Incomplete citric acid cycle obliges aminolevulinic acid synthesis via the C₅ pathway in a methylotroph

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The enzymic activities of the citric acid cycle and the connected pathway of 5-aminolevulinic acid (ALA) formation in the methylotroph *Methylophilus methylotrophus* (strain AS1) have been studied. The organism has the enzymes required for conversion of pyruvate to 2-oxoglutarate. Of these, isocitrate dehydrogenase is unusual because of its preference of NAD as coenzyme over NADP. In addition, the segment of the cycle that oxidizes 2-oxoglutarate to oxaloacetate is incomplete, lacking 2-oxoglutarate and succinate and malate dehydrogenase activities. Furthermore, alternative routes of 2-oxoglutarate oxidation to succinate are undetectable. The enzymes of the glyoxylate cycle are also absent. This suggests that the cycle in *M. methylotrophus* has no catabolic role and is purely biosynthetic. We also show that *M. methylotrophus* uses the C₅ pathway of ALA formation. Cell-free extracts can convert glutamate to ALA in an ATP-, NADPH- and tRNA-dependent manner via the intermediate formation of Glu-tRNA^{Glu} and glutamate 1-semialdehyde. Consistent with the absence of a detectable route by which it could synthesize succinate, *M. methylotrophus* cannot generate ALA from succinyl-CoA and glycine, the pathway found in mammalian cells and yeast.

Introduction

Methylophilus methylotrophus exploits reduced single-carbon compounds such as methanol as sources of energy and cell carbon. The organism is a Gram-negative obligate aerobe (Anthony, 1982; Jenkins *et al.*, 1987). Methanol is oxidized to formaldehyde (Anthony, 1986). NADH, generated by oxidation of formaldehyde by the dissimilatory variant of the ribulose monophosphate cycle, is oxidized by the electron transport chain of the organism (Beardsmore *et al.*, 1982; Anthony, 1986). Consequently, a complete catabolically functioning citric acid cycle has no role in this organism. Indeed, this pathway in *M. methylotrophus* lacks 2-oxoglutarate dehydrogenase, suggesting that its role is purely to generate carbon skeletons for biosynthesis (Taylor, 1977; Large & Haywood, 1981).

Methanol and NADH oxidation involves cytochrome *c*. Thus, there is a large demand for cytochromes which,

indeed, this and other methylotrophs have in large quantities (Anthony, 1986). Thus, the synthesis of the first committed tetrapyrrole precursor, ALA, must proceed at a high rate in *M. methylotrophus*. ALA is synthesized by two pathways stemming from the citric acid cycle (Jahn *et al.*, 1992). In the C₅ pathway of chloroplasts, archaeobacteria and many eubacteria, glutamate, derived from 2-oxoglutarate, is esterified to tRNA^{Glu}. The resulting Glu-tRNA^{Glu} is reduced with NADPH by Glu-tRNA^{Glu} reductase to glutamate 1-semialdehyde which is rearranged to ALA by glutamate-1-semialdehyde 2,1-aminomutase. Alternatively, ALA is synthesized from succinyl-CoA and glycine by ALA synthase. Despite the extensive literature concerning methylotroph cytochrome biochemistry (e.g. Anthony, 1986), synthesis in any methylotroph of the tetrapyrrole cofactors on which these proteins depend has remained unexplored. Likewise, information regarding the citric acid cycle in *M. methylotrophus* is also incomplete. In particular, oxidation of acetyl-CoA to pyruvate and those reactions that interconvert oxaloacetate and succinyl-CoA have yet to be demonstrated.

As *M. methylotrophus* lacks 2-oxoglutarate dehydrogenase, it cannot synthesize succinyl-CoA from 2-oxoglutarate. The ability of *M. methylotrophus* to synthesize these metabolites is germane to the means by which it generates ALA. Thus, our first aim was to

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Abbreviations: ALA, 5-aminolevulinic acid; Glu-tRNA, glutamylated tRNA^{Glu}; PLP, pyridoxal phosphate; SSA, succinic semi-aldehyde.

establish the complete form of the citric acid cycle in *M. methylotrophus*. Stemming from this, our second aim was to characterize the means by which this organism generates ALA.

Methods

Materials. *M. methylotrophus* strain AS1 (Jenkins *et al.*, 1987; NCIB 10515) and *Saccharomyces cerevisiae* strain 3A84 (Weyand-Durasevic *et al.*, 1987) were used in this work. Acetyl-CoA and succinyl-CoA were prepared as in Stadtman (1957) and Simon & Shemin (1953). L-[U-¹⁴C]Glu [281 mCi mmol⁻¹ (10.4 GBq mmol⁻¹)] and [2-¹⁴C]Gly [49 mCi mmol⁻¹ (1.8 GBq mmol⁻¹)] were from Amersham and DuPont NEN respectively. *Escherichia coli* Glu-tRNA synthetase (EC 6.1.1.17), glutamate-1-semialdehyde 2,1-aminomutase (EC 5.4.3.8) and a cell-free extract of *Euglena gracilis* z were generous gifts from Drs K. Rogers, L. Ilag and U. Thomann of Yale University. *E. coli* DH5 α over-expressing the *E. coli* tRNA^{Glu} gene on plasmid (PKR15) was from Dr K. Rogers. *E. coli* tRNA was purified and charged with [¹⁴C]Glu by *E. coli* Glu-tRNA synthetase (Perona *et al.*, 1988). Glutamate 1-semialdehyde was a gift from Dr G. Kanangara (Carlsberg Laboratory, Copenhagen, Denmark).

Culture conditions. *M. methylotrophus* was grown at 37 °C in shake flasks at 250 r.p.m. in minimal salts medium with 0.5% (v/v) methanol (Large & Haywood, 1981).

