METHANOL ACCUMULATION BY RESTING CELLS OF *METHYLOSINUS TRICHOSPORIUM* (I)

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(Received November 21, 1986)

Techniques for measuring methanol accumulated by resting cells of *Methylosinus trichosporium* in the narrow range of 70–80 mM phosphate concentration have been standardized. The maximum amount of methanol, $6 \mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{hr}^{-1}$, was accumulated at a cell density of $2 \text{ mg} \cdot \text{ml}^{-1}$ (w/v) of cells in the early stationary phase at pH 6.5, temperature 35°C . The phosphate-dependent inhibition of methanol dehydrogenase was found to be uncompetitive and reversible. Further, 80 mM phosphate inhibited methane monooxygenase and formate dehydrogenase by 16% and 20% respectively. A possible biochemical explanation of the role of phosphate inhibition is offered.

The methane-utilizing bacteria are versatile and available in a variety of habitats. They are predominantly aerobic, gram-negative bacteria and are collectively called methylotrophs. The methylotrophs occupy a special position among microorganisms utilizing C_1 compounds (methane, methanol, dimethyl ether, methyl formate, etc.) due to their characteristic biochemical properties and physiology (1). On the basis of the morphology of their complex intracytoplasmic paired membranes and the nature of their carbon assimilation pathways (2) these organisms are broadly classified into type I and II groups. It has now been established that methylotrophs oxidize methane to carbon dioxide by a route involving successive two-electron oxidation steps via methanol, formaldehyde, and formate.

The single-step oxidation of methane to methanol by the obligate methanotroph *Methylosinus trichosporium* is a function of the enzyme methane monooxygenase (MMO) (3). MMO is a complex enzyme with broad substrate specificity that oxygenates methane, higher alkanes, *n*-alkenes, ethers, alicyclic, aromatic, and

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heterocyclic compounds(4,14). Some of these substrates have been chosen for monitoring MMO enzyme activity.

The assay of MMO in terms of single step oxidation to methanol has not been possible because of the further oxidation of the methanol. Formate is found to be the first stable identifiable product. This paper reports the assay of MMO according to the methanol accumulated in the presence of 70–80 mM phosphate ions. The kinetics of the inhibition of methanol dehydrogenase by phosphate has been studied. Further, the effect of phosphate ions on other enzymes of the methane oxidation pathway, namely methane monooxygenase and formate dehydrogenase, have also been studied. The data indicate that the target of inhibition by phosphate ions is specifically associated with methanol oxidation.

MATERIALS AND METHODS

Chemicals. Potassium cyanide, sodium azide, ethylene diamine tetraacetate, *p*-nitrophenyl hydrazine, and α,α -bipyridyl were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Methane used as a routine growth substrate was purified from biogas to 95–97% purity after passing through a KOH solution. Standard methane (5.1% methane in nitrogen) was obtained from EDT Research (London). Pure methanol (99.9%) was a gift from Prolabo (Paris). All the other chemicals were of AR grade and were obtained locally.

Culture and growth conditions. The bacterial strain *Methylosinus trichosporium* NCIB 11131 was obtained from NCIB (Aberdeen, U.K.) and maintained on nitrate mineral salts (NMS) agar slant(5) tubes in 'anaerobic jars' under an atmosphere of methane and air (1:1, v/v) at 30°C. The organisms were grown at 30°C in 500- and 1,000-ml Erlenmeyer flasks fitted with stopcocks containing respectively 150 and 300 ml of NMS medium with methane: air (1:1, v/v) as the sole carbon and energy source. Three-day-old seed cultures (7.5%, v/v) were used to inoculate the growth medium. The initial pH was 6.8 and it was left uncontrolled during the growth period. Larger scale cultivation of cells was carried out in a 2-l Bioengineering fermenter containing 1.5 l of NMS medium. Other conditions were the same as those used for shake-flask runs.

To measure growth, a series of parallel cultures were cultivated in 500-ml flasks containing 150 ml of NMS medium. Two flasks were removed at time intervals of 24 up to 144 hr and the optical density (OD) was read at 600 nm. One OD unit corresponds to $2 \text{ mg} \cdot \text{ml}^{-1}$ of dry cell weight.

