrifugation of the particulate fraction into Percoll plus sucrose the RuBisCO activity was located in a carboxysome band in the lower half of the gradient while the phosphoribulokinase activity essentially remained concentrated at the top of the gradient. Comparison of RuBisCO and phosphoribulokinase distributions suggests that the latter is not a carboxysomal enzyme in late exponential phase cells of *C. fritschii*.

INTRODUCTION

The polyhedral bodies of colourless sulphur-oxidizing bacteria, nitrite- and ammonia-oxidizing bacteria and cyanobacteria, all Calvin cycle autotrophs, have been found to contain the CO₂-fixing enzyme of the Calvin cycle, D-ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) (see Shively *et al.*, 1973, 1977; Harms *et al.*, 1981; Codd & Stewart, 1976; Lanaras & Codd, 1981*a*). SDS-dissociation of isolated carboxysomes, followed by SDS-polyacrylamide gel electrophoresis has revealed several polypeptides, the most abundant being the large (L) and small (S) subunits of RuBisCO, which together account for more than 50% of total carboxysome protein (Lanaras & Codd, 1981*a*; Cannon & Shively, 1983). In addition to the RuBisCO L and S subunits, between 5 and about 11 other polypeptides can be seen in stained gels after electrophoresis (Biedermann & Westphal, 1979; Lanaras & Codd, 1981*a*; Cannon & Shively, 1983). With the exception of two polypeptides with molecular weights of 10000 and 15000, which may be *Thiobacillus neapolitanus* carboxysome shell components (Cannon & Shively, 1983), no identities have been ascribed to the remaining carboxysomal polypeptides.

The possibility that *T. neapolitanus* carboxysomes may contain the remaining enzymes of the Calvin cycle, plus malate dehydrogenase, aspartate aminotransferase and adenylate kinase, has been raised (Beudeker & Kuenen, 1981). Such an enzyme complement could enable carboxysomes to function as CO₂-fixing organelles in which the reducing power and ATP needed to drive the Calvin cycle would be generated via an exogenous malate/aspartate shuttle system. Subsequent studies on isolated *T. neapolitanus* carboxysomes have not found ribose-5-phosphate

Abbreviations: RuBisCO, D-ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, D-ribulose 1,5-bisphosphate; Ru5P, D-ribulose 5-phosphate.

isomerase, fructose-1,6-bisphosphatase or phosphoribulokinase activities and the suggestion that the organelles may contain a fully functional Calvin cycle has been refuted (Cannon & Shively, 1983).

We have isolated carboxysomes from the cyanobacterium *Chlorogloeopsis fritschii* and found a 39000 Dal polypeptide among the dissociation products (Lanaras & Codd, 1981a). Since phosphoribulokinase from this organism consists of subunits of molecular weight 40000 (Marsden & Codd, 1984), we have determined whether the enzyme is carboxysomal in this organism. Subcellular localization studies based on enzyme activities have been complemented by quantitative immunoelectrophoresis using antiserum raised to the purified *C. fritschii* phosphoribulokinase.

METHODS

Organism and growth. *Chlorogloeopsis fritschii* 1411/16 from the Culture Centre of Algae and Protozoa, Cambridge, UK, was grown photoautotrophically in pure batch culture in BG-11 medium including nitrate (Stanier *et al.*, 1971) as detailed previously (Codd & Stewart, 1973).

Buffers. Buffer A contained 10 mM-Tris/HCl, 10 mM-EDTA (disodium salt), 50 mM-NaHCO₃ and 80 mM-2-mercaptoethanol, pH 8.0 at 4 °C. Buffer B contained 100 mM-Tris/HCl, 10 mM-EDTA (disodium salt), 50 mM-NaHCO₃ and 80 mM-mercaptoethanol, pH 8.0 at 4 °C.

Preparation of cell-free extracts. Mid to late exponential phase cells were used throughout and all steps were performed at 4 °C unless stated otherwise. Cells were obtained by centrifuging 18 l culture at 2000 g for 10 min. Then 3–5 g wet wt of cells were washed, resuspended in about 5 vols buffer A, and broken by passage through an ice-cold French press at 110 mPa. Unbroken cells and large fragments were removed by centrifugation at 1500 g for 10 min and the supernatant was used for differential centrifugation.