Extract preparation. *S. cerevisiae* mitochondria were isolated (Jazwinski, 1990), frozen at -20 °C and thawed three times in 20 mM-HEPES (pH 7.2), 1 mM-EDTA, 0.1 mM-PLP, 2 mM-DTT, 0.2 mM-PMSF and 10% (v/v) glycerol. They were then sonicated in four 10 s bursts interspersed by 1 min cooling on ice, and centrifuged at 4 °C for 20 min at 12000 g. The supernatant was retained. *M. methylotrophus* was harvested in late exponential phase by centrifugation at 13300 g. One gram of cells was suspended in 1 ml buffer and sonicated in eight 15 s bursts interspersed by 30 s cooling on ice. A sonication buffer consisting of 20 mM-Tris (pH 8.0), 2.4 mM-EDTA and 10 mM-MgCl₂ (MET-8.0) was used for pyruvate dehydrogenase (EC 1.2.4.1), pyruvate carboxylase (EC 6.4.1.1), citrate synthase (EC 4.1.3.7), aconitase (EC 4.2.1.3), isocitrate dehydrogenase (EC 1.1.1.41/42), malate dehydrogenase (EC 1.1.1.37) and 2-oxoglutarate dehydrogenase (EC 1.2.4.2), for which MET-8.0 and 10% (v/v) glycerol was also used. Likewise, MET-8.0 + 10% (v/v) glycerol was used for isocitrate lyase (EC 4.1.3.1), malate synthase (EC 4.1.3.2), malic enzyme (EC 1.1.1.38/40), 2-oxoglutarate decarboxylase (EC 4.1.1.71) and succinate-semialdehyde (SSA) dehydrogenase (EC 1.2.1.16/24). Potassium phosphate buffer (pH 7.0, 20 mM) was used for glutamine synthetase (EC 6.3.1.2), glutamine-oxoglutarate aminotransferase (EC 1.4.1.14/13), succinate thiokinase (EC 6.2.1.5) and malate oxidase (EC 1.1.3.3); 0.1 M-potassium phosphate, pH 7.5, was used for succinate dehydrogenase (EC 1.3.99.1). Fumarase (EC 4.2.1.2) was extracted in 0.1 M-Tris (pH 8.6), 20 mM-MgCl₂, 5 mM-EDTA and 20% (v/v) glycerol. A buffer consisting of 20 mM-HEPES (pH 7.9), 20 mM-KCl, 10 mM-MgCl₂, 1 mM-EDTA, 10% (v/v) glycerol, 3 mM-DTT and 0.2 mM-PMSF was used for enzymes generating ALA.

For studies of ALA synthesis, sonicates were centrifuged at 100000 g for 90 min at 4 °C and the supernatant was retained. For C₅ pathway studies, supernatants, with the exception of those used in glutamate-1-semialdehyde 2,1-aminomutase assays, were dialysed into 20 mM-HEPES (pH 7.2), 10 mM-MgCl₂ and 20% (v/v) glycerol (ALA assay medium). For aminomutase assays, extracts were dialysed in darkness into 30 mM-bisTris (pH 6.5), 10 mM-MgCl₂, 2.5 mM-DTT, 25% (v/v) glycerol, 20 μ M-PLP (GSA-AM assay medium). For ALA

synthase (EC 2.3.1.37) assays, supernatants were dialysed in the dark into ALA assay medium and 0.1 mM-PLP. For other assays, the sonicate was centrifuged at 12000 g for 30 min at 4 °C. The supernatant and as required, the pellet, were retained for assay.

Protein estimations. Protein was estimated according to Bradford (1976).

Units. ALA-synthesizing activities are in pmol ALA formed (mg of protein)⁻¹. As recovery of ALA varied depending on how it was purified (below), all ALA-synthesizing activity is normalized to an ALA recovery of 100%. All other enzyme activities are in units (U) where 1 U is defined as formation or consumption of 1 μ mol product or substrate min⁻¹.

Assays for [¹⁴C]ALA formation. Assays were carried out at 30 °C for 1 h using [¹⁴C]Glu or [¹⁴C]Gly in a final vol. of 0.25 ml in ALA assay medium. Radioactive ALA was quantified by liquid scintillation counting. Counting efficiencies were between 74 and 97%. Whatever the label, no more than 77 c.p.m. accumulated in the buffer blank.

(i) **ALA synthase.** ALA assay medium was supplemented with 5 mM-levulinic acid, 0.56 mM-succinyl-CoA, 1 μ Ci (37 kBq) [¹⁴C]Gly [80 c.p.m. (pmol)⁻¹; 81.2 μ M], 0.1 mM-PLP, 1 mM-DTT and 50 μ g bovine serum albumin ml⁻¹. Assays were initiated by addition of extract and incubated for 1 h at 30 °C. The reaction was terminated by adding 0.25 ml 10% (w/v) SDS, 50 μ l 1 M-citric acid and 20 nmols unlabelled ALA. Samples were boiled for 5 min. ALA was further purified and derivatized exactly as in Avissar *et al.* (1989). The yield of ALA, measured according to Mauzerall & Granick (1956), was 40%.

(ii) **ALA from glutamate.** ALA assay medium was supplemented by 5 mM-levulinic acid, 1 μ Ci (37 kBq) [¹⁴C]Glu [173 c.p.m. (pmol)⁻¹; 50 μ M], 0.1 mM-PLP, 1 mM-DTT and, when required, 2.5 mM-ATP and/or 1 mM-NADPH. Assays were then carried out exactly as described for ALA synthase (i, above).

(iii) **Glu-tRNA reductase.** Assays were done as described for synthesis of ALA from glutamate apart from the following: ATP and [¹⁴C]Glu were replaced with 10000 c.p.m. [152 c.p.m. (pmol)⁻¹; 0.26 μ M] of [¹⁴C]Glu-tRNA and 1 mM-unlabelled Glu. The assays were also supplemented by *E. coli* glutamate-1-semialdehyde 2,1-aminomutase (4 μ g ml⁻¹) and bovine serum albumin (50 μ g ml⁻¹). With the smaller quantities of ¹⁴C used here, it was unnecessary to wash the columns with 0.2 M-sodium citrate, pH 4.25, as in Avissar *et al.* (1989). Under these circumstances, recovery of ALA was 85%. This assay showed lower activity than assay (ii), probably due to the inherent instability of [¹⁴C]Glu-tRNA in the reductase assay, whereas in the assay of ALA formation from glutamate, Glu-tRNA was continually regenerated.