Preparation of cell suspension. Cells were harvested at the end of the exponential growth phase by centrifugation ($10,000 \times g$, 15 min, 4°C), washed twice with 20 mM sodium phosphate buffer (pH 7.0), and resuspended in the same buffer containing 5 mM MgCl_2 at a cell concentration of $70\text{--}80 \text{ mg} \cdot \text{ml}^{-1}$.

Analytical methods. Methane monooxygenase activity was measured in 35-ml conical flasks sealed with 'suba-seals.' The gaseous phase of the flask was replaced with a gas mixture of 50% methane and 50% air. The reaction mixture contained

per ml, 20 μmol sodium phosphate buffer (pH 7.0), 5 μmol MgCl_2 , 2–4 mg of whole cells. The reaction mixture was incubated at 30°C on a temperature-controlled rotary shaker (200 rpm). A 50- μl sample from the gaseous phase was removed with a gas syringe and assayed for methane by gas-chromatography every 90 min. The enzyme activity is reported either in terms of μmol of methane oxidized $\text{mg}^{-1} \cdot \text{hr}^{-1}$ or μmol of methanol accumulated $\text{mg}^{-1} \cdot \text{hr}^{-1}$ (see below). In the experiments on the effect of substrate concentration on MMO activity, the reaction mixture was the same as above except that methane was replaced by varying concentrations of ethylene (3–50%) in the gaseous phase. The product of ethylene oxidation, ethylene oxide, was measured by gas chromatography. Enzyme activity is reported as μmol of ethylene oxide formed $\text{mg}^{-1} \cdot \text{hr}^{-1}$.

To assay methanol, the reaction mixture was the same as above except that 20 mM buffer was replaced by 70–80 mM buffer. Methanol was assayed by removing a 2- to 4- μl sample from the aqueous phase with a syringe and analyzing it by gas chromatography using a 4-foot glass column packed with Porapak Q (80–100 mesh) in a Nucon Series 5700 chromatograph.

Methanol dehydrogenase activity (MDH) was measured by following the disappearance of methanol in the aqueous phase. The reaction mixture was the same as for methane monooxygenase except that the reaction mixture contained 15 $\mu\text{l} \cdot \text{ml}^{-1}$ of methanol in 20 mM phosphate buffer as substrate. The enzyme activity is reported as μmol of methanol consumed $\text{mg}^{-1} \cdot \text{hr}^{-1}$.

Formate dehydrogenase activity was measured by following formate oxidation. The reaction mixture contained per ml, 10 μmol of sodium formate, 5 μmol MgCl_2 , 2–4 mg cells. Samples (10 μl) were obtained with a syringe from the gaseous phase after acidifying the aqueous phase, and analyzed for CO_2 production. The activity is expressed as μmol of CO_2 produced $\text{mg}^{-1} \cdot \text{hr}^{-1}$.

RESULTS

Effect of inoculum size on growth of M. trichosporium

The effect of inoculum size on specific growth rate and yield of *M. trichosporium* was studied in a 2-l Bioengineering fermenter to determine the best conditions for growth. The inoculum levels tested were 3.35, 5.0, 7.5, and 10.0 (% v/v). A maximum yield of 0.29 $\text{g} \cdot \text{g}^{-1}$ of culture medium and a specific growth rate (μ) of 0.136 hr^{-1} was obtained with 7.5% (v/v) inoculum size. Increasing inoculum size to 10% (v/v) decreased both these parameters. Hence, in all further experiments, 7.5% inoculum size was used.

Effect of culture age on MMO activity

The methane monooxygenase activity was measured at different growth periods of *M. trichosporium* and the results are shown in Fig. 1. The exponential phase of growth continues up to 72 hr, followed by a stationary phase. During the exponential phase, the uptake rates for methane were high. Maximum methanol

Fig. 1.

Fig. 2.

Fig. 1. Effect of culture age on methane uptake ($\circ$), methanol formation ($\bullet$), and CO_2 production ($\square$). Cells were harvested at different stages of growth (24, 48 hr etc.) and the three parameters were assayed separately. The experimental variation was 10–12%.

Fig. 2. Effect of phosphate concentration on methanol oxidation in cell suspensions of *M. trichosporium* in suba-sealed 35 ml conical flasks containing 10–12 μmol of methanol.

accumulation of $0.32 \mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{hr}^{-1}$ was obtained from an extracellular reaction mixture of stationary phase cells.

