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A Biochemical Basis for Obligate Methylotrophy: Properties of a Mutant of *Pseudomonas* AM1 lacking 2-Oxoglutarate Dehydrogenase

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SUMMARY

Pseudomonas AMI is a facultative methylotroph which grows on a wide range of carbon compounds. A mutant of *Pseudomonas* AMI (ICT41) grew only on C_1 compounds and is thus an artificial obligate methylotroph. Measurements of activities of the components of the 2-oxoglutarate dehydrogenase complex suggest that the E_2 component (dihydrolipoamide transsuccinylase) is not functional. All other tricarboxylic acid cycle enzymes were present with activities comparable to those in wild-type *Pseudomonas* AMI and cytochrome levels were unchanged in the mutant. Suspensions of the mutant oxidized pyruvate, lactate, β-hydroxybutyrate, acetoacetate and 2-oxoglutarate at very low rates. By contrast, C_1 compounds were oxidized at the same rate as in wild-type bacteria. Two revertants of ICT41 which regained 2-oxoglutarate dehydrogenase activity also regained the ability to oxidize and grow on the same substrates as wild-type bacteria. It is concluded that lack of 2-oxoglutarate dehydrogenase may well be the basis of obligate methylotrophy in some bacteria.

INTRODUCTION

Pseudomonas AMI is a pink, facultative methylotroph capable of growth on a variety of carbon sources including C_1 compounds (Quayle, 1972; Anthony, 1975*a*). Obligate methylotrophs grow only at the expense of compounds containing no carbon–carbon bonds (see Colby & Zatman, 1972) perhaps because they have no 2-oxoglutarate dehydrogenase (Kelly, 1971). Obligate methylotrophs lacking 2-oxoglutarate dehydrogenase include bacterium 4B6 (Colby & Zatman, 1972), organism W1 (Dahl, Mehta & Hoare, 1972) and methane utilizers having type I membranes and the ribulose monophosphate pathway for formaldehyde fixation (Davey, Whittenbury & Wilkinson, 1972). However, although the lack of 2-oxoglutarate dehydrogenase may be important in conferring the characteristic of obligate methylotrophy, other mechanisms may also be involved (Colby & Zatman, 1972; Davey *et al.*, 1972). Furthermore, doubt has been expressed that a single lesion (e.g. loss of 2-oxoglutarate dehydrogenase) could account for the inability of a large number of potential growth substrates to support growth (Ribbons, Harrison & Wadzinski, 1970).

If the lack of 2-oxoglutarate dehydrogenase causes obligate methylotrophy then a 2-oxoglutarate dehydrogenase-deficient mutant of a facultative methylotroph should be able to grow only on C_1 compounds. This paper describes the properties of such a mutant of *Pseudomonas* AMI.

METHODS

Maintenance, growth and harvesting of bacteria. Methods were as previously described (Anthony, 1975*b*). Carbon sources included in the basal salts medium were used at a concentration of 0·2 % except for the following: methanol and ethanol (0·4 %), β-hydroxybutyrate (0·1 %), and methanol (0·4 %) plus succinate (0·1 %).

Isolation and characterization of mutant ICT41 *and revertant organisms.* Mutant ICT41 was isolated by a method based on that of Heptinstall & Quayle (1970), designed to select for mutants able to grow on methanol but unable to grow on β-hydroxybutyrate. Bacteria treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) were expressed in methanol medium and then treated three times with penicillin (1000 i.u./ml of medium) in the presence of β-hydroxybutyrate. Revertant bacteria were isolated by plating about 10^8 bacteria (mutant ICT41) on to β-hydroxybutyrate-agar plates. A small crystal of NTG was placed in the centre of each plate. Revertant colonies which appeared outside the zone of growth inhibition were picked off and purified by single colony isolation. Growth characteristics of mutant strains were determined in liquid media. When growth occurred the strain was subcultured at least twice into the same medium using a wire loop. After the second subculture the growth properties were confirmed on solid media to ensure that contamination by wild-type bacteria had not occurred. In some cases supplements of acetate and succinate (1 mM) were added to the primary carbon source.

When large-scale cultures of the mutant were used for enzyme assays the growth responses of these cultures on methanol-, β-hydroxybutyrate-, ethanol- and succinate-agar plates were tested to ensure that the strains had maintained their original phenotype.

Measurements of oxygen uptake by bacterial suspensions were done as previously described (Anthony, 1975*b*).

Preparation of cell-free extracts and determination of protein. The methods were as described by Anthony (1975*b*). For assay of succinate dehydrogenase the sonic extract was centrifuged at 4000 ***g*** for 10 min to remove unbroken bacteria before centrifuging at 40000 ***g*** for 1 h. The pellet was resuspended in a portion of buffer equal in volume to the original volume of the extract.

