Ribulose-1,5-bisphosphate Carboxylase from *Rhodomicrobium vannielii*

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(Received 23 April 1979; revised 21 June 1979)

The effect of carbon growth substrate on the level of ribulose-1,5-bisphosphate (RuBP) carboxylase in crude cell-free extracts of *Rhodomicrobium vannielii* (RM5) was investigated. The highest specific activity followed growth with sodium hydrogen malate as carbon source. Cells grown autotrophically or on the reduced carbon substrates, butyrate and ethanol, gave extracts of lower activity. RuBP carboxylase was purified from *R. vannielii* (RM5) grown on a mixture of pyruvate and malate as carbon source. The enzyme was homogeneous as judged by electrophoresis on polyacrylamide gels of several acrylamide concentrations and had an apparent molecular weight of 430000 as measured by gel filtration. Sodium dodecyl sulphate polyacrylamide gel electrophoresis suggested the presence of three subunit types of molecular weights 56200, 53300 and 15700. Mg^{2+} was required for maximum enzyme activity although this could be completely replaced by Ni^{2+} and, to a lesser extent, by Mn^{2+} or Co^{2+} . As with other high molecular weight RuBP carboxylases, the enzyme was sensitive to inhibition by 6-phosphogluconate.

INTRODUCTION

Ribulose-1,5-bisphosphate (RuBP) carboxylase (EC 4.1.1.39) is the primary catalyst of the reductive pentose phosphate cycle (Calvin cycle) for CO_2 assimilation (Bassham *et al.*, 1954). Because of its key role in this cycle and the diversity of organisms within which it is found, there is considerable interest in its structure and properties.

All RuBP carboxylases so far examined contain a large polypeptide (mol. wt 52000 to 58000) on which the catalytic site resides (McFadden & Tabita, 1974). Enzymes purified from *Thiocapsa* (McFadden, 1977), *Rhodospirillum rubrum* (Tabita & McFadden, 1974a), *Chlorobium* (Tabita *et al.*, 1974a) and certain cyanobacteria (Tabita *et al.*, 1974b, 1976) are reported to be made up solely of this large polypeptide subunit having one, two, six and eight copies, respectively. The majority of RuBP carboxylases, however, also contain multiple copies of a small polypeptide with a molecular weight between 12000 and 20000 (McFadden, 1973). This small polypeptide is believed to have a regulatory function (Takabe & Akazawa, 1973). Where both are present, the available evidence suggests that there is a 1:1 mole ratio of large to small subunits, as in the RuBP carboxylases of *Chromatium D* (Akazawa *et al.*, 1972), *Ectothiorhodospira halophila* (Tabita & McFadden, 1976), *Anabaena variabilis* (Takabe *et al.*, 1976), the eukaryotic algae and higher plants. These carboxylases all have molecular weights of approximately 550000 and consist of eight large plus eight small subunits. The RuBP carboxylase of *Pseudomonas oxalaticus* contains only six large and six small subunits (McFadden, 1977).

Two forms of RuBP carboxylase have been reported in *Rhodopseudomonas sphaeroides* (Gibson & Tabita, 1977a) and *Rhodopseudomonas capsulata* (Gibson & Tabita, 1977b). One form has a high molecular weight (550000) and contains both large and small subunits; the second has a lower molecular weight (360000) and contains only large subunits.

Rhodomicrobium vannielii exhibits a complex life cycle in which CO_2 has been implicated as a controlling factor (France, 1978). As part of an investigation into the role and regulation of CO_2 fixation in *R. vannielii* and other *Rhodospirillaceae*, we have purified the RuBP carboxylase from this organism and examined some of its properties.

METHODS

Growth. *Rhodomicrobium vannielii* strain RM5 was grown on a salts medium plus 1.5 g each of sodium pyruvate and sodium hydrogen malate, adjusted to pH 6.8 with KOH (Whittenbury & Dow, 1977). For enzyme purification, cultures were grown in 20 l flasks sealed with rubber serum caps, flushed with O_2 -free N_2 and incubated with stirring at 30 °C under constant illumination from tungsten lamps (incident light intensity, 2000 lx). For measuring enzyme activity following growth on various carbon substrates, 200 ml cultures were grown in 250 ml shake flasks on the salts medium supplemented with carbon substrate as indicated. After flushing with O_2 -free N_2 , cultures were incubated as above and harvested in the mid-exponential phase of growth.