Glutamate-1-semialdehyde 2,1-aminomutase. Assays were carried out at 28 °C in a final vol. of 0.5 ml. GSA-AM assay medium was supplemented with 10 mM-levulinic acid and 10 μ M-glutamate 1-semialdehyde. Reactions were initiated by extract, or for controls, by 5 mg bovine serum albumin. Samples were taken at intervals up to 6 min and processed as described by Hooper *et al.* (1988).

Isocitrate lyase. Three methods were employed. (a) By following the isocitrate-dependent generation of phenylhydrazones species at 25 °C in 1 ml 50 mM-MOPS, pH 7.3, 1 mM-EDTA, 4 mM-phenylhydrazine and 4 mM-DL-isocitrate as an increase in absorbance at 324 nm. (b) By carrying out the incubation at 30 °C for 30 min using the incubation mixture in (a) in a final vol. of 3 ml without phenylhydrazine. The reaction was terminated by the addition of 0.1 ml conc. HCl, and the reaction products were then converted to their 2,4-dinitrophenylhydrazones, isolated by ethyl acetate extraction and analysed by TLC on cellulose as described by Salem *et al.* (1973). (c) By carrying out an incubation of the same reaction mixture described in (a) for 30 min at 30 °C. The reaction was terminated by boiling, and any glyoxylate phenylhydrazone formed was converted to 1,5-diphenylformazan

carboxylic acid (Attwood & Harder, 1977). The formazan has an absorbance maximum at 520 nm and a standard calibration curve for glyoxylate 1,5-diphenylformazan carboxylic acid yielded an absorption coefficient of $3.9 \times 10^3 \text{ l mol}^{-1} \text{ cm}^{-1}$.

Other enzyme assays. All other assays were performed at 25 °C. Citrate synthase, aconitase, isocitrate dehydrogenase (NAD^+ or NADP^+ as cofactor), malate dehydrogenase (following malate production), 2-oxoglutarate dehydrogenase and succinate and pyruvate dehydrogenases were assayed according to Barnes & Weitzman (1986). Glutamate-oxoglutarate aminotransferase, glutamine synthetase, malate oxidase, pyruvate carboxylase, succinate thiokinase, malic enzyme (NADH or NADPH as cofactor), SSA dehydrogenase (NAD^+ or NADP^+ as cofactor) and malate synthase were assayed according to Windass *et al.* (1980), Shapiro & Stadtman (1970), Francis *et al.* (1963), Aperghis (1981), Cha (1969), Weitzman & Jaskowska-Hodges (1982), Frenkel (1972), Tokunaga *et al.* (1976) and Dixon & Kornberg (1962).

Fumarase was assayed by following 2-thio-5-nitrobenzoic acid production in MET-8-0 adjusted to pH 8.6, 0.5 mM- NAD^+ , 0.2 mM-acetyl-CoA, 0.1 mM-5,5'-dithiobis(2-nitrobenzoic acid), 5.8 U pig-heart citrate synthase, 60 U pig-heart malate dehydrogenase and 1 mM-fumarate. Malate dehydrogenase was assayed by following the formation of oxaloacetate in 20 mM-potassium phosphate (pH 7.5), 0.5 mM- NAD^+ or 0.2 mM- NADP^+ , 0.2 mM-acetyl-CoA, 0.1 mM-5,5'-dithiobis(2-nitrobenzoic acid), 5.8 U porcine-heart citrate synthase and 1 mM-malate. 2-Oxoglutarate decarboxylase was monitored by coupling the thiamin pyrophosphate and 2-oxoglutarate-dependent generation of SSA to NADP^+ reduction by SSA dehydrogenase. The assay mixture consisted of 50 mM-potassium phosphate and 1 mM- MgCl_2 adjusted to pH 7, 0.2 mM-thiamin pyrophosphate, 0.5 mM- NADP^+ , 0.1 U *Pseudomonas fluorescens* SSA dehydrogenase and 3 mM-2-oxoglutarate.

A wild-type *E. coli* DH5 α cell-free extract was employed as a positive control for assays of 2-oxoglutarate dehydrogenase, malic enzyme, isocitrate lyase (assay c) and malate synthase (data not shown). Likewise, an *E. gracilis* cell-free extract was used as a positive control for 2-oxoglutarate decarboxylase and NAD(P)-linked SSA dehydrogenase (data not shown).

Results

M. methylotrophus synthesizes glutamate but not succinyl-CoA by the citric acid cycle

Extracts of *M. methylotrophus* contained pyruvate carboxylase and pyruvate dehydrogenase activities (Table 1), suggesting that pyruvate can be disproportionated to acetyl-CoA and oxaloacetate. All enzymes, including aconitase, required for the conversion of acetyl-CoA and oxaloacetate to 2-oxoglutarate were also demonstrable (Table 1). The isocitrate dehydrogenase involved in this process was predominantly NAD-linked. The organism contained no 2-oxoglutarate dehydrogenase when extracted in MET-8-0. The presence of glycerol has been shown to stabilize the activity in crude extracts of other organisms (Bessam *et al.*, 1989). However, no activity could be detected even if extracts were prepared in MET-8-0+10% (v/v) glycerol. This suggests that succinyl-CoA cannot be synthesized directly from acetyl-CoA and oxaloacetate by conventional citric acid cycle reactions (Table 1).

