A Possible Alternative Mechanism for the Oxidation of Formaldehyde to Formate

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A survey of the occurrence of a number of enzymes possibly involved in the C₁ dissimilatory pathway in organisms using the serine pathway of carbon assimilation indicated that neither the methanol dehydrogenase nor the NH₄⁺-independent dye-linked formaldehyde dehydrogenase had a specific role in the oxidation of formaldehyde. Conversely, the activities of enzymes involved in the methenyl-THF pathway were shown to be induced during growth on methanol or methylamine, suggesting a potential dissimilatory role for this pathway.

INTRODUCTION

The central pathway for the oxidation of reduced C₁ compounds in methylotrophs has generally been accepted to be a sequence of three successive oxidation reactions:

↑
N-Methyl compounds

Therefore, irrespective of the reduced C₁ compound used as the substrate for growth, formaldehyde is oxidized via formate.

Several enzymes have been reported to be capable of oxidizing formaldehyde (Stirling & Dalton, 1978). These include both NAD(P)-linked and dye-linked enzymes. It is assumed that, in organisms which lack an NAD(P)-linked dehydrogenase, formaldehyde is oxidized by either methanol dehydrogenase (Anthony & Zatman, 1964; Johnson & Quayle, 1964; Patel & Hoare, 1971; Mehta, 1973) which can oxidize, at least *in vitro*, formaldehyde as well as primary alcohols, or the NH₄⁺-independent dye-linked formaldehyde dehydrogenase (Johnson & Quayle, 1964; Mehta, 1975; Marison & Attwood, 1980). However, a mutant of *Pseudomonas* AM1 (*Pseudomonas* M15A) which lacked methanol dehydrogenase but was able to grow unimpaired on methylamine was described by Heptinstall & Quayle (1970), and the formaldehyde oxidation was ascribed to the dye-linked enzyme (Eady & Large, 1968). Studies with *Hyphomicrobium* X have shown that the dye-linked enzyme has a low activity, was not induced during growth on C₁ compounds and was a general aldehyde dehydrogenase unlikely to play a major role in the oxidation of formaldehyde (Marison & Attwood, 1980). Thus, neither the methanol dehydrogenase nor the dye-linked enzyme is necessarily responsible for the oxidation of formaldehyde *in vivo* during C₁ dissimilation, and therefore the mechanism of formaldehyde oxidation is an open question in organisms which lack an NAD(P)-linked formaldehyde dehydrogenase.

There have been reports showing that *Pseudomonas* AM1 and *Hyphomicrobium* X can assimilate formate via a sequence of reactions involving tetrahydrofolate derivatives (Large *et al.*, 1961; Attwood & Harder, 1978). Since the enzymes involved have been reported to be reversible (Rabinowitz & Pricer, 1958; Whiteley & Huennekens, 1962; O'Brien *et al.*, 1973), it seems plausible that they could also function in the oxidation of formaldehyde to formate.

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For clarity this sequence of reactions will be termed, in this paper, 'the methenyl-THF pathway'. To test this possibility, the enzymes involved were examined in crude extracts of bacteria that had been grown on a range of carbon compounds. The results are reported in this paper.

METHODS

Organisms and growth conditions. Organisms were grown in batch culture in an inorganic salts medium (Van Dijken *et al.*, 1975) containing a carbon source (50 mM). Growth in chemostat culture (working volume 75 ml) was in a mineral salts medium containing a carbon source (100 mM). Cultivation was at 30 °C, the pH was controlled at 6.8–7.0 with HCl (1 M) and the dissolved oxygen was maintained at 30–50%. Throughout the experiments a dilution rate of 0.04 h⁻¹ was used.

Substrate transition experiments were performed using a 20 litre batch fermenter. Mineral salts medium containing carbon source (20 mM) was maintained at pH 6.8–7.0 and 30 °C, stirred at 500 rev. min⁻¹ and aerated at 2 litre min⁻¹. On exhaustion of the first carbon source a second and different carbon source was added and growth was monitored. During growth on formate, extended culture was employed (Edwards *et al.*, 1970) in which formic acid (1 M) was used to maintain both the pH and formate concentration (16–18 mM) in the medium.

Growth. This was followed either by measuring the turbidity of the culture at 430 nm or by filtering the culture samples and drying the cells to a constant weight at 104 °C.

Assay of residual substrates. Samples were removed from the chemostat and centrifuged. The supernatants were analysed for ethanol with a gas chromatograph equipped with a flame ionization detector (Pye Series 104); a glass column (1.5 m × 4 mm internal diameter) packed with Porapak Q was used with N₂ as the carrier gas (40 ml min⁻¹) at 160 °C. Formate was measured by the method of Lang & Lang (1972) and methylamine using the method of Large *et al.* (1969).