Enzyme assay and measurement of protein concentration. RuBP carboxylase was assayed at 30 °C in a reaction mixture (0.25 ml final vol.) which contained 15 μmol Tris/HCl, pH 8.2 (unless stated otherwise), 2.5 μmol $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 5 μmol $\text{NaH}^{14}\text{CO}_3$ (sp. act. 0.8 $\mu\text{Ci } \mu\text{mol}^{-1}$) and enzyme. Any further additions were as indicated in Results. After preincubation for 5 min, the reaction was started by adding 0.2 μmol RuBP, and it was stopped after a further 5 min by adding 100 μl 12 M-formic acid. Any precipitate was removed by centrifugation. A 200 μl sample of the final reaction mixture was evaporated to dryness in a scintillation vial, resuspended in 1 ml water and radioactivity (fixed ^{14}C) was measured in a liquid scintillation spectrometer with a Triton/toluene scintillation fluid. One unit of enzyme activity catalyses the RuBP-dependent fixation of 1 $\mu\text{mol } \text{CO}_2 \text{ min}^{-1}$. Specific activity is expressed in units $(\text{mg protein})^{-1}$. Protein concentrations were determined with the Folin-Ciocalteu reagent (Kennedy & Fewson, 1968) with dried crystalline bovine serum albumin as standard.

Preparation of cell-free extracts. All purification steps and centrifugations were done at 4 °C. Bacteria in the late-exponential phase of growth were harvested by centrifuging at 10000 g, washed twice and finally resuspended in 20 mM-Tris/HCl buffer, pH 8.0 containing 10 mM- $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 50 mM- NaHCO_3 , 5 mM-2-mercaptoethanol and 1 mM-EDTA (TEMMB buffer) (Tabita & McFadden, 1974a). The cell suspension was passed twice through a pre-chilled French press at 137 MPa and unbroken cells and debris were removed by centrifuging at 10000 g for 10 min. The supernatant was then centrifuged at 120000 g for 90 min to yield soluble and particulate fractions. The soluble fraction was the source of crude RuBP carboxylase.

Purification procedure. The crude soluble extract was slowly taken to 35 % saturation with solid $(\text{NH}_4)_2\text{SO}_4$ and stirred for 30 min. The precipitate was removed by centrifuging at 30000 g for 20 min and discarded. The supernatant was then taken to 60 % saturation with $(\text{NH}_4)_2\text{SO}_4$ and stirred for 1 h. After centrifuging at 30000 g for 20 min, the pellet obtained was dissolved in TEMMB buffer and dialysed overnight against the same buffer. The enzyme solution was then applied to an Ultragel Aca 22 column (3.5 $\times$ 100 cm) equilibrated with TEMMB buffer. This was eluted at a flow rate of 10 ml h^{-1} and 5 ml fractions were collected. Active fractions were pooled, taken to 60 % saturation with $(\text{NH}_4)_2\text{SO}_4$ and stirred for 1 h. Following centrifugation at 30000 g for 20 min, the pellet was dissolved in TEMMB buffer and dialysed against the same buffer overnight. This solution was then applied to a DEAE-cellulose column (2 $\times$ 10 cm) prewashed with Tris/HCl buffer, pH 8.0 containing 5 mM- $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and 5 mM-2-mercaptoethanol (TMM buffer). Enzyme was eluted at a flow rate of 50 ml h^{-1} by a linear 0 to 1 M-NaCl gradient prepared in TMM buffer. Active fractions were pooled and concentrated in an Amicon ultrafiltration apparatus with an Amicon PM10 membrane. This enzyme solution (2 ml) was finally loaded on to a discontinuous sucrose gradient (Tabita & McFadden, 1974b) comprising four successive layers (each 14 ml) of 0.8, 0.6, 0.4 and 0.2 M-sucrose in TEMMB buffer. After centrifugation at 80000 g for 24 h, 2 ml fractions were collected by piercing the base of the tube. Active fractions were combined and concentrated by ultrafiltration.