Recently, it has become apparent that the phytoflagellate *E. gracilis* converts 2-oxoglutarate to succinate by the decarboxylation of 2-oxoglutarate to SSA and oxidation of the latter to succinate (Tokunaga *et al.*, 1976; Shigeoka & Nakano, 1991). The enzymes catalysing these reactions, 2-oxoglutarate decarboxylase and NADP- or NAD-linked SSA dehydrogenase, were therefore assayed in *M. methylotrophus* to determine if they could provide a mechanism for the generation of succinate and thus, succinyl-CoA from 2-oxoglutarate. However, both enzymes were undetectable in *M. methylotrophus* (Table 1).

Of the enzymes required for interconversion of succinyl-CoA and oxaloacetate, a low level of ADP-linked succinate thiokinase was detectable by both the spectrophotometric assay of Cha (1969) and the polarographic assay of Weitzman & Jaskowska-Hodges (1982). No other nucleoside diphosphate supported thiokinase activity (Table 1). Fumarase was detectable; however succinate dehydrogenase was not in either the supernatant or the insoluble material of *M. methylotrophus* extracts (Table 1). NAD- and NADP-linked malate dehydrogenase activities were not detectable with any of the assays used. Likewise, malate oxidase was absent from the supernatant and insoluble fraction (Table 1). The possibility that malate could be generated from carboxylation of pyruvate by malic enzyme remained. However, neither enzyme could be detected in *M. methylotrophus* (Table 1). Thus, it seems *M. methylotrophus* cannot synthesize succinyl-CoA from either reduction of oxaloacetate or reductive carboxylation of pyruvate.

2-Oxoglutarate was converted to glutamate by glutamine synthetase and glutamine oxoglutarate aminotransferase. The latter was found to be specific for NADH.

Succinate is not generated by the glyoxylate cycle in M. methylotrophus

The foregoing suggested that the citric acid cycle could not generate succinate. It was, however, conceivable that the presence of the glyoxylate cycle enzymes, isocitrate lyase and malate synthase, might perform this function. Given that the ability to synthesize either glutamate or succinate could be germane to the mechanism of ALA synthesis in *M. methylotrophus*, assessment of glyoxylate cycle enzyme activity was pursued.

Initial assays of *M. methylotrophus* extracts for isocitrate lyase, ostensibly following the isocitrate-dependent generation of glyoxylate phenylhydrazone (assay a), gave an apparently low activity of $0.4 \pm 0.1 \text{ mU (mg protein)}^{-1}$ ($n = 3$). However, as Anthony (1982) and Attwood & Harder (1977) have noted, such assays are

Table 1. *Citric acid cycle and glutamate-synthesizing enzymes of M. methylotrophus*Data shown are means $\pm$ standard error of the mean; n = number of cultures assayed.

Enzyme	Specific activity [mU (mg protein) ⁻¹]
Pyruvate carboxylase	6.1 $\pm$ 0.7 (n = 3)
Pyruvate dehydrogenase	36.1 $\pm$ 9.0 (n = 3)
Citrate synthase	28.0 $\pm$ 4.6 (n = 3)
Aconitase	2.0 $\pm$ 0.4 (n = 3)
Isocitrate dehydrogenase:	
NAD-linked	19.4 $\pm$ 4.1 (n = 3)
NADP-linked	2.0 $\pm$ 0.1 (n = 3)
2-Oxoglutarate dehydrogenase:	
extracted with or without glycerol	0.0 (n = 3) and 0.0 (n = 3)
2-Oxoglutarate decarboxylase	0.0 (n = 3)
Succinate thiokinase:	
ADP-linked (polarographic assay)	1.0 (n = 2)
ATP-linked (spectrophotometric assay)	1.7 (n = 2)
GDP- and IDP-linked (polarographic assay)	0.0 (n = 2)
Succinate dehydrogenase:	
supernatant and pellet	0.0 (n = 1 and 1)
Succinic semialdehyde dehydrogenase:	
NAD-linked	0.0 (n = 3)
NADP-linked	0.0 (n = 3)
Fumarase	47.3 $\pm$ 9.7 (n = 3)
Malate dehydrogenase, linked to:	
NAD ⁺ or NADP ⁺ (malate oxidation)	0.0 (n = 1)
NADH or NADPH (oxaloacetate reduction)	0.0 (n = 2)
Malic enzyme:	
NADH-linked	0.0 (n = 3)
NADPH-linked	0.0 (n = 3)
Malate oxidase (supernatant or pellet)	0.0 (n = 1 and 1)
Glutamine synthetase	1086 $\pm$ 87 (n = 3)
Glutamine-oxoglutarate aminotransferase:	
NADH-linked	47.2 (n = 2)
NADPH-linked	0.0 (n = 2)

difficult to interpret, as contamination of the extract with NAD(P)⁺ would, with isocitrate, allow isocitrate dehydrogenase to synthesize 2-oxoglutarate. This would also generate a phenylhydrazone which, like glyoxylate phenylhydrazone, would absorb at 324 nm.

Therefore, the assay was repeated such that after incubation with 12.6–14.4 mg protein, 2,4-dinitrophenylhydrazones of the products of the reaction were formed and analysed by TLC (assay *b*). Standard R_f values for the *cis* and *trans* isomers of glyoxylate 2,4-phenylhydrazone (Kun & Garcia-Hernandez, 1957) and 2-oxoglutarate 2,4-dinitrophenylhydrazone were 0.42 ± 0.03 , 0.61 ± 0.02 and 0.24 ± 0.03 (n = 3 runs each), respectively. Analysis of incubations of three separate extracts with or without isocitrate showed that the only 2,4-dinitrophenylhydrazone formed on addition of isocitrate migrated with an R_f of 0.21 ± 0.03 (n = 3 extracts). The data seemed to suggest that the result of assay (*a*) was due to generation of 2-oxoglutarate phenylhydrazone.

