Ribulose-1,5-bisphosphate Carboxylase/Oxygenase and Carbon Assimilation in *Methylococcus capsulatus* (Bath)

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Intact cells of chemostat-grown *Methylococcus capsulatus* (Bath) assimilated CO₂ such that approximately 2.5% (w/w) of cell carbon arose from CO₂ during growth with methane as carbon substrate. Radiolabelling studies suggested that CO₂ was fixed by both ribulose-1,5-bisphosphate (RuBP) carboxylase and known heterotrophic mechanisms. The pattern of CO₂ fixation was similar to that in heterotrophically grown autotrophs. Enzyme analysis suggested the presence of a complete Calvin cycle but attempts to grow the organism autotrophically were unsuccessful. A specific phosphoglycollate phosphatase, required for the metabolism of phosphoglycollate arising as a result of RuBP oxygenase activity, was present. The product of this reaction, glycollate, was further metabolized by reactions of the serine cycle, used for C₁ assimilation in type II methylotrophs. The possession of this pathway explains both the presence of hydroxypyruvate reductase and the results of [¹⁴C]formate uptake reported by other workers. The relationship between RuBP carboxylase/oxygenase and net carbon assimilation is discussed.

INTRODUCTION

Most methane-oxidizing bacteria can be conveniently classified into two types depending on the pathway of carbon assimilation (Whittenbury *et al.*, 1970). Type I organisms have a ribulose monophosphate (RMP) cycle whilst type II organisms have a serine cycle. In having an RMP cycle as its main carbon assimilation route (Strom *et al.*, 1974), *Methylococcus* was originally considered to be a type I organism. However, the possible existence of an auxiliary serine pathway (Reed, 1976), the presence of ribulose-1,5-bisphosphate (RuBP) carboxylase in at least two *Methylococcus* strains (Taylor, 1979) and a DNA base composition of 62.5 mol% GC has led Whittenbury & Dalton (1980) to re-classify *Methylococcus* as a type X methylotroph.

The presence of RuBP carboxylase and phosphoribulokinase in *M. capsulatus* (Bath) has been demonstrated (Taylor, 1977). However, as reported here, this organism does not appear to grow autotrophically at the expense of CO₂. This paper attempts to establish a possible role for RuBP carboxylase in relation to the overall carbon metabolism of this organism.

METHODS

Growth of organisms and preparation of cell-free extracts. *Methylococcus capsulatus* (Bath) (Whittenbury *et al.*, 1970) was grown as a chemostat culture in a 3 l fermenter (L.H. Engineering, Stoke Poges, Bucks.) on ammonium mineral salts (AMS) medium (Dalton & Whittenbury, 1976). The chemostat was operated under oxygen limitation with methane (20%, v/v, in air at a flow rate of 50 ml min⁻¹) as carbon source. Temperature and

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pH were maintained at 45 °C and 6.8, respectively. A dilution rate (D) of 0.15 h^{-1} was used and at steady state the dry weight of the culture was 4 mg ml^{-1} . *Methylobomonas methanica* (SI) was grown under the same conditions at 30 °C.

Cells were harvested by centrifugation at 10000 g , washed twice with, and resuspended in 20 mM-Tris/HCl, pH 8.0, containing 10 mM-MgCl₂, 50 mM-NaHCO₃, 1 mM-EDTA (di-sodium salt) and 5 mM-2-mercaptoethanol (Tabita & McFadden, 1974). Crude cell extracts were prepared by two passages of the suspension through a pre-cooled French press (American Instrument Co., Maryland, U.S.A.) at 137 MPa. Unbroken cells and debris were removed by centrifugation (10000 g for 10 min) and the supernatant was further centrifuged (120000 g for 1.5 h) to yield soluble and particulate fractions.

For the attempted autotrophic growth of *M. capsulatus* (Bath), cultures were prepared in 250 ml Erlenmeyer flasks, containing 20 ml of either AMS or nitrate mineral salts (NMS) medium (Dalton & Whittenbury, 1976) and sealed with rubber serum caps. CO₂ was added as filter-sterilized NaHCO₃ (final concentration 0.01 to 0.1%, w/v). Hydrogen (5 to 50 ml) was added to the gas phase and other potential electron donors were added directly to the culture (final concentration 0.005 to 0.1%, w/v). Inocula were taken from a chemostat culture and flasks were incubated with shaking at 45 °C.