Enzyme and cytochrome assays. All spectrophotometric assays were done in a Pye-Unicam SP1800 recording spectrophotometer at 25 °C. The following enzymes were assayed by published procedures: citrate synthase, EC. 4.1.3.7 (Srere, 1969); isocitrate dehydrogenase (NAD^+), EC. 1.1.1.41 (Cox, 1969); isocitrate dehydrogenase ($NADP^+$), EC. 1.1.1.42 (Ochoa, 1955*a*); succinate dehydrogenase, EC. 1.3.99.1 (Veeger, DerVartanian & Zeylemaker, 1969); fumarase, EC. 4.2.1.2 (Racker, 1950); malate dehydrogenase, EC 1.1.1.37 (Ochoa, 1955*b*).

Acetyl-CoA synthetase (EC. 6.2.1.1) and succinyl-CoA synthetase (EC. 6.2.1.4) were assayed by the hydroxamate methods of Jones & Lipmann (1955) and Kaufmann (1955), respectively.

Overall 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase activities were assayed by the method of Guest & Creaghan (1973). The decarboxylase (E_1) components of the 2-oxoacid dehydrogenase complexes were assayed spectrophotometrically at 420 nm with ferricyanide as the electron acceptor (modified from Hager & Kornberg, 1961). The reaction mixture contained (μmol/1·0 ml): potassium phosphate pH 6·3, 100; thiamine pyrophosphate, 0·5; potassium ferricyanide, 1·5; sodium 2-oxoglutarate or sodium pyruvate, 20; and extract up to 1 mg protein. After incubation for 2 to 3 min the reactions were started by adding ketoacid. Lipoamide dehydrogenase (lpdh) was assayed by recording the

lipoamide-dependent oxidation of NADH at pH 6·5 according to the modified method of Massey (1966). Specific activities (nmol substrate transformed/mg protein per min) were determined in the region of proportionality between reaction velocity and protein concentration.

Cytochromes were determined as described previously (Anthony, 1975*b*; Widdowson & Anthony, 1975).

Chemicals. All chemicals were obtained from BDH except for the following: CoA (lithium salt) was from P-L Biochemicals Inc., Milwaukee, U.S.A.; 3-acetyl-NAD^+ (grade I), $NADP^+$, NADPH, NAD^+, NADH and DL-6,8-thioctic acid amide (DL-lipoamide) were from Sigma; sodium benzylpenicillin was from Glaxo; NTG, phenylhydrazine hydrochloride and glyoxylic acid monohydrate were from Koch-Light; Bacto-agar was from Difco. Acetyl-CoA was prepared by the method of Srere (1969).

RESULTS

Properties of mutant ICT41

Growth properties

Mutant ICT41 grew at the same rate as wild-type *Pseudomonas* AMI on C_1 compounds (methanol, methylamine and formate) and on oxalate. However, this mutant did not grow on β-hydroxybutyrate, malonate, ethanol, lactate, pyruvate, malate, fumarate, succinate or nutrient broth. Supplements (1 mM) of succinate did not promote growth of mutant ICT41 on malate, β-hydroxybutyrate or lactate, and supplements of succinate plus acetate (1 mM each) did not allow growth on β-hydroxybutyrate or ethanol.

Oxidative properties of mutant ICT41

Ethanol, succinate, malate, malonate and C_1 compounds were oxidized at rates similar to those measured in wild-type bacteria (Table 1). By contrast, pyruvate, lactate, β-hydroxybutyrate, acetoacetate and 2-oxoglutarate were oxidized at only 9 to 20 % of the wild-type rates.

Activities of TCA cycle enzymes and cytochrome levels

The low rates of oxidation of many substrates suggested that mutant ICT41 was deficient in one or more of the tricarboxylic acid (TCA) cycle enzymes. No 2-oxoglutarate dehydrogenase activity was detected in any extract of mutant ICT41 using either NAD^+ or 3-acetyl NAD^+ as the electron acceptor, but the specific activities of all the other TCA cycle enzymes (Table 2) were at least as high as wild-type levels (aconitase was not measured). The levels of cytochromes *a*, *b* and *c*, and of carbon monoxide-binding cytochromes were the same as in wild-type *Pseudomonas* AMI.

Enzymic components of 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase in Pseudomonas AMI *and mutant* ICT41

2-Oxoglutarate dehydrogenase is usually a multi-enzyme complex consisting of three enzymic components, the 2-oxoglutarate decarboxylase (E_1), the dihydrolipoamide transsuccinylase (E_2) and the lipoamide dehydrogenase (E_3); together, these catalyse the oxidative decarboxylation of 2-oxoglutarate to succinyl-CoA (Reed & Cox, 1970). Pyruvate is converted to acetyl-CoA by an analogous series of reactions.