As assay (*b*) involved hydrazone formation after termination of the incubation, it was possible that any

glyoxylate formed was further metabolized during the incubation. Therefore, assay (*c*), which includes phenylhydrazine in the incubation and therefore traps carbonyl compounds as they are formed, was used. This procedure is specific in that glyoxylate phenylhydrazone, but not 2-oxoglutarate phenylhydrazone will react to generate a formazan, thereby preventing interference in the assay by isocitrate dehydrogenase (Kramer *et al.*, 1959). In agreement with the TLC data, no isocitrate lyase activity could be detected in *M. methylotrophus* extracts (n = 3 cultures). Likewise, malate synthase was undetectable in *M. methylotrophus* extracts (n = 3 cultures).

Thus, it seems that *M. methylotrophus* cannot generate succinate via either the citric acid cycle or glyoxylate cycle.

M. methylotrophus uses the C₅ pathway of ALA synthesis from glutamate

Our data suggested that the logical precursor for ALA was glutamate. Indeed, incubation of *M. methylotrophus* extracts with ATP, NADPH and [¹⁴C]Glu led to

Table 2. *ALA synthesis from glutamate by M. methylotrophus extracts*

Activities are the mean of duplicate incubations. Incubations were carried out with 3.2 mg of protein.

Incubation conditions	ALA synthesized [pmol (mg protein) ⁻¹]
Extract + [¹⁴ C]Glu + ATP + NADPH	18.7
Extract + [¹⁴ C]Glu + ATP + NADPH + RNAase A*	0.3
Extract + [¹⁴ C]Glu - ATP + NADPH	1.1
Extract + [¹⁴ C]Glu + ATP - NADPH	2.5

* Extract was pre-incubated for 15 min in the presence of 2.9 µg RNAase A ml⁻¹ at 30 °C. The assay was then initiated by addition of appropriately supplemented assay medium.

significant incorporation of label into ALA (Table 2). Omission of ATP, which prevents the formation of Glu-tRNA, almost abolished ALA formation. Likewise, omission of NADPH, the cofactor for Glu-tRNA reductase, caused a 85% drop in ALA synthesis (Table 2). Pre-incubation of the extract with RNAase A abolished activity (Table 2). This shows that *M. methylotrophus* can synthesize ALA from glutamate in an RNA-dependent manner. To expand this observation we wanted to test if the two enzymes of the C₅ pathway could be detected. When *M. methylotrophus* extracts were incubated with *E. coli* [¹⁴C]Glu-tRNA, significant ALA synthesis was observed (Table 3). ALA formation was heavily dependent on NADPH (the low activity in the absence of the cofactor was probably due to tightly bound endogenous NADPH). Again, the [¹⁴C]Glu to [¹⁴C]ALA conversion was abolished by RNAase A treatment (Table 3), demonstrating the critical role of intact tRNA. Taken together the data show that Glu is acylated onto tRNA which then is used as a substrate for Glu-tRNA reductase. Furthermore, we were able to detect glutamate-1-semialdehyde 2,1-aminomutase, which was present at a specific activity of 5.2 pmol min⁻¹ (mg protein)⁻¹. Thus, the data clearly demonstrate that *M. methylotrophus* converts Glu to ALA, using the C₅ pathway, via the intermediate formation of Glu-tRNA and glutamate 1-semialdehyde.

There is no or negligible ALA synthase activity in M. methylotrophus

The operation of the C₅ pathway in the absence of 2-oxoglutarate dehydrogenase and other citric acid cycle enzymes led us to suspect that this organism lacked ALA synthase. It was crucial, therefore, that the ALA synthase assays included a positive control. Thus, the *S. cerevisiae* mitochondrial supernatant showed that the assay

Table 3. *M. methylotrophus extract Glu-tRNA reductase activity*

Activities are the mean of duplicate incubations; 3.2 mg protein was added per assay.

Incubation conditions	ALA synthesized [pmol (mg protein) ⁻¹]
Extract + [¹⁴ C]Glu-tRNA + NADPH	3.2
Extract + [¹⁴ C]Glu-tRNA + NADPH + RNAase A*	0.01
Extract + [¹⁴ C]Glu-tRNA - NADPH	0.4

* [¹⁴C]Glu-tRNA in the assay mix was incubated with 2.9 µg of RNAase A ml⁻¹ for 15 min at 30 °C. The assay was then initiated by the addition of extract.

Table 4. *ALA synthesis from succinyl-CoA and glycine*

Activities are the mean of values from duplicate incubations. The *M. methylotrophus* assays were carried out with 2.8 mg of protein. The *S. cerevisiae* assays were carried out with 1.53 mg protein.

Incubation conditions	ALA synthesized [pmol (mg protein) ⁻¹]	
	<i>M. methylotrophus</i>	<i>S. cerevisiae</i> mitochondrial extract
Extract + 0.56 mM-succinyl-CoA + 81.2 µM-[¹⁴ C]Gly	1.5	43.4
Extract + 0.50 mM-succinate + 0.5 mM-CoA + 81.2 µM-[¹⁴ C]Gly	1.0	13.7
Extract + 0.5 mM-CoA + 81.2 µM-[¹⁴ C]Gly	0.9	4.0

detected ALA synthase. With succinyl-CoA, glycine was incorporated into ALA by the extract (Table 4) in a manner largely dependent on the integrity of the succinyl-CoA thioester bond, as succinate and CoA supported less than one-third of the activity supported by succinyl-CoA (Table 4). This latter activity may have been due to tightly bound mitochondrial ATP which, with the succinate and CoA supplied, would have enabled endogenous succinate thiokinase to support succinyl-CoA synthesis. Evidence for this possibility is that with CoA alone, ALA synthesis was less than 10% of that obtained with succinyl-CoA (Table 4).