Enzyme assays and units. RuBP carboxylase [3-phospho-D-glycerate carboxy-lyase (dimerizing); EC 4.1.1.39] was assayed by the RuBP-dependent incorporation of NaH¹⁴CO₃ into acid stable products (Taylor, 1977). Fructose-1,6-bisphosphatase [D-fructose-1,6-bisphosphate 1-phosphohydrolase; EC 3.1.3.11] was assayed by measuring the liberation of inorganic phosphate from fructose 1,6-bisphosphate by the method of Pontremoli (1966). Inorganic phosphate was estimated with the amidol reagent of Allen (1940). Phosphoglycollate phosphatase [2-phosphoglycollate phosphohydrolase; EC 3.1.3.18] was assayed by the method of Anderson & Tolbert (1966).

Assays were done at 45 °C for *M. capsulatus* (Bath) and 30 °C for *Methylobomonas methanica* (SI). One enzyme unit is the amount of enzyme that catalyses the transformation of $1\text{ }\mu\text{mol}$ substrate min^{-1} under the assay conditions described.

Measurement of CO₂ fixation by intact cells. Cells were harvested by centrifugation (10000 g for 10 min) washed once with, and resuspended to an A_{540} of 0.1 in AMS medium at 45 °C. Methane (25 ml) was added to a 20 ml suspension in a 250 ml Erlenmeyer flask sealed with a rubber serum cap, and equilibrated at 45 °C for 10 min on a shaking water bath (Grant Instruments, Cambridge) at 80 cycles min^{-1} . Control flasks either lacked methane or contained heat-killed cells. NaHCO₃ (final concentration 2.5 mM) was then added, followed, after a further 10 min, by $10\text{ }\mu\text{Ci}$ (370 kBq) NaH¹⁴CO₃ to give a final specific activity of $0.2\text{ }\mu\text{Ci }\mu\text{mol}^{-1}$ (7.4 kBq μmol^{-1}). At 10 min intervals a 1 ml sample was transferred to a scintillation vial containing 2.0 ml 95% (v/v) ethanol/5% (v/v) glacial acetic acid. Samples were evaporated to dryness, resuspended in 1.0 ml distilled water and radioactivity (fixed ¹⁴C) was measured.

Radiotracer studies. Cells were harvested by centrifugation (10000 g for 10 min), washed once with, and resuspended in 10 ml AMS medium to a density of either 10 mg ml^{-1} (for NaH¹⁴CO₃ incorporation) or 5 mg ml^{-1} (for [^{1-¹⁴C]}glycollate incorporation). The suspension, in a 50 ml round-bottomed flask sealed with a rubber serum cap and partially submerged in a water bath at 45 °C, was aerated by stirring with a magnetic follower. After 5 min equilibration, 10 ml methane was injected into the flask followed, after 10 min, by the ¹⁴C substrate [$200\text{ }\mu\text{Ci}$ (7.4 MBq) NaH¹⁴CO₃ or $50\text{ }\mu\text{Ci}$ (1.85 MBq) [^{1-¹⁴C]}glycollate]. At various intervals a 1 ml sample was removed by syringe and added to 4 ml hot (60 °C) absolute ethanol. The resulting precipitate was removed by centrifugation and further extracted with two changes of 50% (v/v) hot ethanol. The supernatants were combined and the volume was reduced to 0.5 ml in a vacuum rotary evaporator (Quickfit).

Radioactive compounds were separated by two-dimensional ascending paper chromatography on Whatman no. 1 chromatography paper. The solvent system was phenol/water/glacial acetic acid/EDTA (1 M) (840:149:10:1, by vol.) in the first dimension and equal volumes of propionic acid/water (620:790, v/v) and 1-butanol/water (1246:84, v/v) in the second dimension. The position of labelled metabolites was determined by exposing the dried chromatograms to 'Kodirex' X-ray film for 1 month. The radioactivity per metabolite was measured by cutting out radioactive areas of the chromatograms and counting these in a liquid scintillation spectrometer using a Triton/toluene scintillation fluid [61 toluene, 31 Triton X-100, 36 g 2,5-diphenyloxazole, 0.45 g 1,4-di-2-(5-phenyloxazoly)benzene]. Radioactive areas from duplicate chromatograms were cut out, eluted with 50% (v/v) ethanol and re-chromatographed with a series of standards. Metabolites were positively identified when they co-chromatographed with the authentic standard. Amino acids were detected with 0.25% (w/v) ninhydrin in acetone, carboxylic acids with 0.1% (w/v) bromocresol green (pH 5.0) in ethanol and phosphorylated compounds by the method of Bandurski & Axelrod (1951).

