Enzyme Regulation in *Methylobacterium organophilum*

By MARY L. O'CONNOR AND RICHARD S. HANSON

Department of Bacteriology, University of Wisconsin,
Madison, Wisconsin 53706, U.S.A.

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Several enzymes have been assayed in *Methylobacterium organophilum* grown on different substrates. The enzymes which are involved in growth on C₁ compounds were induced by methanol and not repressed by succinate. When succinate-grown bacteria were resuspended in medium containing methanol, four enzymes unique to growth on C₁ compounds (hydroxypyruvate reductase, serine-glyoxylate aminotransferase, methanol dehydrogenase and glycerate kinase) were fully induced by the time growth began. When methanol-grown bacteria were resuspended in medium containing succinate, all four enzyme activities decreased. Several mutants unable to grow on C₁ compounds were examined for deficiencies in the enzymes specific for growth on these compounds. Seven of the mutants were pleiotropic, and six were not revertible by chemical mutagens, suggesting the possibility of genetic linkage or the presence of a regulon for the genes involved in C₁ metabolism.

INTRODUCTION

In facultative methanol- and methylamine-utilizing bacteria, enzymes that are uniquely required for growth on C₁ compounds are inducible by the C₁ compounds on which the bacteria are grown (Large & Quayle, 1963; Dunstan, Anthony & Drabble, 1972; Large & Carter, 1973). In the facultative methanol-utilizing bacterium *Pseudomonas* AM1, some C₁-specific enzymes are repressed by succinate and some are not (Dunstan *et al.*, 1972; Large & Quayle, 1963). These enzymes appear to be coordinately controlled, and Dunstan *et al.* (1972) have suggested that they are under operonic control in *Pseudomonas* AM1.

The isolation and characterization of *Methylobacterium organophilum* (Patt *et al.*, 1974; Patt, Cole & Hanson, 1976) has permitted the study of enzyme regulation in a methane-utilizing bacterium for the first time. Previous studies have shown that the ability of *M. organophilum* to oxidize methane and the synthesis of intracytoplasmic membranes occurred only in organisms grown on methane (Patt *et al.*, 1974). Such studies are important in defining markers for biochemical and genetic analyses of C₁ metabolism, methane oxidation and membrane synthesis. This paper describes the response of several enzyme activities in *M. organophilum* to changes in the substrates used for growth.

METHODS

Organism. *Methylobacterium organophilum* (ATCC27886; Patt *et al.*, 1976) was used in all experiments.

Media and growth conditions. The modified nitrate mineral salts medium (NMS) previously described (Patt *et al.*, 1976) was used for all studies. Succinate was dissolved in distilled water, sterilized separately and added directly to the growth medium at 0.1 % (w/v). Methanol (96 %, v/v) was filter-sterilized and added separately to the growth medium at 0.5 % (v/v). All cultures were incubated at 30 °C. Liquid cultures were incubated on rotary shakers.

Preparation of extracts. Extracts used for enzyme assays were prepared as described previously (O'Connor & Hanson, 1975). Protein was estimated by the method of Lowry *et al.* (1951).