M. methylotrophus extracts behaved completely differently. In the presence of succinyl-CoA, ALA synthesis was below 4% of that by *S. cerevisiae* mitochondria (Table 4). Replacement of succinyl-CoA by succinate and CoA or CoA alone only slightly reduced accumulation of [¹⁴C]ALA (Table 4). Thus, the negligible activities in Table 4 were largely independent of the thioester bond of succinyl-CoA and so unlikely to be due to ALA synthase. A comparison of the data in Tables 2

Fig. 1. Synthesis of ALA and its precursors in *M. methylotrophus*.

and 4 clearly suggests that only the C₅ pathway of ALA formation functions *in vivo* in *M. methylotrophus*.

Discussion

The results above complement earlier work on the citric acid cycle of *M. methylotrophus*. The demonstration of pyruvate dehydrogenase and aconitase activities extends current data (Aperghis, 1981; Taylor, 1977; Windass *et al.*, 1980). Thus *M. methylotrophus* has all the enzymes needed to oxidize pyruvate to 2-oxoglutarate and convert the latter to glutamate (Fig. 1). Failure to detect 2-oxoglutarate dehydrogenase agrees with previous data from this and similar methylotrophs (Large & Haywood, 1981; Taylor, 1977; Davey *et al.*, 1972; Colby & Zatman, 1975; Trotsenko, 1976). There are two alternative routes

of 2-oxoglutarate oxidation to succinate: the concerted activities of 2-oxoglutarate decarboxylase and SSA dehydrogenase, and the 4-aminobutyric acid bypass that links glutamate to the citric acid cycle at succinate. Both routes operate in *E. gracilis* and both are terminated by SSA dehydrogenase (Shigeoka & Nakano, 1991; Tokunaga *et al.*, 1979). However, the absence of these enzymes from *M. methylotrophus* makes the operation of either pathway unlikely. The apparent inability to oxidize 2-oxoglutarate is consistent with the idea that in this and related organisms, the citric acid cycle is purely biosynthetic (Colby & Zatman, 1975; Anthony, 1982).

The presence of isocitrate dehydrogenase activity is novel for a prokaryote as it is mostly NAD-linked (Ragland *et al.*, 1966). The low NADP-linked activity in *M. methylotrophus* is due to the same enzyme responsible

for the NAD-linked activity (A. J. Lloyd & P. D. Weitzman, unpublished). This specificity is very rare amongst prokaryotes (Ragland *et al.*, 1966), but it is found among metabolically related methylotrophs with incomplete citric acid cycles (e.g. Davey *et al.*, 1972; Colby & Zatman, 1975; Trotsenko, 1976).

The sequence of enzymes needed to interconvert succinyl-CoA and oxaloacetate lacked succinate and malate dehydrogenases. Malic enzyme, which allows a bypass of malate dehydrogenase in organisms such as *Pseudomonas aeruginosa* that lack the latter enzyme (Golovina & Maltseva, 1985), was also undetectable in *M. methylotrophus*. Furthermore, *M. methylotrophus* appeared to lack isocitrate lyase and malate synthase. Thus, how succinate thiokinase and fumarase obtain their substrates is unclear (Fig. 1). Similar situations exist for other closely related methylotrophs (Davey *et al.*, 1972; Colby & Zatman, 1975; Trotsenko, 1976). It is thus difficult to envisage how *M. methylotrophus* synthesizes succinyl-CoA.

Consistent with the ability of the *M. methylotrophus* citric acid cycle to generate glutamate, ALA was synthesized via the C₅ pathway (Fig. 1). The evidence for this is that conversion of glutamate to ALA was dependent on ATP, NADPH and endogenous RNA satisfying the substrate and coenzyme requirements of Glu-tRNA synthetase and Glu-tRNA reductase (Jahn *et al.*, 1992). *M. methylotrophus* Glu-tRNA reductase accepted pre-charged *E. coli* Glu-tRNA as a substrate, which is consistent with the heterologous tRNA recognition of other reductases (reviewed in Jahn *et al.*, 1992). Finally, *M. methylotrophus* could convert glutamate 1-semialdehyde to ALA, demonstrating the presence of the final enzyme of the C₅ pathway, glutamate-1-semialdehyde 2,1-aminomutase.

Consistent with the metabolic architecture of the citric acid cycle is the lack of ALA synthase in *M. methylotrophus* extracts. This prevents synthesis of ALA from succinyl-CoA and is consistent with the apparent inability of the citric acid cycle to generate succinate (Fig. 1).

It is becoming increasingly clear that the microbial C₅ pathway is widely distributed. It has been found in photosynthetic bacteria, cyanobacteria, methanogenic archaeobacteria and obligately aerobic and anaerobic heterotrophic bacteria (Avissar *et al.*, 1989; Friedmann & Thauer, 1986; Oh-hama *et al.*, 1991; O'Neill *et al.*, 1989; Jahn *et al.*, 1992). However, this is the first report we are aware of concerning the functioning of the C₅ pathway of ALA synthesis in methylotrophs. The mechanism of ALA synthesis in *M. methylotrophus* is consistent with the phylogenetic distribution of the pathways of ALA synthesis. 16S rRNA sequence analysis has assigned *M. methylotrophus* to the β -subdivision

of the purple bacteria (Tsuji *et al.*, 1990). Other bacteria in this subdivision use only the C₅ pathway (Avissar *et al.*, 1989). It will be of interest to see if these phylogenetic relationships extend to other methylotrophs.

Metabolic rationales have been devised for the distribution of bacterial ALA synthesis pathways. Possession of ALA synthase has been linked by Avissar *et al.* (1989) to an anticipated ability of CO₂-fixing organisms to maintain high intracellular pools of glycine, derived from by-products of the Calvin cycle, relative to glutamate. Unfortunately, Avissar *et al.* (1989) provided no data to support this hypothesis. Oh-hama *et al.* (1986) proposed that the C₅ pathway operated in those organisms that did not generate succinyl-CoA from 2-oxoglutarate. However, studies with aerobically grown *E. coli*, *Bacillus subtilis* and the anaerobe *Propionibacterium shermanii* indicate that the ALA synthesis pathway employed does not always reflect the ability of an organism to generate 2-oxoglutarate from succinyl-CoA (Menon & Shemin, 1967; Gottschalk, 1986; O'Neill *et al.*, 1989). As not only lack of 2-oxoglutarate dehydrogenase, but also the lack of other enzymes precludes succinyl-CoA synthesis in *M. methylotrophus*, we maintain that simply the inability of this methylotroph to synthesize succinyl-CoA prevents it from employing ALA synthase as a means of generating ALA.