Chemicals. All chemicals were the best grade available and were obtained from Sigma or BDH. Radiochemicals were purchased from The Radiochemical Centre, Amersham. Before use sodium [^{1-¹⁴C]}glycollate was purified by paper chromatography on Whatman no. 1 paper (Long *et al.*, 1951).

RESULTS AND DISCUSSION

CO₂ fixation by methane-grown M. capsulatus (Bath)

Intact cells, incubated in the presence of methane, showed a linear rate of CO₂ fixation over a 10 to 60 min period of 0.23 $\mu\text{mol CO}_2$ fixed h^{-1} (mg dry wt^{-1}). No significant fixation was observed in the absence of methane indicating the probable energy dependence of CO₂ fixation in this organism. This measured rate is probably an underestimate of the true fixation rate because of (i) dilution of radioactive CO₂ during assay by endogenously produced CO₂ and (ii) the effect of washing and resuspension of cells in fresh growth medium. However, assuming a protein content of 60 to 70% of the dry weight, the measured rate of CO₂ fixation agrees with the specific activity of RuBP carboxylase in cell-free extracts [6 m-unit (mg protein^{-1})]. Taking the formula for cell material to be C₄H₈NO₂ (MacLennan *et al.*, 1971; Goldberg *et al.*, 1976) then the measured rate of CO₂ fixation suggests that approximately 2.5% (w/w) of cell carbon originates from CO₂.

This measure of CO₂ fixation does not differentiate between that catalysed by known heterotrophic reactions and that catalysed by RuBP carboxylase. To investigate further the fate of CO₂, the early time course of labelling of pool metabolites following fixation of ¹⁴CO₂ was examined. The amounts of radioactivity in various metabolites, expressed as percentages of the total radioactivity in the sample at various time intervals, are shown in Table 1.

The decrease with time of the percentage of incorporated label in 3-phosphoglycerate and total phosphate esters indicates that RuBP carboxylase does function *in vivo*. Similar decreases in the percentage of incorporated label in malate, citrate and aspartate indicate that CO₂ is also fixed by carboxylation of C₃ metabolites yielding C₄ dicarboxylic acids and hence, by transamination, aspartate. The pattern of labelling (Table 1) is similar to that reported for many autotrophic bacteria when grown heterotrophically (Fuller *et al.*, 1961; Anderson & Fuller, 1967; Slater & Morris, 1973). Furthermore, the specific activity of RuBP carboxylase in cell-free extracts [6 m-unit (mg protein^{-1})] was similar to that found in cell-free extracts of heterotrophically grown autotrophs.

The potential for autotrophic growth in M. capsulatus (Bath)

Apart from RuBP carboxylase and phosphoribulokinase, the only other essential enzyme of the Calvin cycle which is not present in *M. capsulatus* (Bath) as part of the RMP cycle is

Table 1. *Percentage incorporation of ¹⁴CO₂ into pool metabolites of M. capsulatus (Bath) at 45 °C*

Metabolite	Radioactivity in metabolite (% of total in sample)					
	Sample time:					
	10 s	20 s	40 s	60 s	2 min	4 min
3-Phosphoglycerate	27.3	13.0	20.1	13.7	15.6	4.5
Aspartate	37.7	26.0	23.5	15.8	9.3	3.5
Citrate	14.6	8.5	7.4	7.1	5.3	3.3
Malate	20.4	18.2	15.8	20.5	15.6	8.2
Phosphoenolpyruvate	—	5.9	4.9	3.8	2.8	1.5
Other phosphate esters	—	6.5	4.5	9.7	6.5	6.3
Glutamate	—	8.4	11.4	17.4	26.7	27.5
Alanine	—	7.6	7.4	6.9	6.9	5.6
Succinate	—	5.9	5.0	5.1	5.2	2.5
Glutamine	—	—	—	—	2.0	2.7
Leu/Ile/Phe	—	—	—	—	4.1	28.6
Threonine	—	—	—	—	—	1.5
Serine + Glycine	—	—	—	—	—	1.9
Unidentified compounds	—	—	—	—	—	2.4