Electrophoresis. Polyacrylamide gel electrophoresis (PAGE) was done using the discontinuous system of Laemmli (1970). Gels were prepared in Tris buffer, pH 8.8, and Tris/glycine, pH 8.3 was used as the running buffer. Where required, sodium dodecyl sulphate (SDS) at 0.1 % (w/v) was incorporated into both buffers. Enzyme purity was assessed using non-denaturing gels polymerized from 4, 4.5, 5, 6 and 7.5 % (w/v) acrylamide. Quaternary structure was determined by SDS-PAGE using 10 % (w/v) acrylamide gels or, for higher resolution, 7.5 to 20 % (w/v) exponential gradient acrylamide gels (O'Farrell, 1975). Proteins were dissociated by boiling for 3 min in 1 % (w/v) SDS plus 0.1 % (w/v) 2-mercaptoethanol.

Determination of molecular weight. A Sephadex G-200 column (1 $\times$ 90 cm) was equilibrated at 4 °C with TEMMB buffer and then a 0.5 ml sample containing *R. vannielii* (RM5) RuBP carboxylase and marker proteins was applied to the column. The marker proteins were *Escherichia coli* β -galactosidase (mol. wt

Table 1. *Effect of carbon growth substrate on the specific activity of ribulose-1,5-bisphosphate carboxylase in crude extracts of Rhodomicrobium vannielii (RM5)*

Unless otherwise indicated, substrates were each present at a final concentration of 10 mM; CO₂ was provided as filter-sterilized NaHCO₃ (final concn 0.05 %, w/v). All cultures were harvested in the mid-exponential growth phase, determined by following the increase in *A*₅₄₀.

Carbon growth substrate	Specific activity [m units (mg protein) ⁻¹]
Sodium pyruvate	19.3
Sodium pyruvate + CO ₂	19.1
Sodium malate	39.2
Sodium succinate	25.6
Sodium pyruvate + sodium malate	25.7
Ethanol + CO ₂	20.8
Sodium butyrate + CO ₂	16.8
Sodium propionate + CO ₂	16.7
Sodium acetate + CO ₂	13.1
Sodium lactate + CO ₂	15.3
Hydrogen (30 %, v/v) + CO ₂	16.0

540000), horse spleen apoferritin (mol. wt 443000), beef liver glutamate dehydrogenase (mol. wt 320000), bovine liver catalase (mol. wt 232000), yeast alcohol dehydrogenase (mol. wt 141000) and *E. coli* alkaline phosphatase (mol. wt 86000) (Darnall & Klotz, 1975). Proteins were eluted at a flow rate of 10 ml h⁻¹ and 1 ml fractions were collected. Enzymes were located by assaying fractions for activity and apoferritin was located by its absorbance at 412 nm.

Determination of subunit molecular weights. This was done by SDS-PAGE using 10 % (w/v) acrylamide gels. The marker proteins were bovine serum albumin (mol. wt 67000), γ -globulins (mol. wts 25000 and 50000), ovalbumin (mol. wt 43500), horse heart myoglobin (mol. wt 16890) and horse heart cytochrome *c* (mol. wt 13400).

Cation requirement. The requirement for a divalent cation for activity was examined using freshly purified enzyme dialysed overnight at 4 °C against 20 mM-Tris/HCl buffer, pH 8.0 containing 5 mM-EDTA. The standard assay was then used, except that Mg²⁺ was replaced by other cations at final concentrations of 10 mM.

Chemicals. All enzymes and biochemicals were of the highest grade available and obtained from Sigma. Chemicals were supplied by BDH and acrylamide came from Fluka.

RESULTS

Levels of RuBP carboxylase

The effect of carbon growth substrate on RuBP carboxylase activity in crude cell extracts of *R. vannielii* (RM5) was examined (Table 1). The highest specific activity [0.039 units (mg protein)⁻¹] was obtained after growth on malate. Extracts of cells grown on the reduced carbon substrates, butyrate and ethanol, had much lower RuBP carboxylase activities; however, growth on these substrates was poor.

Purification of RuBP carboxylase

The purification procedure adopted (Table 2) gave a 13 % recovery of RuBP carboxylase with a final specific activity of 1.9 units (mg protein)⁻¹. Enzyme eluted from both Ultrogel AcA 22 and DEAE-cellulose columns as a single peak of activity indicating the presence of just one form of the enzyme. After sedimentation into a 0.8 to 0.2 M-sucrose gradient the enzyme was electrophoretically homogeneous, only one stained band being evident on gels polymerized from 4 to 7.5 % (w/v) acrylamide (Fig. 1). An overall 38-fold purification from cell-free extracts indicates that the active protein constituted about 2.5 % (w/v) of the total soluble protein present in the 120000 g supernatant.