*Isolation of mutants of *Hyphomicrobium* X.* Mutants lacking methanol dehydrogenase activity, but capable of growth on methylamine, were obtained using the mutation and selection procedure described by O'Connor *et al.* (1977), followed by replica platings on agar plates containing either methanol or methylamine.

Preparation of crude extracts. Organisms were disrupted using an ultrasonic disintegrator (MSE model 60 W) as previously reported (Marison & Attwood, 1980). After centrifugation at 30000 g for 15 min at 4 °C the supernatant was used for enzyme assays.

Enzyme assays. All assays were performed at 30 °C spectrophotometrically using a double-beam spectrophotometer. Enzyme activities are expressed as nmol electron acceptor reduced or product formed min⁻¹ (mg protein)⁻¹. Methanol dehydrogenase (EC 1.4.99.-), formaldehyde dehydrogenase and formate dehydrogenase (EC 1.2.1.2) activities were measured using the assay procedures of Marison & Attwood (1980) and Johnson & Quayle (1964). Formyl-THF synthetase (EC 6.3.4.3), methenyl-THF cyclohydrolase (EC 3.5.4.9) and methylene-THF dehydrogenase (EC 1.5.1.5) activities were measured using the procedures of Rabinowitz & Pricer (1963), Greenberg (1971) and Scrimgeour & Huennkens (1963), respectively.

Protein determination. Protein was measured using the Lowry method with bovine serum albumin as standard.

Chemicals. 10-Formyltetrahydrofolate (formyl-THF) and 5,10-methenyltetrahydrofolate (methenyl-THF) were prepared from 5-formyltetrahydrofolate (calcium leucovorin) as described by Greenberg (1971).

RESULTS AND DISCUSSION

Organisms which were known not to contain an NAD(P)-linked formaldehyde dehydrogenase were grown in batch culture on C₁ and non-C₁ compounds. Crude extracts were prepared and analysed for the activity of the enzymes involved in the direct dissimilation pathway: methanol dehydrogenase, dye-linked formaldehyde dehydrogenase and formate dehydrogenase. All extracts prepared from wild-type organisms, irrespective of the carbon source for growth, contained detectable levels of all the enzymes; methanol dehydrogenase and formate dehydrogenase, but not the dye-linked formaldehyde dehydrogenase, were further induced during growth on reduced C₁ compounds. The mutants *Pseudomonas* M15A (Heptinstall & Quayle, 1970) and *Hyphomicrobium* X mutant 1 lacked methanol dehydrogenase (Table 1a). The activity of the dye-linked formaldehyde dehydrogenase was always low and was no higher in extracts prepared from the mutants than in extracts prepared from wild-type organisms. This indicated that the enzyme was not induced to account for the formaldehyde oxidation necessary for growth on methylamine in the absence

Table 1. Activities of the enzymes in the direct dissimilation pathway for C_1 compounds and the 'methenyl-THF pathway' in crude extracts of methylotrophs grown on a range of carbon substrates in batch culture and continuous culture

Organism	Growth substrate	Specific activity [nmol min ⁻¹ (mg protein) ⁻¹]						
		Methanol dehydrogenase		Dye-linked formaldehyde dehydrogenase	Formate dehydrogenase	Methylene-THF dehydrogenase	Methenyl-THF cyclohydrolase	Formyl-THF synthetase
		MeOH	HCHO					
(a) Batch culture								
<i>Hyphomicrobium</i> X	MeOH	764	892	21	316	222	5	766
	MeNH ₂	808	793	15	305	231	4	1775
	EtOH	60	82	28	26	118	11	250
<i>Hyphomicrobium</i> X mutant 1	MeNH ₂	0	0	6	349	233	3	1821
	EtOH	0	0	11	35	105	5	287
<i>Pseudomonas</i> AM1	MeOH	251	254	5	37	339	100	880
	MeNH ₂	283	352	5	34	288	97	908
	Succinate	49	52	4	3	97	90	244
<i>Pseudomonas</i> M15A	MeNH ₂	0	0	4	47	217	90	784
	Succinate	0	0	4	3	72	90	201
	MeOH	316	374	33	67	386	4	571
<i>Hyphomicrobium vulgare</i> 3	EtOH	146	144	21	6	292	3	295
(b) Continuous culture								
<i>Hyphomicrobium</i> X	MeOH	748	757	17	304	220	4	634
	MeNH ₂	752	985	15	379	256	2	1504
	HCOOH	271	308	5	240	215	4	836
	EtOH	78	76	30	67	78	2	238

