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The Distribution in the Methylobacteria of Some Key Enzymes Concerned with Intermediary Metabolism

John F. Davey*, Roger Whittenbury**, and John F. Wilkinson

Department of Microbiology, University of Edinburgh, Scotland

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Summary. Cell-free extracts of methane-utilizing bacteria (methylobacteria) were examined for the presence of enzymes of the tricarboxylic acid cycle. Representative organisms of the Type II group had a complete set of enzymes for the operation of this cycle. Members of the Type I group lacked α-ketoglutarate dehydrogenase. All the methylobacteria examined had an NADH-oxidase which had the properties of a flavoprotein type enzyme. Glucose-6-phosphate dehydrogenase and gluconate-6-phosphate dehydrogenase was NADP-specific and could only be detected in cell extracts of Type I methylobacteria.

Smith *et al.* (1967) proposed a biochemical basis for obligate autotrophy, based on the absence of NADH-oxidase and α-ketoglutarate dehydrogenase in a selected group of autotrophic microorganisms. This idea was extended to explain the obligate dependence of *Methylococcus capsulatus* on methane or methanol as growth substrate (Patel *et al.*, 1969; Hoare *et al.*, 1970). This report describes an enzymic analysis of the tricarboxylic acid cycle and NADH-oxidizing system in methane-utilizing bacteria (methylobacteria). The purpose of the investigation was to see if the absence of some enzyme(s) necessary for the metabolism of organic compounds could account for the obligate C-1 dependence of these microorganisms.

Over a 100 methylobacteria which could only use methane or methanol as growth substrates were isolated by Whittenbury, *et al.* (1970). These isolates were divided into two major groups (Type I and Type II) on the basis of internal membrane arrangement (Davies and Whittenbury, 1970). This group division was supported by Lawrence and Quayle (1970), who showed that these bacteria used one of two pathways of carbon assimilation, the serine pathway (Heptinstall and Quayle, 1970)

* Present address: Departments of Biochemistry and Microbiology, University of Miami School of Medicine and Howard Hughes Medical Institute, Miami, Florida 33152, U.S.A.

** Present address: School of Biological Sciences, University of Warwick, Coventry, Warwickshire CV4 7AL, England.

or the ribose phosphate cycle of formaldehyde fixation (Kemp and Quayle, 1967). The activities of some enzymes of the pentose phosphate and Embden-Meyerhof pathways in a member from each major group of methylobacteria were compared in order to see if any differences could be detected.

Materials and Methods

Microorganisms and Growth Conditions. The following organisms were used: *Methylosinus sporium* (12); *Methylosinus trichosporium* (PG) and (OB 3B); *Methylocystis parvus* (OB BP); *Methylomonas albus* (BG 8); *Methylomonas methanica* (25); *Methylococcus capsulatus* (MC); and *Methylococcus minimus* (TMC). The nomenclature was that suggested by Whittenbury *et al.* (1970), and the symbols in parenthesis denote the particular strain. Bacterial cultures were grown in 5 l "Quickfit" pots each containing 1 l of the nitrate mineral salts medium of Whittenbury *et al.* (1970). The growth from 4-day old agar slopes was used as inoculum and incubation was at 30° C. A bladder of pure methane gas attached to a part in the lid was allowed to diffuse into the culture vessel.

Preparation of Cells and Cell Extracts. Bacterial cells were harvested by centrifugation at 23000× g for 20 min and washed once with distilled water. Cell-free extracts were prepared by twice passing organisms through a pre-cooled French pressure cell at 3000 psi. Broken cells were centrifuged at 10000× g for 20 min and the supernatant used to assay NADH-oxidase, succinate dehydrogenase and α-ketoglutarate dehydrogenase. The supernatant obtained after centrifugation at 38000× g for 30 min was used to assay enzymes of the pentose phosphate and Embden-Meyerhof pathways. Other enzymes of the tricarboxylic acid cycle were assayed with the supernatant obtained after centrifugation of broken cells at 38000× g for 1 h.

Enzyme Assays. All enzyme activities were measured at 35° C according to well-established methods or modifications of currently used techniques, as indicated below: NADH-oxidase (EC 1.6.99.3), Smith *et al.* (1967); citrate synthase (EC 4.1.3.7), Srere *et al.* (1963); aconitase (EC 4.2.1.3), Ochoa (1948); isocitrate dehydrogenase (EC 1.1.1.41, EC 1.1.1.42), Kornberg (1955); α-ketoglutarate dehydrogenase (EC 1.2.4.2), Amarishingham and Davis (1965); succinate dehydrogenase (EC 1.3.99.1), Arrigoni and Singer (1962); fumarase (EC 4.2.1.2), Racker (1950); malate dehydrogenase (EC 1.1.1.37), Mehler *et al.* (1948); glucose-6-phosphate dehydrogenase (EC 1.1.1.49), Kornberg and Horecker (1955); gluconate-6-phosphate dehydrogenase (EC 1.1.1.43), Horecker and Smyrniotis (1955); ribose-phosphate isomerase (EC 5.3.1.6), Axelrod and Jang (1954); transketolase (EC 2.2.1.1), Haba and Racker (1955); glucokinase (EC 2.7.1.2), Di Pietro and Weinhouse (1960); glucosephosphate isomerase (EC 5.3.1.9), Wu and Racker (1959); phosphofructokinase (EC 2.7.1.11), Sols and Salas (1966); aldolase (EC 4.1.2.13), Rutter and Hunsley (1966).