Methanol accumulation by M. trichosporium

To determine factors optimizing methanol accumulation in whole cells, it is necessary to inhibit further oxidation of methanol in culture medium. Only small quantities of methanol (when assayed by gas chromatography) accumulated when washed cell-suspensions of *M. trichosporium* were incubated with methanol and air for 2–3 hr in 5 ml of reaction mixture containing 20 mM phosphate. A number of inhibitors such as potassium cyanide, iodoacetate, ethylene diamine tetra-acetate, and *p*-nitrophenyl hydrazine (a known specific inhibitor of methanol dehydrogenase)(6), were screened for their ability to inhibit methanol dehydrogenase in whole cell suspensions of *M. trichosporium* but none led to an accumulation of methanol in extracellular reaction mixtures (data not shown). Therefore, following the work of HIGGINS and QUAYLE(7) in *Pseudomonas* sp. and TONGE et al. (8) in *M. trichosporium* cell-free extracts, attempts were made to cause methanol accumulation in whole-cell suspensions of *M. trichosporium*. A 20 mM phosphate concentration was found to result in some methanol accumulation in the extracellular reaction mixture by inhibiting methanol dehydrogenase activity, and methanol

accumulation could be progressively increased by increasing the phosphate concentration in the reaction mixture (Fig. 2). The maximum inhibition of methanol oxidation occurs at 120 mM phosphate concentration.

Factors affecting methanol accumulation

A time course experiment with whole cells indicated that methanol accumulation is linear up to 3.5 hr after which it slows down (Fig. 3a). Thus, in all further experiments the 3-hr incubation period was used. The effect of temperature on methanol accumulation by resting whole-cell suspensions indicated the optimum temperature to be 35°C. Increasing the temperature resulted in a decrease in methanol accumulation, possibly due to instability of the enzyme monooxygenase (Fig. 3b). Further, methane monooxygenase was found to retain over 80% of its activity when stored at 4°C for 8–10 days (data not shown).

Figure 3c shows the effect of pH on methanol accumulation. Sodium phosphate buffer (80 mM) was used for pH values from 5.5 to 8.5. The optimum pH was 6.5. It resulted in 2.7 μmol methanol accumulation $\text{mg}^{-1} \cdot \text{hr}^{-1}$.

The cell concentration in the reaction medium also influences methanol accumulation. The amount of methanol accumulated in 3 hr increased to 6.4 μmol as cell concentration was increased to 4 $\text{mg} \cdot \text{ml}^{-1}$. At higher cell concentrations there was no further increase in methanol accumulation. The effect of increased substrate concentration on enzyme activity was studied in terms of ethylene to ethylene oxide conversion. An ethylene concentration of 15% (220 μmol) supported

Fig. 3a. Effect of time of incubation on accumulation of methanol in resting cell suspensions.

Fig. 3b. Effect of temperature on methanol accumulation in resting cell suspensions.

Fig. 3c. Effect of pH on methanol accumulation in resting cell suspensions.

Fig. 4. Linearization of data of the kinetics of phosphate inhibition of methanol dehydrogenase activity. Whole cell suspensions of *M. trichosporium* supplemented with varying phosphate levels were used. Cell density of $2 \text{ mg}^{-1} \cdot \text{ml}^{-1}$ was employed.

a maximum ethylene oxide production of $0.15 \mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{hr}^{-1}$.

Kinetics of phosphate inhibition of methanol dehydrogenase

The kinetics of phosphate inhibition of methanol dehydrogenase was studied at three phosphate concentrations. Figure 4 gives a Lineweaver-Burk plot of enzyme activity at various substrate concentrations. A series of parallel lines with the same slope ($K_m/V_{\max}$) was obtained with 20, 60, 80 mM phosphate in the reaction mixture. The degree of inhibition i (9) was calculated from Eq. (1) and rose from 40 to 53% as the substrate concentration was increased from 2.4 to $11.9 \mu\text{M}$ in the reaction mixture.

$$i = \frac{(S)(I/K_I)}{K_m + (1 + I/K_I)(S)} \quad (1)$$

The calculated K_I value is 66 mM for the phosphate-caused inhibition of methanol oxidation.

Effect of phosphate on methane monooxygenase and formate dehydrogenase

The effect of 80 mM phosphate ions was studied on methane monooxygenase and formate dehydrogenase. Table 1 indicates that only 16% of MMO and 20% of formate dehydrogenase activity was inhibited by this phosphate level.