Mutant ICT41 had no detectable 2-oxoglutarate dehydrogenase when grown on methanol or on methanol plus succinate (Table 3). The E_1 component was present with 26 to 50 % of the activity measured in wild-type bacteria. The K_m value for 2-oxoglutarate of the

Table 1. *Comparison of rates of oxygen uptake by suspensions of methanol-grown mutant* ICT41 *and the parent strain (Pseudomonas* AMI)

The results are expressed as percentages of the rate of the parent strain. Figures in parentheses indicate absolute values of oxygen uptake (μl O_2/mg dry wt per h). Results given are the averages of a number of determinations with different cell suspensions. The endogenous rate of oxidation was subtracted.

Substrate	*Pseudomonas* AMI	ICT41	ICT41-R_1	ICT41-R_2
Methanol	100 (120)	70	75	90
Formate	100 (120)	97	—	—
Formaldehyde	100 (110)	103	—	—
Ethanol	100 (84·6)	80	—	—
Succinate	100 (21·0)	82	75	62
Malate	100 (35·0)	93	—	—
Malonate	100 (17·7)	70	—	—
Pyruvate	100 (14·0)	9	—	—
Lactate	100 (19·5)	20	120	100
β-Hydroxybutyrate	100 (15·0)	13	65	90
Acetoacetate	100 (20·0)	10	—	—
2-Oxoglutarate	100 (21·5)	13	75	87
Endogenous substrate	100 (11·0)	10	130	70

—, Not measured.

Table 2. *Specific activities of TCA-cycle enzymes in extracts of methanol-grown Pseudomonas* AMI *and mutant* ICT41

Enzymes were assayed as described in Methods.

Enzyme	Specific activity (nmol/min per mg protein)	
	Pseudomonas AMI	Mutant ICT41
Citrate synthase	41·7	38·2
Isocitrate dehydrogenase ($NADP^+$)	142	163
Isocitrate dehydrogenase (NAD^+)	ND	ND
2-Oxoglutarate dehydrogenase	25	ND
Succinyl-CoA synthetase	80	72
Succinate dehydrogenase	38·3	46·8
Fumarase	28·2	28·2
Malate dehydrogenase	1080	2650
NADH dehydrogenase	8·3	16·3

ND, Not detected.

E_1 component in extracts of mutant ICT41 (59 μM) was the same as that measured in extracts of wild-type bacteria (60 μM).

E_3 activity was present in extracts of mutant ICT41 at higher levels than in wild-type bacteria and this may account for the high pyruvate dehydrogenase activity measured in mutant ICT41.

For technical reasons it was not possible to measure the E_2 component in crude extracts of *Pseudomonas* AMI or in crude extracts of other bacteria such as *Escherichia coli* (Dr J. R. Guest, personal communication).

Revertant strains derived from mutant ICT41

Two revertants of mutant ICT41 were obtained as described in Methods. Both revertants (ICT41-R_1 and ICT41-R_2) had regained 2-oxoglutarate dehydrogenase activity (Table 3) and

Table 3. *Specific activities of the 2-oxoglutarate and pyruvate dehydrogenase complexes and component enzymes in wild-type Pseudomonas* AMI *and derived mutants*

Enzymes were assayed in extracts as described in Methods. The average specific activities for determinations with several different extracts are quoted (nmol/min per mg protein); all values were within 10 % of the average value quoted.

		Enzyme specific activities				
Strain	Growth substrate	2-ogdh	E_1(2-og)	lpdh(E_3)	pyrdh	E_1(pyr)
Wild-type	Methanol	24·6	6·7	25·0	2·3	2·0
	Methanol + succinate	43·4	16·6	58·5	11·2	3·3
	Succinate	138·0	21·7	143·0	21·7	4·7
	Lactate	93·5	21·7	139·0	40·0	7·0
	β-Hydroxybutyrate	58·5	10·0	108·0	17·1	4.7
ICT41	Methanol	ND	3·3	42·0	7·7	1·8
	Methanol + succinate	ND	4·3	190·0	14·2	2·7
ICT41-R_1	Methanol	11·7	6·7	18·3	2·3	—
	Succinate	48·4	23·4	58·5	10·8	—
ICT41-R_2	Methanol	10·0	15·0	10·8	4·0	—
	Succinate	26·8	33·2	108·0	33·2	—

2-ogdh and pyrdh, activity of the overall 2-oxoglutarate and pyruvate dehydrogenase complexes; E_1(2-og) and E_1(pyr), 2-oxoglutarate and pyruvate decarboxylases respectively; lpdh(E_3), lipoamide dehydrogenase; —, not assayed; ND, not detected.

both had concomitantly regained the ability to oxidize and grow on the same substrates as the wild-type *Pseudomonas* AMI (Table 1). These results suggest that the characteristics of mutant ICT41 are not a result of multiple lesions.