Enzymes were assayed on the same day as cell extracts were prepared. Specific activities are reported as nanomoles substrate converted/mg protein/min. The following millimolar extinction coefficients were used in the calculations: reduced NAD(P) 340 nm, 6.2; fumarate 240 nm, 2.1; 3-acetylpyridine-NAD 363, 9.1; mercaptide ion formation 412 nm, 13.6; 2,6-dichlorophenol indophenol 600 nm, 20.6. The protein content of cell-free extracts was estimated according to the procedure of Lowry *et al.* (1951) using crystalline bovine serum albumin as standard.

Chemicals. Pure methane gas was obtained from Middlesex County Council Main Drainage Department, Isleworth, Middlesex, U.K. Enzyme substrates and pure

enzyme preparations were purchased from The Boehringer Corporation (London) Ltd. Other chemicals were obtained from Sigma Chemical Company Ltd. (London).

Results

Cell-free extracts of methylobacteria with the serine pathway of carbon assimilation (Type II) contained all of the tricarboxylic acid cycle enzymes (Table 1). Citrate synthase, aconitase and fumarase were easily detected in extracts of all Type II strains. Isocitrate dehydrogenase was predominantly NADP-specific and no activity was detected with NAD. Succinate dehydrogenase activity was high and comparable to rates reported for some facultative autotrophs (see Smith *et al.*, 1967). Malate dehydrogenase was assayed by using oxaloacetate as substrate and gave a high and similar specific activity for the four Type II organisms (Table 1). The presence of α-ketoglutarate dehydrogenase was easily demonstrated in crude extracts when 3-acetylpyridine-NAD(AP-NAD) was the electron acceptor; reproducible results were not obtained when NAD was used as the electron acceptor.

The specific activities of the tricarboxylic acid cycle enzymes in extracts of methylobacteria with the ribose phosphate pattern of carbon assimilation (Type I) are presented in Table 2. Citrate synthase and malate dehydrogenase were present at about the same level of activity as found in the Type II organisms (Table 1); the only exception was the relatively low activity of malate dehydrogenase in *M. capsulatus*. Aconitase and succinate dehydrogenase gave low activities. Isocitrate dehydrogenase showed equal specificity for NAD and NADP except in *M. capsulatus* in which this enzyme was strictly NAD-dependent. The enzyme α-ketoglutarate dehydrogenase could not be detected in cell extracts of any of the Type I methylobacteria. Control experiments with mixed extracts of Type I and Type II organisms showed that the negative results could not be attributed to the presence of an inhibitor of α-ketoglutarate dehydrogenase activity in the extracts of the Type I microorganisms. Further, pyruvate dehydrogenase was easily detected in cell-free extracts of all the methylobacteria.

The oxidation of NADH under aerobic conditions was a property common to crude cell extracts of all the methylobacteria (Table 3). Sodium cyanide (0.001 M) or sodium azide (0.01 M) caused no inhibition of the reduced nicotinamide adenine dinucleotide oxidase. Cell extracts of all the methylobacteria oxidized NADPH at the same low rate (2—4 nmoles/mg protein/min).

The specific activities of some enzymes involved in carbohydrate metabolism are shown in Table 4. Glucose-6-phosphate dehydrogenase and glucose-6-phosphate isomerase were present in all Type I organisms investigated and were NADP specific; no activity was found in any of the Type II methylobacteria used in this investigation.

Table 1. Specific activities of the tricarboxylic acid cycle enzymes in extracts of methylobacteria with a Type II membrane system[a]

Enzyme	Microorganism			
	Methylosinus trichosporium (PG)	*Methylosinus trichosporium* (OB3B)	*Methylosinus sporium*	*Methylocystis parvus*
Citrate synthase	50	35	32	37
Aconitase	32	16	17	4
Isocitrate dehydrogenase (NADP-specific)	47	30	53	38
α-ketoglutarate dehydrogenase	7	20	10	8
Succinate dehydrogenase	51	38	59	64
Fumarase	38	52	43	50
Malate dehydrogenase	450	400	360	480

[a] Enzyme activities are expressed as nmoles/mg protein/min.

Table 2. Specific activities of the tricarboxylic acid cycle enzymes in extracts of methylobacteria with a Type I membrane system[a]

Enzyme	Microorganism			
	Methylomonas methanica	*Methylomonas albus*	*Methylococcus minimus*	*Methylococcus capsulatus*
Citrate synthase	38	20	18	16
Aconitase	10	3	6	8
Isocitrate dehydrogenase				
(NAD-specific)	14	17	18	22
(NADP-specific)	15	19	22	0
α-ketoglutarate dehydrogenase	0	0	0	0
Succinate dehydrogenase	12	12	10	2
Fumarase	34	37	52	32
Malate dehydrogenase	430	340	440	74

[a] Enzyme activities are expressed as nmoles/mg protein/min.