Differential centrifugation. The 1500 g cell-free supernatant was centrifuged at 40000 g for 1 h. The pellet was resuspended in 3 vols buffer B and ultrasonicated for 4 × 30 s with 30 s intermittent cooling in an MSE Soniprep 150 (9.5 mm probe). The volumes of the supernatant and resuspended ultrasonicated pellet fractions were recorded to permit estimation of enzyme distribution and they were retained for enzyme activity and immunology assays.

Density gradient centrifugation. The 40000 g pellet was resuspended in buffer A and 1.7 ml vols were loaded onto 20 ml density gradients of Percoll plus sucrose in buffer A, prepared as before (Lanaras & Codd, 1981a). The gradients were spun in a 3 × 25 ml swing-out rotor at 27000 g in an MSE PrepSpin 75 centrifuge for 2.5 h. Fractions of 1 ml were collected from the bottom of the gradients by suction. Density was determined as before (Lanaras & Codd, 1981a) and fractions were retained for assays of total protein, chlorophyll, RuBisCO and phosphoribulokinase.

Enzyme assays. RuBisCO (EC 4.1.1.39) carboxylase activity was measured by preincubation of samples for 10 min in a final volume of 0.1 ml containing 100 mM-Bicine, pH 7.8 at 30 °C, 10 mM-MgCl₂, 50 mM-NaH¹⁴CO₃ (74 kBq μmol⁻¹), 1 mM-EDTA (disodium salt) and 1 mM-DTT. Catalysis was initiated by the addition of 50 μl RuBP to a final concentration of 0.5 mM and was allowed to run for 2 min at 30 °C before termination by the addition of 50 μl 50% (w/v) TCA. Vials were heated to 100 °C in a water bath for 1 h and the acid-stable radioactivity that was incorporated was measured by standard liquid scintillation counting. Specific radioactivity of the NaH¹⁴CO₃ was determined; controls lacked RuBP.

Phosphoribulokinase (EC 2.7.1.19) activity was measured by coupling Ru5P- plus ATP-dependent RuBP formation to the RuBisCO assay run in the presence of excess exogenous spinach RuBisCO, as detailed earlier (Marsden & Codd, 1984).

Phosphoribulokinase purification and antiserum production. The *C. fritschii* phosphoribulokinase was purified according to Marsden & Codd (1984). A 0.5 ml sample of electrophoretically homogeneous enzyme containing 320 μg protein was emulsified with an equal volume of Complete Freund's Adjuvant and injected subcutaneously into a New Zealand White rabbit. Null serum was collected by bleeding prior to injection. Three booster injections of 200, 120 and 120 μg phosphoribulokinase protein in 0.5 ml were emulsified with equal volumes of Incomplete Freund's Adjuvant and injected subcutaneously at 14 d intervals. Blood was taken 14 d after the last injection. Null serum and antiserum were prepared by allowing blood to clot overnight at 4 °C followed by centrifugation at 2000 g for 10 min. The serum was concentrated threefold by precipitation with 50% saturation (NH₄)₂SO₄. The precipitated material was recovered by spinning at 40000 g for 30 min and resuspended in 10 mM-phosphate buffer, pH 7.5 at 4 °C, containing 0.15 M-NaCl, and dialysed overnight against the same buffer. The sera were dispensed into 100 μl volumes and stored at -20 °C.

Immunological methods. Ouchterlony double immunodiffusion using *C. fritschii* phosphoribulokinase antiserum was carried out on 5 × 5 cm glass plates supporting 4.4 ml Indubiose Agarose A37 in 0.1 M-potassium phosphate buffer, pH 7.5, containing 0.005 g sodium azide l⁻¹. Wells of 10 μl were loaded with sera and enzyme preparations

and incubated overnight at 4 °C. The gels were fixed in 0.1 M-NaCl for 30 min and washed in distilled water for 30 min prior to pressing and drying. Gels were stained with Coomassie Blue G250 (5 g l⁻¹) in 45% (v/v) methanol, 10% (v/v) acetic acid in water. Destaining was done in the same mixture minus Coomassie Blue.