of methanol dehydrogenase activity, and therefore it is unlikely that methanol dehydrogenase has a role in formaldehyde oxidation *in vivo* during growth on methanol or methylamine. Furthermore, the results confirm those previously reported which suggested that the dye-linked formaldehyde dehydrogenase was a general aldehyde dehydrogenase not responsible for formaldehyde oxidation during the dissimilation of C_1 compounds (Marison & Attwood, 1980). Since neither methanol dehydrogenase nor dye-linked formaldehyde dehydrogenase appears to be responsible for formaldehyde oxidation *in vivo*, the extracts were analysed for methylene-THF dehydrogenase, methenyl-THF cyclohydrolase and formyl-THF synthetase — enzymes of the methenyl-THF pathway. All activities were detected and, except for the cyclohydrolase, shown to be induced during growth on reduced C_1 compounds (Table 1a). These results were consistent with the enzymes being involved in the maintenance of the cell's C_1 pool for amino acid and purine biosynthesis and suggest an additional role when the cells were grown on reduced C_1 compounds. The consistently low activity of the freely reversible enzyme, methenyl-THF cyclohydrolase was probably due to the necessity of using an inefficient assay procedure (Greenberg, 1971).

When extracts were prepared from cells grown in continuous culture and analysed for the enzymes, all were detected at levels similar to those measured in extracts prepared from batch culture cells (Table 1b). Methylamine-grown cells always contained a high level of formyl-THF synthetase which is difficult to explain. More importantly, methylene-THF dehydrogenase and formyl-THF synthetase were induced during growth on C_1 compounds and furthermore the activities were similar in extracts prepared from cells grown on methanol, methylamine or formate. When the cells were grown on formate the induced enzyme level could be accounted for by the two known roles for the enzymes: the requirement to maintain the C_1 pool and involvement in the C_1 assimilation pathway for growth (Large & Quayle, 1963; Attwood & Harder, 1978). To explain the equally high induced level of enzymes in cells grown on methanol or methylamine one has to postulate that growth on any reduced C_1 compound induces all C_1 enzymes irrespective of whether or not an enzyme has a maintenance role during growth on the specific compound present or a second assimilatory role over and above the maintenance of the cell's C_1 pool. This maintenance role during growth on methanol or methylamine should be equally as important as its C_1 assimilation role during growth on formate. We propose that this second role is the oxidation of formaldehyde to formate as part of the *in vivo* dissimilation pathway. This idea was supported when enzyme levels were measured during transition experiments in which the organism was grown initially on one substrate and on its exhaustion a second different substrate was added to the medium. When *Hyphomicrobium* X was initially grown on formate and subsequently on ethanol, the activities of methylene-THF dehydrogenase and formyl-THF synthetase fell rapidly by 4.5- and 2-fold, respectively. A differential plot (Monod *et al.*, 1952) for both enzymes was biphasic and showed an abrupt change in the differential rate of enzyme synthesis when the formate was exhausted and ethanol added. Similar results (Fig. 1a, b) were seen when transition experiments were performed using *Hyphomicrobium* X mutant 1 growing initially on methylamine and then ethanol. The reverse was seen if the organisms were grown on ethanol initially rather than on a reduced C_1 compound. Transition experiments using wild-type *Hyphomicrobium* X grown initially on methanol or methylamine were not possible. This was because the methanol dehydrogenase induced during the growth on C_1 compounds rapidly oxidized the added ethanol to acetate. The acetate accumulated in the medium (up to 16 mM) and since no energy was generated by this conversion a long lag phase occurred. In some experiments further growth was inhibited (I. W. Marison & M. M. Attwood, unpublished data).

To obtain direct evidence for the proposed alternative formaldehyde oxidation pathway, attempts were made to isolate mutants of *Hyphomicrobium* X or *Pseudomonas* AM1 which would not grow on formate because they lacked formyl-THF synthetase activity. Such a mutant could be tested for the ability to grow on methanol or methylamine; if it grew then the

Fig. 1. Activities of methylene-THF dehydrogenase and formyl-THF synthetase in *Hyphomicrobium* X mutant 1 transferred from growth on methylamine to ethanol. (a) The activities [$\text{nmol min}^{-1} (\text{mg protein})^{-1}$] of methylene-THF dehydrogenase (●) and formyl-THF synthetase (□) and cell density (○) were measured as described in Methods. (b) A differential plot of the activities of the methylene-THF dehydrogenase (●) and formyl-THF synthetase (□): the plot was prepared from the data in (a).

proposed pathway would be incorrect. To date, such a mutant has not been isolated. However, stable mutants isolated from methylotrophs are difficult to obtain.

Despite the lack of direct evidence for the role of methenyl-THF pathway enzymes in formaldehyde oxidation during growth on reduced C_1 compounds, the enzyme levels in cells reported in this paper are consistent with the idea that the oxidation of formaldehyde to formate cannot be accounted for by the activity of either methanol dehydrogenase or the dye-linked formaldehyde dehydrogenase but can be accounted for by the induced activities of the methenyl-THF pathway enzymes.

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