From a biotechnological viewpoint, ALA synthesis is important as it is the initial step in the synthesis of vitamin B₁₂. Some methylotrophs do excrete vitamin B₁₂ (Large & Bamforth, 1988; Anthony, 1982) but in quantities that are less than 10% of those excreted by *P. shermanii* and *Pseudomonas denitrificans*, both of which are exploited industrially for this purpose (Florent, 1986). Interestingly, the latter organisms possess ALA synthase (Florent, 1986). No data are available regarding vitamin B₁₂ production by *M. methylotrophus*. Whether the C₅ pathway would allow synthesis of significant amounts of vitamin B₁₂ in a methylotroph remains to be investigated.

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References

- ANTHONY, C. (1982). *The Biochemistry of Methylotrophs*. London: Academic Press.
- ANTHONY, C. (1986). Bacterial oxidation of methane and methanol. *Advances in Microbial Physiology* **27**, 113–210.
- APERGHIS, P. N. G. (1981). *Studies on the intermediary metabolism of Methylophilus methylotrophus*. PhD thesis, University of Sheffield, UK.
- ATTWOOD, M. M. & HARDER, W. (1977). Isocitrate lyase activity in *Hyphomicrobium* spp.: a critical reappraisal. *FEMS Microbiology Letters* **1**, 25–30.