Rocket immunoelectrophoresis was carried out using *C. fritschii* phosphoribulokinase antiserum and RuBisCO antiserum against the enzyme from the cyanobacterium *Microcystis* PCC 7820 (provided by S. Murray of this laboratory). Experimental details were as given by Lanaras & Codd (1981b).

Protein determination. Protein concentrations were measured according to Bradford (1976) with 10 µl samples, 1 ml reagent and crystalline BSA as a standard.

Chemicals. NaH¹⁴CO₃ was obtained from Amersham. Agarose C was from Pharmacia and DTT, BSA, Ru5P, RuBP and spinach RuBisCO from Sigma. ATP was from Boehringer and Coomassie Brilliant Blue G250 was from Serva, Heidelberg, FRG. Indubiose A37 was from L'Industrie Biologique Française, Genevilliers, France. All other chemicals were from BDH and were used at the highest purity commercially available.

RESULTS AND DISCUSSION

Cell-free extracts were prepared from *Chlorogloeopsis fritschii* under conditions which favour carboxysome isolation and the separation of soluble (cytoplasmic) and particulate (carboxysomal) RuBisCO (Lanaras & Codd, 1981a). The distribution of RuBisCO and phosphoribulokinase activities and the enzyme protein content in 40000 g supernatant and particulate (carboxysome-containing) extracts is shown in Table 1. About 40% of the extractable RuBisCO activity was particulate, whereas only 5% of the total phosphoribulokinase activity was sedimented. Exhaustive dialysis against fresh buffer B did not influence either enzyme activity, precluding reversible inhibition by low molecular weight metabolites in the fractions.

Since rates of RuBisCO activity per unit RuBisCO protein can vary widely in such preparations (Lanaras & Codd, 1982), levels of enzyme protein were also measured using antisera to RuBisCO purified from *Microcystis* PCC 7820 and to phosphoribulokinase from *C. fritschii*. As before, the RuBisCO antiserum cross-reacted to form single immunoprecipitation bands versus *C. fritschii* extracts in Ouchterlony gels and formed rockets in immunoelectrophoresis gels to enable quantification of RuBisCO protein (Lanaras & Codd, 1981b). Phosphoribulokinase antiserum has not been produced previously against the cyanobacterial enzyme; the behaviour of the antiserum against the homologous pure antigen and against the 40000 g supernatant and particulate extracts of *C. fritschii* is shown in Fig. 1(a). Single immunoprecipitation bands were formed in all cases. Immunochemical identity between the soluble and particulate phosphoribulokinase preparations was indicated by the complete fusion between respective bands.

A linear relationship was established between rocket height and antigen concentration which enabled the enzyme protein to be quantified in the cell-free extracts (Fig. 1b). The levels of RuBisCO and phosphoribulokinase protein were thereby measured and are compared in Table 1. Over 20% of total RuBisCO protein was particulate (carboxysomal) in the differential centrifugation fractions, whereas less than 2% of phosphoribulokinase protein was sedimented. Comparison of the relative levels of the enzymes within each fraction reveals considerable

Table 1. *Distribution of RuBisCO and phosphoribulokinase activities and proteins in soluble and particulate fractions of Chlorogloeopsis fritschii*

Enzyme	Fraction*	Specific activity†	Distribution of activity (%)‡	Enzyme protein (mg)	Distribution of enzyme protein (%)	Distribution of enzyme protein as a percentage of protein in the fraction
RuBisCO	Supernatant	0.867	62.20	5.14	78.50	12.80
	Pellet	0.383	38.80	0.47	21.50	23.41
Phosphoribulokinase	Supernatant	1.261	95.08	0.18	98.29	0.67
	Pellet	3.752	4.92	0.00313	1.71	0.08

* Supernatant and particulate fractions obtained after centrifugation of a 1500g broken cell-free supernatant at 40000g for 1 h. Total soluble and particulate fractions contained 26.15 and 4.04 mg protein, respectively.

† nmol substrate transformed min⁻¹ (µg enzyme protein)⁻¹.