Enzyme assays. Hydroxypyruvate reductase [D-glycerate:NAD⁺ oxidoreductase, EC 1.1.1.29], formate-tetrahydrofolate ligase [formate:tetrahydrofolate ligase (ADP-forming), EC 6.3.4.3] and methylenetetrahydrofolate dehydrogenase [5,10-methylenetetrahydrofolate:NADP⁺ oxidoreductase, EC 1.5.1.5] were assayed by the methods of Large & Quayle (1963). Other assays were: serine-glyoxylate aminotransferase [L-serine:glyoxylate aminotransferase, EC 2.6.1.45], Blackmore & Quayle (1970); glycerate kinase [ATP:D-glycerate 3-phosphotransferase, EC 2.7.1.31], Heptinstall & Quayle (1970); phosphoenolpyruvate carboxylase [orthophosphate:oxaloacetate carboxy-lyase (phosphorylating), EC 4.1.1.31], Canovas & Kornberg (1969); serine transhydroxymethylase [5,10-methylenetetrahydrofolate:glycine hydroxymethyltransferase, EC 2.1.2.1], O'Connor & Hanson (1975); *N*⁵-formyltetrahydrofolate cyclodehydrase, Greenberg (1971); malate synthase [L-malate glyoxylate-lyase (CoA-acetylating), EC 4.1.3.2], Dixon & Kornberg (1959); citrate synthase [citrate oxaloacetate-lyase (*pro*-3S-CH₂COO⁻→acetyl-CoA), EC 4.1.3.7], Srere, Brazil & Gonen (1963); and 2-oxoglutarate dehydrogenase [2-oxoglutarate:lipoate oxidoreductase (decarboxylating and acceptor-succinylating), EC 1.2.4.2], Tanaka & Hanson (1975).

Methanol dehydrogenase and formaldehyde dehydrogenase were assayed by measuring the methanol- or formaldehyde-dependent oxygen uptake in the presence of phenazine methosulphate (PMS). Oxygen uptake was measured by adding 0.8 ml buffer (0.015 M-Tris/0.02 M-NH₄Cl, pH 9.0) and 0.1 ml PMS (1 mg ml⁻¹) to the chamber of a Rank oxygen electrode (Rank Bros., Bottisham, Cambridgeshire). Extract and buffer were added to bring the volume to 0.95 ml. Methanol or formaldehyde [0.05 ml of a 1% (v/v) solution] was added after methanol-independent oxygen consumption had stopped.

Mutant isolation. Mutants unable to grow on C₁ compounds were isolated using the ultraviolet light procedure described previously (O'Connor, Wopat & Hanson, 1977).

Reversion tests. A heavy suspension of each methanol⁻ mutant (i.e. unable to grow on methanol) was spread on to a plate of NMS plus methanol plus 0.03% (w/v) Casamino acids. A filter soaked in *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG, 2 mg ml⁻¹) or ethyl methanesulphonate (EMS, 1:10 dilution) was placed in the centre of each plate.

RESULTS

Enzyme activities

Of the enzymes in bacteria grown on methanol, succinate or methanol plus succinate, those specific for C₁ assimilation (hydroxypyruvate reductase, serine-glyoxylate aminotransferase, glycerate kinase and glyoxylate-activated serine transhydroxymethylase) (Quayle, 1972; O'Connor & Hanson, 1975) showed high activities in methanol-grown bacteria, low activities in succinate-grown bacteria and intermediate activities (30 to 50% lower) in bacteria grown on methanol plus succinate (Table 1). Of the enzymes involved in, but not specific for, C₁ assimilation (Quayle, 1972), phosphoenolpyruvate carboxylase and methylenetetrahydrofolate dehydrogenase activities showed a trend similar to the C₁-specific enzymes, while formate-tetrahydrofolate ligase and *N*⁵-formyltetrahydrofolate cyclodehydrase were not repressed by succinate and were present in methanol-grown bacteria. 2-Oxoglutarate dehydrogenase, citrate synthase and malate synthase activities were highest in succinate-grown organisms and lower with the other two growth conditions. Methanol dehydrogenase and formaldehyde dehydrogenase are involved in both carbon assimilation and energy production (Quayle, 1972). These enzymes had similar activities in organisms grown on methanol or methanol plus succinate, but only low activities in succinate-grown organisms. Table 2 gives values from the literature for the specific activities of these enzymes in some facultative methanol-utilizing bacteria.

Induction and dilution curves

When succinate-grown bacteria were washed and resuspended in medium containing methanol, four enzymes specific for C₁ assimilation were fully induced before noticeable growth occurred (Fig. 1*a*). With methanol-grown bacteria exposed to succinate, the reverse situation occurred (Fig. 1*b*). Formaldehyde dehydrogenase was not included in this particular study, since the observed activity may be due to two separate enzymes, one of which is the methanol dehydrogenase (Patel, Mandy & Hoare, 1973; H. Wolf, unpublished data).