Table 2. *Purification procedure for ribulose-1,5-bisphosphate carboxylase from Rhodomicrobium vannielii (RM5)*

Purification step	Protein (mg)	Activity (units)	Specific activity [units (mg protein) ⁻¹]	Yield (%)
10000 g (10 min) supernatant	2100	56.7	0.027	—
120000 g (90 min) supernatant	1008	50.4	0.050	88.9
(NH ₄) ₂ SO ₄ precipitation (35 to 60 % satn)	446	37.5	0.084	66.1
Gel filtration on Ultrogel AcA 22 (pooled and concd active fractions)	49.4	28.3	0.57	49.7
DEAE-cellulose chromatography (pooled and concd active fractions)	14.0	21.0	1.5	37.0
Sucrose gradient (0.2 to 0.8 M)	3.9	7.4	1.9	13.0

Fig. 1

(a) (b)
Fig. 2

Fig. 1. Polyacrylamide gel electrophoresis of *Rhodomicrobium vannielii* (RM5) ribulose-1,5-bisphosphate carboxylase. A 50 µg sample was applied to each gel polymerized from (left to right) 7.5, 6, 5, 4.5 and 4 % (w/v) acrylamide.

Fig. 2. SDS-polyacrylamide gel electrophoresis [7.5 to 20 % (w/v) exponential gradient acrylamide gel] of purified *Rhodomicrobium vannielii* (RM5) ribulose-1,5-bisphosphate carboxylase (a) following storage for 14 d at 4 °C after (NH₄)₂SO₄ treatment (30 µg protein) and (b) without storage (45 µg protein). The enzyme in (a) and (b) was derived from the same batch of cell-free extract.

Molecular weight of RuBP carboxylase

The molecular weight was determined by gel filtration as described in Methods. A value of 430000 was obtained using both pure enzyme and partially purified material (after (NH₄)₂SO₄ precipitation).

Quaternary structure of RuBP carboxylase

Rhodomicrobium vannielii (RM5) RuBP carboxylase was dissociated by SDS in the presence of 2-mercaptoethanol into three types of polypeptide as revealed by SDS-PAGE

Table 3. *Effect of various cations on the activity of purified Rhodomicrobium vannielii* (RM5) ribulose-1,5-bisphosphate carboxylase

Cation in assay (final concn 10 mM)	Specific activity [units (mg protein) ⁻¹]	Percentage of activity with Mg ²⁺
None	0.34	21
Mg ²⁺	1.62	100
Co ²⁺	0.64	39
Ni ²⁺	2.15	133
Zn ²⁺	0.20	12
Mn ²⁺	0.52	32
Ca ²⁺	0.19	12
Fe ²⁺	0.32	20
Mo ²⁺	0.38	23
Cu ²⁺	0.13	8
Al ³⁺	0.13	8
Cd ²⁺	0.16	10

Fig. 3. Effect of 6-phosphogluconate on the activity of *Rhodomicrobium vannielii* (RM5) ribulose-1,5-bisphosphate carboxylase. The maximum activity was 1.9 units (mg protein)⁻¹.

on 7.5 to 20 % (w/v) exponential gradient gels (Fig. 2*b*). Two slowly moving bands of similar mobility indicated two types of large polypeptide subunit and a faster moving band indicated a small polypeptide subunit. Some preparations contained a minor protein contaminant of molecular weight intermediate between the large and small polypeptides, although this may have represented associated subunits. Molecular weights of the polypeptides were 56 200, 53 300 and 15 700 when measured as described in Methods. An approximate mole ratio of the subunits was determined by scanning the stained bands on gels at 656 nm. The average ratio of small to total large subunits was 1:1.14. With a molecular weight of 430 000, the most likely quaternary structure of this enzyme consists of six large and six small subunits. During purification, some enzyme preparations were stored for up to one month at 4 °C following 35 to 60 % (NH₄)₂SO₄ precipitation. The subunit composition of enzyme purified following storage for 14 d was also examined by SDS-PAGE (Fig. 2*a*). Heterogeneity of the large subunit was not evident and, furthermore, no small polypeptide subunits were detected. The specific activity of this pure enzyme preparation was 0.7 units (mg protein)⁻¹.

Catalytic properties

The purified enzyme was fully stable for at least 1 month when stored at -80 °C. Activity was lost during storage at either 4 or -20 °C, only 50 % of the initial activity remaining after 21 d.