Table 3. Specific activities of NADH-oxidase in crude extracts of both types of methylobacteria

Microorganism	nmoles NADH oxidised/mg protein/min
Type I	
Methylomonas methanica	16
Methylomonas albus	10
Methylococcus capsulatus	29
Methylococcus minimus	23
Type II	
Methylosinus trichosporium (PG)	33
Methylosinus trichosporium (OB3B)	18
Methylosinus sporium	18
Methylocystis parvus	15

Discussion

Methylomonas albus and the other Type I methylobacteria operate the ribose phosphate cycle of formaldehyde fixation (see Kemp and Quayle, 1966) with the resulting formation of allulose-6-phosphate as the initial condensation product. A constant supply of ribose-5-phosphate is therefore required for the operation of the cycle. The allulose-6-phosphate formed may be converted to glucose-6-phosphate via fructose-6-phosphate. The enzyme phosphoglucose isomerase which is responsible for the latter reaction was readily detected in extracts of *M. albus* (Table 4). Dehydrogenation of glucose-6-phosphate by glucose-6-phosphate dehydrogenase and gluconate-6-phosphate dehydrogenase, respectively yields ribulose-5-phosphate and both these enzymes were present in all the Type I methylobacteria (Table 4). The highly active phosphoribose isomerase enzyme (Table 4) is responsible for the convertion of ribulose-5-phosphate to ribose-5-phosphate, thus completing the cycle. Such a constant recycling of ribose-5-phosphate is not required in the Type II methylobacteria which operate the serine pathway of carbon assimilation (Heptinstall and Quayle, 1970) and it is interesting that the enzymes which convert glucose-6-phosphate to ribulose-5-phosphate could not be detected in these organisms. Further, the phosphoribose isomerase activity in *Methylosinus trichosporium* (OB3B) was extremely low by comparison with the Type I organism. These enzymic differences further substantiate the division of methylobacteria into two distinct groups.

The analogy between autotrophs and methylobacteria proposed by Hoare *et al.* (1970) was based on data obtained from an examination

Table 4. Specific activities of some enzymes of intermediary carbohydrate metabolism in methylobacteria

Microorganism	Specific activity (nmoles converted/mg protein/min)	
	Glucose-6-phosphate dehydrogenase	Gluconate-6-phosphate dehydrogenase
Type I		
Methylomonas albus	12	36
Methylomonas methanica	14	14
Methylococcus minimus	10	10
Methylococcus capsulatus	7	28
Type II		
Methylosinus trichosporium (OB3B)	0	0
Methylosinus trichosporium (PG)	0	0
Methylosinus sporium	0	0
Methylocystus parvus	0	0

Microorganism	Specific activity (nmoles converted/mg protein/min)	
	Phosphoribose isomerase	Phosphoglucose isomerase
Type I		
Methylomonas albus	1620	15
Methylomonas methanica	n. t.	n. t.
Methylococcus minimus	n. t.	n. t.
Methylococcus capsulatus	n. t.	n. t.
Type II		
Methylosinus trichosporium (OB3B)	185	6
Methylosinus trichosporium (PG)	n. t.	n. t.
Methylosinus sporium	n. t.	n. t.
Methylocystus parvus	n. t.	n. t.

n. t. = not tested.

of only one member of the latter group (*Methylococcus capsulatus*). The results presented here indicate that the Type I methylobacteria, which may have a very similar evolutionary origin to the autotrophic bacteria, have an incomplete tricarboxylic acid cycle. The lesion appears to involve a loss of part or all of the α-ketoglutarate dehydrogenase enzyme complex. No such lesion was evident in the Type II organisms; this fact, in conjunction with already established data, suggests a separate evolution of these two major groups of methylobacteria.

The inability to couple the breakdown of organic substrates with the generation of ATP as a result of the absence of NADH-oxidase, was proposed as the basic obstacle to heterotrophic growth in the obligate autotrophs (Smith *et al.*, 1967). This does not seem to be the basis of the obligate C-1 dependence of methylobacteria, as NADH-oxidase was present in all the organisms tested (Table 3). The insensitivity of NADH-oxidase to inhibitors does, however, suggest that the oxidation of pyridine nucleotide by these organisms may not be energy yielding. This could account for the failure of methylobacteria to grow on a variety of organic acids and amino acids tested (Whittenbury *et al.*, 1970). It would seem from the information available at present, that the fastidious growth requirement of this group of bacteria, is due to an unusual energy conservation mechanism as suggested by Ribbons *et al.* (1970), rather than to isolated lesions in the central pathways of carbohydrate metabolism.

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Prof. Dr. J. F. Wilkinson
Dept. of Microbiology
School of Agriculture
University of Edinburgh
West Mains Road
Edinburgh EH9 3JG, Scotland