Table 1. Enzyme activities in extracts of *M. organophilum* grown on methanol, methanol plus succinate or succinateEnzyme activities are expressed as $\mu\text{mol product min}^{-1} (\text{mg protein})^{-1}$, except where indicated.

Enzyme	Growth substrate		
	Methanol	Methanol + succinate	Succinate
Hydroxypyruvate reductase	0.58	0.38	0.14
Serine-glyoxylate aminotransferase	0.17	0.11	0.01
Serine transhydroxymethylase (glyoxylate-activated)	0.10	0.06	0.005
Methanol dehydrogenase	0.09	0.08	0.03
Formaldehyde dehydrogenase	0.11	0.11	0.02
Glycerate kinase	0.15	0.07	ND
Phosphoenolpyruvate carboxylase	0.03	0.01	0.003
Formate-tetrahydrofolate ligase	0.12*	0.03*	0.03*
<i>N</i> ⁵ -Formyltetrahydrofolate cyclodehydrase	4.7*	4.4*	4.9*
Methylenetetrahydrofolate dehydrogenase	0.29	0.27	0.05
Malate synthase	0.007	0.006	0.016
Citrate synthase	0.02	0.03	0.10
2-Oxoglutarate dehydrogenase	0.001	0.013	0.086

ND, Not detectable.

* Expressed as $\text{nmol product min}^{-1} (\text{mg protein})^{-1}$.Table 2. Published values of enzyme activities in *Pseudomonas* AM1 and *Pseudomonas* 3A2Enzyme activities are expressed as $\mu\text{mol product min}^{-1} (\text{mg protein})^{-1}$, except where indicated.

Enzyme	Growth substrate			Ref.†
	Methanol	Methanol + succinate	Succinate	
<i>Pseudomonas</i> AM1				
Hydroxypyruvate reductase	1.57	0.32	0.35	1, 2
Serine-glyoxylate aminotransferase	0.083	0.008	0	2
Serine transhydroxymethylase (total activity)	0.042	—	0.014	1
Methanol dehydrogenase	0.54	0.49	0.06	2
Formaldehyde dehydrogenase (dye-linked)	0.003	—	—	3
Glycerate kinase	0.05	0.03	0.016	2
Phosphoenolpyruvate carboxylase	0.13	0.087	0	4
Formate-tetrahydrofolate ligase	10*	—	1.2*	1
Methylenetetrahydrofolate dehydrogenase	0.4	—	0.22	1
Malate synthase	0.08	—	0.07	4
Citrate synthase	0.067	—	0.12	1
<i>Pseudomonas</i> 3A2				
2-Oxoglutarate dehydrogenase	0.002	—	—	5

* Expressed as $\text{nmol product min}^{-1} (\text{mg protein})^{-1}$.† 1, Large & Quayle (1963); 2, Dunstan *et al.* (1972); 3, Johnson & Quayle (1964); 4, Salem *et al.* (1973); 5, Colby & Zatman (1972).

Fig. 1. (a) Induction and (b) repression of four enzymes specific for C₁ metabolism. (a) Succinate-grown bacteria were washed and resuspended in medium containing methanol at time zero; (b) methanol-grown bacteria were washed and resuspended in medium containing succinate at time zero. ○, Hydroxypyruvate reductase; ●, serine-glyoxylate aminotransferase; ■, methanol dehydrogenase; △, glycerate kinase; ▲, bacterial growth measured as extinction at 600 nm.

Similar results for the glyoxylate-activated serine transhydroxymethylase were published previously (O'Connor & Hanson, 1975).