‡ Percentage of activity originally present in a 1500g × 10 min supernatant after further separation into the 40000g × 1 h supernatants and pellets.

Fig. 1. Immunological detection and assay of phosphoribulokinase from *Chlorogloeopsis fritschii*. (a) Ouchterlony double immunodiffusion gel. Centre well, phosphoribulokinase antiserum. Peripheral wells were loaded as follows: PP, purified homologous phosphoribulokinase antigen; S, 40000 g supernatant; P, 40000 g resuspended ultrasonicated pellet of *C. fritschii* extract. (b) Typical rocket immunoelectrophoresis calibration gel of purified *C. fritschii* phosphoribulokinase versus homologous antiserum. Wells were loaded with 1 to 7 μ l purified enzyme containing up to 540 ng protein. The 3.3 ml Agarose C gels contained 75 μ l antiserum and were electrophoresed at 4 mA for 18 h ($\circ$, $\bullet$, duplicate calibrations).

Fig. 2. Distribution of *Chlorogloeopsis fritschii* RuBisCO (carboxylase) and phosphoribulokinase activities after centrifugation of 40000 g pellet material into Percoll plus sucrose density gradients (for details see Lanaras & Codd, 1981a). Fractions of 1 ml were collected from the bottom of the gradients. $\circ$, RuBisCO carboxylase activity; $\bullet$, chlorophyll (A_{663}); $\square$, phosphoribulokinase activity. Fractions are numbered in the order in which they were withdrawn from the gradient, fraction 1 being from the bottom.

disparity: the ratio of RuBisCO to phosphoribulokinase is 28:1 in the soluble fraction and 150:1 in the particulate fraction (calculated from Table 1). On a total protein basis, phosphoribulokinase was about 10 times more abundant in the soluble fraction than in the particulate, while RuBisCO was twice as abundant in the soluble fraction compared to the particulate fraction. Total RuBisCO protein accounted for 18.2% of the total cellular *C. fritschii* protein, a level which is similar to total RuBisCO levels in other autotrophic prokaryotes (Beudeker *et al.*, 1981; Leadbeater *et al.*, 1982). Total phosphoribulokinase protein amounted to 0.6% of the total protein in these extracts of photoautotrophically-grown *C. fritschii*, which compares with values determined in chemolithoautotrophic cultures of *Alcaligenes eutrophus* (Leadbeater *et al.*, 1982).

Further localization studies were made to determine whether the small amount of particulate

C. fritschii phosphoribulokinase was associated with carboxysomes. Profiles of RuBisCO and phosphoribulokinase activities are shown in Fig. 2, after centrifugation of the resuspended 40000 g pellet material into density gradients of Percoll plus sucrose. As before (Lanaras & Codd, 1981a), the RuBisCO activity was focussed in a carboxysome band in the lower half of the gradient below the chlorophyll-containing thylakoid band. The phosphoribulokinase activity originally associated with the 40000 g pellet fraction essentially remained concentrated at the top of the gradients (Fig. 2). It is thus possible that the particulate phosphoribulokinase represents soluble enzyme molecules which may be adsorbed onto cell fragments during cell disruption.

In conclusion, the possibility that phosphoribulokinase is associated with carboxysomes in *C. fritschii* is not favoured since enzyme activity and protein distributions do not mimic those of RuBisCO. The data show no enrichment of crude particulate fractions or isolated *C. fritschii* carboxysomes with phosphoribulokinase. Indeed the distribution of this enzyme in the cyanobacterial extracts closely resembles that found in similarly prepared fractions from the purple non-sulphur bacterium *Rhodospseudomonas palustris* 1850, which does not appear to contain polyhedral bodies (96% soluble, 4% particulate phosphoribulokinase; W. J. N. Marsden, unpublished observations). The possibilities that other Calvin cycle enzymes, in addition to RuBisCO, may be present in cyanobacterial carboxysomes, and that the inclusion bodies may be active sites of CO₂ fixation, are not discounted. However, the absence of phosphoribulokinase alone, due to its specific and essential role in the regeneration of the CO₂ acceptor RuBP, would preclude a fully functional Calvin cycle in *C. fritschii* carboxysomes.

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