- AVISSAR, Y. J., ORMERON, J. G. & BEALE, S. I. (1989). Distribution of δ -aminolevulinic acid biosynthetic pathways among phototrophic bacterial groups. *Archives of Microbiology* **151**, 513–519.
- BARNES, S. J. & WEITZMAN, P. D. J. (1986). Organization of citric acid cycle enzymes into a multienzyme cluster. *FEBS Letters* **201**, 267–269.
- BEARDSMORE, A. J., APERGHIS, P. N. G. & QUAYLE, J. R. (1982). Characterization of the assimilatory and dissimilatory pathways of carbon metabolism during growth of *Methylophilus methylotrophus* on methanol. *Journal of General Microbiology* **128**, 1423–1439.
- BESSAM, H., MARECK, A. & FOUCHER, B. (1989). *Neurospora crassa* α -ketoglutarate dehydrogenase complex: description, resolution of components and catalytic properties. *Biochimica et Biophysica Acta* **990**, 66–72.
- BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.
- CHA, S. (1969). Succinate thiokinase from pig heart. *Methods in Enzymology* **13**, 62–69.
- COLBY, J. & ZATMAN, L. J. (1975). Tricarboxylic acid cycle and related enzymes in restricted facultative methylotrophs. *Biochemical Journal* **148**, 505–511.
- DAVEY, J. F., WHITTENBURY, R. & WILKINSON, J. F. (1972). The distribution in the methylotrophs of some key enzymes concerned with intermediary metabolism. *Archives of Microbiology* **87**, 359–366.
- DIXON, G. H. & KORNBERG, H. L. (1962). Malate synthase from baker's yeast. *Methods in Enzymology* **5**, 633–637.
- FLORENT, J. (1986). Vitamins. In *Biotechnology*, vol. 4, pp. 115–158. Edited by H. Pape & H. J. Rehm. Weinheim: VCH Verlagsgesellschaft.
- FRANCIS, M. J. O., HUGHES, D. E., KORNBERG, H. L. & PHIZACKERLY, P. J. R. (1963). The oxidation of L-malate by *Pseudomonas* sp. *Biochemical Journal* **89**, 430–438.
- FRENKEL, R. (1972). Bovine heart malic enzyme. II. The reversibility of the reaction. *Journal of Biological Chemistry* **247**, 5569–5572.
- FRIEDMANN, H. C. & THAUER, R. K. (1986). Ribonuclease-sensitive δ -aminolevulinic acid formation from glutamate in cell extracts of *Methanobacterium thermoautotrophicum*. *FEBS Letters* **207**, 84–87.
- GOLOVENA, L. A. & MALTSEVA, O. V. (1985). Characterization of the central metabolism of *Pseudomonas* degrading xenobiotics. In *Environmental Regulation of Microbial Metabolism*, pp. 97–104. Edited by I. S. Kulaev, E. A. Dawes & D. W. Tempest. London: Academic Press.
- GOTTSCALK, G. (1986). *Bacterial Metabolism*. New York: Springer-Verlag.
- HOOBER, J. K., KAHN, A., ASH, D. E., GOUGH, S. & KANANGARA, C. G. (1988). Biosynthesis of δ -aminolevulinic acid in greening barley. IX. Structure of the substrate, mode of gabaculine inhibition, and the catalytic mechanism of glutamate 1-semialdehyde aminotransferase. *Carlsberg Research Communications* **53**, 11–25.
- JAHN, D., VERKAMP, E. & SÖLL, D. (1992). Glutamyl-transfer RNA: a precursor of heme and chlorophyll biosynthesis. *Trends in Biochemical Sciences* **17**, 215–218.
- JAZWINSKI, S. M. (1990). Preparation of extracts from yeast. *Methods in Enzymology* **182**, 154–174.
- JENKINS, O., BYROM, D. & JONES, D. (1987). *Methylophilus*: a new genus of methanol-utilizing bacteria. *International Journal of Systematic Bacteriology* **37**, 446–448.
- KRAMER, D. N., KLEIN, N. & BASELICE, R. A. (1959). Quantitative determination of glyoxylic acid. *Analytical Chemistry* **31**, 250–252.
- KUN, E. & GARCIA-HERNANDEZ, M. (1957). Identification and quantitative determination of keto acids by paper chromatography. *Biochimica et Biophysica Acta* **23**, 181–186.
- LARGE, P. J. & HAYWOOD, G. W. (1981). *Methylophilus methylotrophus* grows on methylated amines. *FEMS Microbiology Letters* **11**, 207–209.
- LARGE, P. J. & BAMFORTH, C. W. (1988). *Methylotrophy and Biotechnology*. Essex, UK: Longman Scientific and Technical.
- MAUZERALL, A. & GRANICK, S. (1956). The occurrence and determination of δ -aminolevulinic acid and porphobilinogen in urine. *Journal of Biological Chemistry* **219**, 435–446.
- MENON, A. & SHEMIN, D. (1967). Concurrent decreases in enzyme activities concerned with the synthesis of coenzyme B₁₂ and of propionic acid in propionibacteria. *Archives of Biochemistry and Biophysics* **121**, 304–310.
- OH-HAMA, T., SETO, H. & MIYACHI, S. (1986). ¹³C NMR evidence for bacteriochlorophyll *c* formation in green sulfur bacterium, *Prosthecochloris*. *European Journal of Biochemistry* **159**, 189–194.
- OH-HAMA, T., STOLOWICH, N. J. & SCOTT, A. I. (1991). Characterization of the process of 5-aminolevulinic acid formation from glutamate via the C₅ pathway in *Clostridium thermoaceticum*. *International Journal of Biochemistry* **23**, 1417–1420.
- O'NEILL, G. P., CHEN, M.-W. & SÖLL, D. (1989). δ -Aminolevulinic acid biosynthesis in *Escherichia coli* and *Bacillus subtilis* involves formation of glutamyl-tRNA. *FEMS Microbiology Letters* **60**, 255–260.
- PERONA, J. J., SWANSON, R., STEITZ, T. A. & SÖLL, D. (1988). Overproduction and purification of *Escherichia coli* tRNA₂^{Gln} and its use in the crystallization of the glutamyl-tRNA synthetase: tRNA^{Gln} Complex. *Journal of Molecular Biology* **202**, 121–126.
- RAGLAND, T. E., KAWASAKI, T. & LOWENSTEIN, T. M. (1966). Comparative aspects of some bacterial dehydrogenases and transhydrogenases. *Journal of Bacteriology* **91**, 236–244.
- SALEM, A. R., HACKING, A. J. & QUAYLE, J. R. (1973). Cleavage of malyl-CoA into acetyl-CoA and glyoxylate by *Pseudomonas* AM1 and other C₁-unit-utilizing bacteria. *Biochemical Journal* **136**, 89–96.
- SHAPIRO, M. & STADTMAN, E. R. (1970). Glutamine synthetase (*Escherichia coli*). *Methods in Enzymology* **17A**, 910–922.
- SHIGEOKA, S. & NAKANO, Y. (1991). Characterization and molecular properties of 2-oxoglutarate decarboxylase from *Euglena gracilis*. *Archives of Biochemistry and Biophysics* **288**, 22–28.
- SIMON, E. J. & SHEMIN, D. (1953). The preparation of S-succinyl coenzyme A. *Journal of the American Chemical Society* **75**, 2520.
- STADTMAN, E. R. (1957). Preparation and assay of acyl coenzyme A and other thioesters. Use of hydroxylamine. *Methods in Enzymology* **3**, 931–932.
- TAYLOR, I. J. (1977). Carbon dissimilation by *Methylophilus methylotrophus* – the I.C.I. SCP organism. In *Microbial Growth on C₁ Compounds. Proceedings of the 2nd International Symposium*, pp. 52–54. Edited by G. K. Skyrabin. Pushchino: USSR Academy of Sciences.
- TOKUNAGA, M., NAKANO, Y. & KITAOKA, S. (1976). Separation of the NAD-linked and NADP-linked isoenzymes of succinic semialdehyde dehydrogenase in *Euglena gracilis* z. *Biochimica et Biophysica Acta* **429**, 55–62.
- TOKUNAGA, M., NAKANO, Y. & KITAOKA, S. (1979). Subcellular localization of the GABA-shunt enzymes in *Euglena gracilis* strain z. *Journal of Protozoology* **26**, 471–473.
- TROTSSENKO, Y. A. (1976). Isolation and characterization of obligate methanotrophic bacteria. In *Microbial Utilization and Production of Gases (H₂, CH₄, CO)*, pp. 329–336. Edited by H. G. Schlegel, G. Gottschalk and N. Pfennig. Göttingen: Erich Goltze, K.G.
- TSUJI, K., TSUEN, H. C., HANSON, R. S., DEPALMA, S. R., SCHOLTZ, R. & LAROCHE, S. (1990). 16S ribosomal RNA sequence analysis for determination of phylogenetic relationship among methylotrophs. *Journal of General Microbiology* **136**, 1–10.
- WEITZMAN, P. D. J. & JASKOWSKA-HODGES, H. (1982). Patterns of nucleotide utilization in bacterial succinate thiokinases. *FEBS Letters* **143**, 237–240.
- WEYGAND-DURASEVIC, I., JOHNSON-BURKE, D. & SÖLL, D. (1987). Cloning and characterization of the gene coding for cytoplasmic seryl-tRNA synthetase from *Saccharomyces cerevisiae*. *Nucleic Acids Research* **15**, 1887–1904.
- WINDASS, J. D., WORSEY, M. J., PIOLI, E. M., PIOLI, D., BARTH, P. T., ATHERTON, K. T., DART, E. C., BYROM, D., POWELL, K. & SENIOR, P. J. (1980). Improved conversion of methanol to single cell protein by *Methylophilus methylotrophus*. *Nature, London* **287**, 392–401.