Mutants

Several mutants were isolated which grew on succinate but showed little or no growth when C₁ compounds were used as sole carbon and energy sources. Table 3 shows enzyme activities for these mutants. Strain 17M is deficient in methanol dehydrogenase and strain 8Z is deficient in glycerate kinase. Strains 8A, 4M, 7A, 1M, 2M, 3M and 17A all lack methanol dehydrogenase activity and show little or no activity of two or more of the other enzymes specific for growth on C₁ compounds. Strains 7A, 17A, 17M, 1M, 2M and 3M were not revertible by NTG or EMS.

DISCUSSION

The regulation of the synthesis of enzymes uniquely utilized for growth on C₁ compounds in the facultative methylotroph *M. organophilum* differed in some ways from that of the methanol-utilizing bacterium *Pseudomonas* AM1. In both bacteria, enzymes involved in growth on C₁ compounds were induced by methanol, and methanol dehydrogenase was not repressed by growth in medium containing methanol plus succinate (Dunstan *et al.*, 1972). However, three of the key assimilatory enzymes (hydroxypyruvate reductase, serine-glyoxylate aminotransferase and glycerate kinase) were strongly repressed by growth on methanol plus succinate in *Pseudomonas* AM1 (Dunstan *et al.*, 1972).

None of the enzymes specific for growth on C₁ compounds tested was strongly repressed by growth on methanol plus succinate in *M. organophilum*, although both substrates are utilized (O'Connor & Hanson, 1975). In addition, five enzymes specific for C₁ metabolism (hydroxypyruvate reductase, methanol dehydrogenase, serine-glyoxylate aminotransferase, glycerate kinase and the glyoxylate-activated serine transhydroxymethylase) were induced when bacteria were changed from succinate medium to methanol medium, and were diluted out when bacteria were changed from methanol medium to succinate medium (Fig. 1, and

Table 3. Enzyme activities in methanol⁻ mutants and wild-type *M. organophilum*

All strains were grown on media containing 0.1 % succinate plus 0.5 % methanol. Activities are expressed as $\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$.

Strain	Hydroxy-pyruvate reductase	Serine-glyoxylate amino-transferase	Methanol dehydro-genase	Glycerate kinase	Serine transhydroxymethylase	
					+ Glyoxylate	- Glyoxylate
Wild type	0.38	0.11	0.08	0.07	0.16	0.13
17M	0.28	0.08	ND	0.06	0.19	0.16
8Z	0.27	0.08	0.09	0.006	0.12	0.07
8A	0.05	0.03	ND	0.06	0.20	0.01
4M	0.03	0.01	ND	0.01	0.08	0.10
7A	ND	ND	ND	0.10	0.016	0.011
17A	0.03	0.001	ND	0.03	0.03	0.03
1M	0.06	0.02	ND	ND	0.004	0.019
2M	0.15	0.05	ND	ND	0.019	0.024
3M	0.10	0.04	ND	ND	0.011	0.017

ND, Not detectable.

O'Connor & Hanson, 1975). The results suggest that these enzymes are inducible, but not repressed when succinate is added as an additional carbon and energy source.

Two Krebs cycle enzymes were repressed in methanol-grown bacteria, indicating that the Krebs cycle is not as important in *M. organophilum* during growth on methanol as it is during growth on succinate.

Coordinate control of the above five C₁-specific enzymes involving a common inducer is suggested by the data presented. The methanol dehydrogenase may be under separate control, since it is not repressed by succinate and the other enzymes are moderately repressed.

Possibly some or all of the genes coding for the five C₁-specific enzymes belong to a single regulon. The enzyme activities in the methanol⁻ mutants tested suggest this possibility. Seven separate isolates are pleiotropically negative. Five of these are not revertible by NTG or EMS, and might contain frameshift mutations. The other two, which are revertible by NTG and EMS, might contain nonsense mutations or mutations in a regulatory gene or a gene coding for an inducer. The question of operonic control can only be answered by genetic mapping, a tool which is now available in this organism (O'Connor *et al.*, 1977).

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