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Incomplete Tricarboxylic Acid Cycle in a Type I Methylotroph, *Methylococcus capsulatus*

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α-Ketoglutarate dehydrogenase was undetectable in extracts of *Methylococcus capsulatus*. Cells incorporated [1-^{14}C]acetate into only four protein amino acids (glutamate, proline, arginine, and leucine) and the C5, but not C1, of glutamate.

The methane-oxidizing bacteria are divisible into two types on the basis of ultrastructural and biochemical characteristics (6, 10). *Methylococcus capsulatus* belongs to type I because it has bundles of intracytoplasmic vesicles distributed throughout its cells (5), assimilates C_1 compounds by the hexose-phosphate pathway (6), and lacks α-ketoglutarate dehydrogenase (1; R. Patel, D. S. Hoare, and B. F. Taylor, Bacteriol. Proc., p. 128, 1969). Davey et al. (1) detected α-ketoglutarate dehydrogenase in methylotrophs of type II but not in *M. capsulatus* MC and other methylotrophs of type I. Negative results for enzymatic assays are always open to question and, indeed, fumarate hydratase and α-ketoglutarate dehydrogenase initially were undetectable in *Methanomonas methanooxidans*, a type II methylotroph which was not investigated by Davey et al. (A. M. Wadzinski and D. W. Ribbons, Bacteriol. Proc., p. 166, 1972). Patel et al. (Bacteriol. Proc., p. 128, 1969) first reported the absence of a complete tricarboxylic acid cycle in *M. capsulatus* Texas strain (2), based on enzymatic assays and the fate of assimilated [^{14}C]acetate. The data for this conclusion are documented here and firmly establish the inability of *M. capsulatus* to oxidize acetate via the tricarboxylic acid cycle.

M. capsulatus was grown, with shaking, in a mineral salts medium with an atmosphere of methane (50% by volume) in air at 37 C (4). Cells were harvested in the exponential phase of growth and washed twice in 0.05 M potassium phosphate (pH 7.0) by centrifugation. Cells were disrupted by sonic treatment, and crude extracts were prepared by centrifugation for 10 min at 12,000 × *g* at 5 C. Crude extracts were separated, by ultracentrifugation for 2 h at 96,000 × *g* (5 C), into "soluble" (supernatant) and "particulate" (pellet) fractions. The particulate fraction was then suspended in 0.05 M potassium phosphate (pH 7.0). Enzymatic assays were performed at 25 C by spectrophotometric procedures (8). The enzymes of the tricarboxylic acid cycle and related enzymes were either absent or present at very low levels in cell-free extracts of *M. capsulatus* (Table 1). Activities for aconitate hydratase and α-ketoglutarate dehydrogenase were undetectable; however, fumarate hydratase, citrate synthase, and dehydrogenase activities for isocitrate, succinate, malate, and pyruvate were present. Isocitrate dehydrogenase was specific for nicotinamide adenine dinucleotide, similar to the

TABLE 1. *Enzymes of the tricarboxylic acid cycle and related enzymes in cell-free extracts in M. capsulatus grown on methane*

Enzyme	Sp act[a]
Citrate synthase	41
Aconitate hydratase	0
Isocitrate dehydrogenase (NAD[b] specific)	20
α-Ketoglutarate dehydrogenase	0
Succinate dehydrogenase[c]	+
Fumarate hydratase	68
Malate dehydrogenase (NAD)	52
Pyruvate dehydrogenase	6
NADH[d] oxidase—crude extract	38
NADH oxidase—soluble fraction	25

[a] Nanomoles of substrate used or product formed/minute per milligram protein of soluble fraction.
[b] NAD, Nicotinamide adenine dinucleotide.
[c] Particulate fraction. t, Enzyme present but not in reproducibly quantitable amounts.
[d] NADH, Reduced nicotinamide adenine dinucleotide.

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TABLE 2. *Selective decarboxylation of glutamic acid isolated from the hydrolyzed hot trichloroacetic acid-insoluble fraction of M. capsulatus grown on methane in the presence of [1-^{14}C]acetate*[a]

Decarboxylation	Glutamic acid		Sp act (counts/min per μmol)	CO_2 recovered		Sp act (counts/min per μmol)
	μmol	counts/min (× 10^{-3})		μmol	counts/min (× 10^{-3})	
C1 (Chloramine T)	40.95[b]	60	1,500	40.5	0	0
C5 (Schmidt)	200.95[b]	60	300	178	56.8	313

[a] A 50-ml culture was grown with shaking at 37 C for 48 h on a mineral salts medium containing 5 mM sodium acetate (specific activity, 0.1 μCi/μmol) under an atmosphere of methane (50% by volume) in air. Cells were harvested and washed at 5 C by centrifugation in 50 mM sodium acetate and then fractionated by the procedure of Roberts et al. (7). The hot trichloroacetic acid-insoluble fraction was hydrolyzed with HCl (9), and the glutamic acid was separated by electrophoresis on Whatman 3MM paper (3).

[b] A 0.95-μmol amount of labeled glutamic acid was isolated from the hydrolyzed hot trichloroacetic acid-insoluble fraction supplemented with cold glutamate to facilitate the determinations. Glutamate was assayed with ninhydrin (11), and the degradations and assay of $^{14}CO_2$ were performed as described by Hoare and Gibson (3).

enzyme from *M. capsulatus* MC (1). Reduced nicotinamide adenine dinucleotide oxidation, measured by a decrease in extinction at 340 nm, was catalyzed by cell-free extracts, and only about ⅓ of this activity was sedimented by ultracentrifugation.

Intact cells of *M. capsulatus* assimilated either [1-^{14}C]acetate or [2-^{14}C]acetate. The endogenous rate of acetate uptake was stimulated by the concomitant oxidation of either methane (10-fold), methanol (40-fold), or formate (15-fold). $^{14}CO_2$ production was not detectable from either [1-^{14}C]acetate or [2-^{14}C]acetate, thereby indicating a lack of acetate oxidation via the tricarboxylic acid cycle. Experiments were performed, therefore, to determine the fate of the acetate. Cultures were grown in the presence of [1-^{14}C]acetate, and the cells were harvested and fractionated into the major biochemical components (7). About 60% of the radioactivity recovered was incorporated into lipids and about 25% was incorporated into the hot trichloroacetic acid-insoluble fraction. Analysis by paper chromatography and radioautography (3) of samples of the hydrolyzed hot trichloroacetic acid-insoluble fraction revealed that [1-^{14}C]acetate contributed to the biosynthesis of only four amino acids (glutamate, proline, arginine, and leucine). Glutamic acid was isolated from the hydrolysate by preparative high-voltage electrophoresis and then selectively decarboxylated by chemical methods that liberated either the C1 (chloramine T reaction) or C5 (Schmidt reaction). The C1 of acetate did not contribute to the C1 of glutamate, and radioactivity in glutamate was predominantly recovered (95%) from C5 (Table 2). This result demonstrates the absence of a complete tricarboxylic acid cycle in *M. capsulatus* Texas strain and that, in conjunction with the enzymatic assays, the missing enzyme is α-ketoglutarate dehydrogenase. The incorporation of [1-^{14}C]acetate into glutamate indicates the presence of aconitate hydratase, which was not detected by the conventional assays in cell-free extracts of *M. capsulatus*. The labeling patterns of glutamate, derived from cells grown in the presence of [1-^{14}C]acetate, prove that an enzymatic lesion in the tricarboxylic acid cycle exists in *M. capsulatus*, a type I methylotroph, but not in *M. methanooxidans* (10), a type II methylotroph. These results fully corroborate the observations of Davey et al. (1).

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