Reversal of phosphate inhibition

The inhibition of methanol oxidation by phosphate ions could be reversed by washing 80 mM phosphate-treated cells twice with buffer containing 20 mM phos-

Table 1. Effect of phosphate on enzymes of the methane oxidation pathway.

Enzyme	Specific activity ($\mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{hr}^{-1}$)		% Inhibition
	20 mM	80 mM	
1. Methane monooxygenase (MMO) ^a	0.15	0.126	16
2. Methanol dehydrogenase (MDH) ^b	2.57	0.51	81
3. Formate dehydrogenase (FDH) ^c	3.83	3.04	20

^a MMO was measured by following the μmol of ethylene oxide formed $\text{mg}^{-1} \cdot \text{hr}^{-1}$.

^b MDH was measured by following the μmol of methanol oxidized $\text{mg}^{-1} \cdot \text{hr}^{-1}$.

^c FDH activity is expressed as μmol of CO_2 produced $\text{mg}^{-1} \cdot \text{hr}^{-1}$.

Fig. 5. Reversal of phosphate inhibition of methanol oxidation in cell suspension of *M. trichosporium*. Cells were washed twice with 20 mM phosphate buffer after the addition of 80 mM phosphate ions to the reaction mixture (●). Controls were incubated with 80 mM phosphate ions without washing (■) or by not adding excess phosphate and not washing (○).

phate ions (Fig. 5). Whole cell suspensions washed twice with 20 mM phosphate buffer after incubation with 80 mM phosphate buffer oxidized methanol at a rate equivalent to that of controls receiving only basal 20 mM phosphate.

DISCUSSION

The selective inhibition of methanol oxidation by high phosphate concentration in whole cell suspensions of *M. trichosporium* has been demonstrated. Although methanol oxidation has been reported to be partially inhibited by compounds like potassium cyanide, sodium azide, ethylene diamine tetraacetic acid (7), these compounds have also been found to affect methane monooxygenase (10). But the use of phosphate ions overcomes the problem of co-inhibition of methane monooxygenase and this can be used effectively for extracellular accumulation of methanol.

The detailed study of conditions favoring methanol accumulation indicate a pH optimum of 6.5 at 35°C. The apparent decrease in methanol accumulation above 40°C could possibly be due to instability of the monooxygenase system. The enzyme was found to be stable at 4°C for over 10 days when stored in 20 mM phosphate ions containing 5 mM MgCl₂. Mg²⁺ ions were necessary to retain enzyme activity. Cell densities above 3 mg · ml⁻¹ do not favor proportional increase in methanol accumulation, possibly due to limitation of methane and oxygen transfer.

The kinetic data (Fig. 4) of phosphate inhibition of methanol dehydrogenase indicates an uncompetitive mode of inhibition. The degree of inhibition was found to increase from 40 to 53% as the substrate concentration was increased from 2.4 to 11.4 μM, characteristic of uncompetitive inhibition. This indicates that phosphate binds to a site on the enzyme (or enzyme-substrate complex) other than the active site. Further, the phosphate-caused inhibition was reversible and the enzyme activity was completely restored on washing the cells with basal buffer.

The specificity of inhibition by high phosphate levels on methanol oxidation is interesting as it is found to affect methane monooxygenase (16%) and formate dehydrogenase (20%) only marginally. Hence phosphate ions can be used in large scale studies involving methanol accumulation.

At this time the mode of inhibition of methanol dehydrogenase by phosphate ions can only be conjectured. It may inhibit enzyme activity either (i) by binding to certain metal ions essential for enzyme activity, as in desacetoxycephalosporin C synthetase and cyclase enzymes of cephalosporin synthesis in *Streptomyces clavuligerus* (11), or (ii) by phosphorylation of certain amino acid residues critical to enzyme activity as in some enzymes of the energy transduction pathway (12, 13). Both of these possibilities are being examined in detail.

NOMENCLATURE

I = inhibitor concentration (mol · l⁻¹),

i = degree of inhibition,

K_i = equilibrium constant ($=k_{-i}/k_{+i}$) for

K_m = Michaelis constant,

S = substrate concentration (mol · l⁻¹),

$Y_{X/S}$ = overall yield factor, $\Delta X / -\Delta S$ (g · g⁻¹),

μ = specific growth rate (hr⁻¹).

This work was supported by a grant from Department of Non-Conventional Energy Sources, GOI, New-Delhi.

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