Maximum enzyme activity was dependent on the presence of a divalent cation (Table 3). The reaction was not specific for Mg^{2+} , with Mn^{2+} , Co^{2+} and Ni^{2+} giving 32, 39 and 133 %, respectively, of the activity with Mg^{2+} .

Effect of 6-phosphogluconate on enzyme activity

6-Phosphogluconate has been shown to inhibit RuBP carboxylases from various sources (Tabita & McFadden, 1972), sensitive enzymes having a molecular weight of approximately 500000. When added to the assay mixture simultaneously with RuBP, 6-phosphogluconate inhibited *R. vannielii* (RM5) RuBP carboxylase, the enzyme having only 50 % of its original activity in the presence of 0.3 mM-6-phosphogluconate (Fig. 3).

DISCUSSION

The derepression of RuBP carboxylase synthesis following growth with butyrate as carbon substrate, as reported for *Rhodospirillum rubrum* (Tabita & McFadden, 1974a), *Rhodopseudomonas sphaeroides* (Gibson & Tabita, 1977a) and *Rhodopseudomonas capsulata* (Gibson & Tabita, 1977b), was not observed in *Rhodomicrobium vannielii* (RM5). The specific activity of RuBP carboxylase in cell-free extracts of malate-grown cells was, however, some five-fold greater than the equivalent activity reported for *Rhodospirillum rubrum* (Tabita & McFadden, 1974a). These results may be indicative of a different mechanism of regulation of CO_2 fixation in *Rhodomicrobium vannielii* from that in other *Rhodospirillaceae*.

The requirement for a divalent cation for activity is consistent with data for all other RuBP carboxylases that have been investigated. With plant RuBP carboxylases, substitution of Mg^{2+} by Ni^{2+} or Co^{2+} has been shown to depend on the age and purity of the enzyme preparation (Kawashima & Wildman, 1970). The high activity of freshly isolated *Rhodomicrobium vannielii* carboxylase, assayed in the presence of Ni^{2+} , may be novel and is particularly interesting in view of the reported specificity of the *Rhodospirillum rubrum* enzyme for Mg^{2+} (Tabita & McFadden, 1974a).

Two types of large polypeptide subunit in the pure *Rhodomicrobium vannielii* (RM5) RuBP carboxylase were clearly resolved by electrophoresis of dissociated enzyme on exponential gradient acrylamide gels. Large subunit heterogeneity has previously been reported to occur in the RuBP carboxylases of tobacco (Kung *et al.*, 1974) and *Hydrogenomonas eutropha* (Purohit *et al.*, 1976) (being evident in *Hydrogenomonas* in the presence of protease inhibitors). Proteolysis, however, cannot be ruled out as a possible cause of heterogeneity of the *Rhodomicrobium vannielii* carboxylase, particularly in view of the apparent instability of its quaternary structure. The loss of the small polypeptide subunits during storage may be compared with the reported loss of the small subunits from the *Aphanocapsa* RuBP carboxylase on acid precipitation (Codd & Stewart, 1977) and suggests a relatively weak quaternary structure. Although enzyme activity did decrease on storage, the presence of some activity despite loss of detectable small subunits, suggests that the catalytic activity of the *Rhodomicrobium vannielii* RuBP carboxylase resides with the large subunits, as has been found with the carboxylases of *Chromatium* (Takabe & Akazawa, 1973) and *Aphanocapsa* (Codd & Stewart, 1977).

Apart from the *Rhodomicrobium vannielii* carboxylase, the only other RuBP carboxylases so far found that probably comprise six large and six small subunits are those of *Pseudomonas oxalaticus* (McFadden, 1977) and *Methylococcus capsulatus* (Bath) (Taylor, 1977, 1979). Elucidation of the spatial arrangement of the subunits in these carboxylases merits further investigation.

Results presented here have demonstrated the instability of the quaternary structure of the *Rhodomicrobium vannielii* (RM5) RuBP carboxylase. This may explain the reported

presence of two forms of the enzyme in *Rhodopseudomonas sphaeroides* (Gibson & Tabita, 1977a) and *Rhodopseudomonas capsulata* (Gibson & Tabita, 1977b) where one form lacks detectable small polypeptide subunits. Care should therefore be taken in the determination of RuBP carboxylase structure.

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