Table 2. *Percentage incorporation of [1-¹⁴C]glycollate into pool metabolites of M. capsulatus (Bath)*

Metabolite	Radioactivity in metabolite (% of total in sample)				
	Sample time:				
	1 min	4 min	8 min	12 min	16 min
Aspartate	6.0	10.3	10.1	12.5	15.3
Serine + Glycine	27.4	23.9	17.0	15.1	18.5
Glutamate	15.3	27.5	22.0	21.0	17.7
Glutamine	13.5	3.6	—	—	—
Proline	8.9	—	—	—	—
Valine	6.8	—	—	—	—
Leucine	5.9	—	—	—	—
3-Phosphoglycerate	—	4.8	2.9	1.4	—
Phosphoenolpyruvate	—	4.0	2.3	1.9	1.7
Other phosphate esters	6.5	20.9	28.0	30.6	32.3
Unidentified compounds	9.7	5.0	17.7	17.5	14.5

fructose-1,6-bisphosphatase. This enzyme was detected in the soluble fraction of cell-free extracts at a specific activity of 5 m-unit (mg protein)⁻¹.

Formate, ethanol and hydrogen are all oxidized by *M. capsulatus* (Bath) and may be considered potential electron donors for autotrophic growth (Dalton & Whittenbury, 1976). However, none of these compounds in combination with CO₂ supported detectable growth on incubation for 3 weeks. Therefore, although the enzymes of the Calvin cycle are present in this organism, evidence so far suggests that CO₂ cannot be utilized as the sole source of carbon for growth.

Metabolism of glycollate by M. capsulatus (Bath)

The previously unexplained formation of glycollate from [¹⁴C]methanol (Reed, 1976) may result from the oxygenase activity of RuBP carboxylase (Taylor *et al.*, 1980) in generating phosphoglycollate from RuBP.

The soluble fraction of cell-free extracts of *M. capsulatus* (Bath) does contain a specific phosphoglycollate phosphatase with a specific activity of 37 m-unit (mg protein)⁻¹. This enzyme was not detected in *Methylobacterium methanica* (SI), a type I methylotroph with an RMP cycle but with no RuBP carboxylase.

The further metabolism of glycollate by *M. capsulatus* (Bath) was investigated by examining the incorporation of [1-¹⁴C]glycollate by intact cells. A linear rate of incorporation was observed over 20 min corresponding to 3000 c.p.m. incorporated min⁻¹ (mg dry wt)⁻¹. The time course of labelling is shown in Table 2. The majority of label appearing initially in serine plus glycine probably represents the formation of glycine from glycollate via glyoxylate, the presence of which would not be shown by the chromatography system used. The absence of large amounts of label initially in phosphate esters argues against the tartronic semialdehyde pathway (Kornberg & Gotto, 1961; Codd & Stewart, 1973) being an important route for glycollate metabolism in this organism. The increase in the percentage of incorporated label in phosphate esters probably indicates the conversion of glycine and serine to these compounds. The lack of label in the tricarboxylic acid cycle intermediates suggests that glycollate is not significantly metabolized via either a glyoxylate cycle or β -hydroxyaspartate pathway (Kornberg & Morris, 1965). This then leads to the possibility that glycollate may be incorporated into cell carbon by a pathway similar to that found in higher plants (Fig. 1) and analogous to part of the serine cycle for C₁ assimilation (Large & Quayle, 1963) in type II methylotrophs.

Although the mechanism of a glycine to serine conversion has not been elucidated, the

Fig. 1. Postulated pathway of incorporation of glycollate into cell carbon.

labelling of serine after incorporation of [^{14}C]formate, recorded by both Eccleston & Kelly (1973) and Whittenbury *et al.* (1976), suggests that formate, probably as a tetrahydrofolate derivative, may combine with glycine to give serine.

The presence of hydroxypyruvate reductase in *M. capsulatus* (Bath) (Whittenbury *et al.*, 1976; J. R. Quayle, personal communication), with activities of 40 to 60 m-unit (mg protein) $^{-1}$, has previously been unexplained. This enzyme may be involved in the conversion of serine to phosphate esters, and its requirement in phosphoglycollate metabolism in *M. capsulatus* (Bath) may also be the reason for its apparent 'transvestite' appearance in *Paracoccus denitrificans* growing autotrophically on methanol (Bamforth & Quayle, 1977).

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