DISCUSSION

2-Oxoglutarate and pyruvate dehydrogenases are relatively unimportant during growth of wild-type *Pseudomonas* AMI on methanol compared with growth on β-hydroxybutyrate, lactate or succinate (Table 3). Both E_1 and E_2 components, as well as overall dehydrogenase activities, were low during growth on methanol. The lack of 2-oxoglutarate dehydrogenase activity in mutant ICT41 is unlikely to be due to the lower activity of the E_1 component. Wild-type bacteria and revertant ICT41-R_1 have only twice as much E_1 activity as the lowest value recorded in the mutant, and yet they have significant overall 2-oxoglutarate dehydrogenase activity. It is more probable that mutant ICT41 has an altered (inactive) E_2 component and that this also has some effect on the activities of the E_1 and E_3 components in the multienzyme complex.

The growth properties of mutant ICT41 show that loss of 2-oxoglutarate dehydrogenase is sufficient to convert a typical facultative methylotroph into an obligate methylotroph. The lack of this enzyme may well be the basis, therefore, of obligate methylotrophy in some bacteria. Although growth of mutant ICT41 does occur on oxalate this compound is assimilated by way of formate and the serine pathway in *Pseudomonas* AMI (Blackmore & Quayle, 1970) and can thus be classed as a substrate for methylotrophic growth.

The 'obligate mutant' of *Pseudomonas* AMI is most similar to those obligate methane-utilizers having type 2 membranes, because they share the same pathway for the assimilation of C_1 compounds (the serine pathway). Davey *et al.* (1972) suggested that there must be an alternative basis for the obligate methylotrophy of these bacteria, because they do have some 2-oxoglutarate dehydrogenase activity (average specific activity 12 nmol/min per mg

protein). However, this is lower than the specific activity of the dehydrogenase in *Pseudomonas* AMI growing on methanol. Perhaps in type 2 methane-utilizers there is no mechanism for the induction of the higher levels required for growth on multicarbon compounds (specific activity of 60 to 140 nmol/min per mg protein). The results with mutant ICT41 support previous suggestions that the lack of 2-oxoglutarate dehydrogenase could be the basis for obligate methylotrophy in bacteria having the ribulose monophosphate pathway for formaldehyde fixation (type 1 methane-utilizers, organism W1 and bacterium 4B6). Lack of this enzyme is also the probable reason for the limited range of substrates supporting growth of the restricted facultative methylotrophs described by Colby & Zatman (1975). They pointed out that this would be a very similar situation to that obtaining in *Hyphomicrobium* (a restricted facultative methylotroph lacking pyruvate dehydrogenase), which has a complete TCA cycle but which is unable to grow on any compound metabolized exclusively by way of pyruvate (Harder, Martin & Attwood, 1975).

Having shown that lack of 2-oxoglutarate dehydrogenase is able to confer the characteristic of obligate methylotrophy (in *Pseudomonas* AMI) it is necessary to consider how this one lesion might have such a far-reaching effect. The complete oxidation of compounds with more than one carbon atom is not possible without a complete TCA cycle and the resulting energy deficiency may in itself lead to failure to grow on multi-carbon compounds. In addition, the resulting accumulation of intermediates of central metabolic pathways may lead to inhibition of key enzymes. Such inhibitory accumulations may have caused the slower growth of mutant ICT41 on methanol when succinate was also included in the growth medium. This effect is not observed with wild-type bacteria.

It may, at first sight, be surprising that rates of oxygen consumption in the presence of ethanol, succinate, malate and malonate were not greatly affected by the lack of a complete TCA cycle in mutant ICT41. However, the measured rates depend on the interaction of a number of factors, any one of which may be rate-limiting and which may or may not be affected by a single metabolic lesion. Such factors include the rate of transport into the bacteria, the activities of primary dehydrogenases and electron transport chains, the equilibrium constants of the reactions leading to the TCA cycle, and the rate of flux through reactions of the TCA cycle. For most substrates (including malate and malonate) it is not known which factor is rate-limiting; it is consequently impossible to predict in detail how the loss of 2-oxoglutarate dehydrogenase would be expected to affect the rate of oxygen uptake with these substrates. Succinate oxidation is catalysed by the irreversible, membrane-bound succinate dehydrogenase and changes in the rate of further metabolism (or lack of it) of the oxidation product is then unlikely to affect markedly the rate of oxygen uptake measured with succinate as substrate. A similar argument applies to the oxidation of ethanol by the irreversible NAD-independent methanol dehydrogenase which interacts directly with the cytochrome chain to oxygen at the level of cytochrome *c* and is the only enzyme for oxidation of ethanol in *Pseudomonas* AMI (Anthony, 1975*b*; Widdowson & Anthony, 1975).

In summary, it is the rate and extent of total oxidation of a substrate that determines the amount of energy available to the bacteria, and this will be altered in mutant ICT41 for all substrates whose total oxidation to CO_2 depends on a complete TCA cycle.

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