Energy Coupling in *Methylosinus trichosporium*

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INTRODUCTION

Methane-utilizing bacteria are of potential value for production of single-cell protein and the efficiency with which they convert methane to biomass is therefore of practical as well as theoretical interest. Several reports of cell-yield determinations have recently been collated (Barnes *et al.*, 1977), but there is some variation in the values obtained. Recent studies with several methylotrophs (Davey & Mitton, 1973; Tonge *et al.*, 1974; Anthony, 1975*a*; Widdowson & Anthony, 1975) have led to schemes being proposed for their electron transport and energy transducing systems (Tonge *et al.*, 1975; Higgins, Knowles & Tonge, 1977). Theoretical analyses of the relationships between cell-yield data, sites of ATP synthesis and P/O ratios have also been published (Van Dijken & Harder, 1975; Barnes *et al.*, 1977). However, there are no direct experimental data concerning either the sites of ATP synthesis or the P/O ratios associated with substrate oxidation in these bacteria.

The chemiosmotic theory of oxidative phosphorylation (Mitchell, 1966*a, b*) postulates that a transmembrane proton gradient is generated as a result of electron flow down the respiratory chain and that this gradient can drive ATP synthesis such that 1 mol ATP is synthesized per 2 g-equiv. H⁺ translocated. Therefore, the P/O ratio can be calculated from measurements of the →H⁺/O ratios (g-equiv. H⁺ translocated per g-atom O consumed). Recently such ratios have been estimated for a range of heterotrophic bacteria (Jones *et al.*, 1975), the chemoautotrophs, *Hydrogenomonas eutropha* (Beatrice & Chappell, 1974), *Thiobacillus neapolitanus* (Drozd, 1974) and *Nitrosomonas europaea* (Drozd, 1976), and indirectly in *Nitrobacter winogradskyi* (Cobley, 1976). In all these studies the →H⁺/O ratios generally indicated a P/O ratio which was in good agreement with that predicted from previous work (Peck, 1968; Suzuki, 1974). We describe here similar studies with the obligate methylotroph *Methylosinus trichosporium*.

METHODS

Micro-organism, source, maintenance and culture. *Methylosinus trichosporium* OB 3b was a gift from Professor R. Whittenbury, Department of Biological Sciences, University of Warwick, and was maintained and grown on methane in continuous culture as described previously (Tonge, Harrison & Higgins, 1977).

Preparation of washed bacterial suspensions. Organisms were harvested (6000 g, 30 min, 4 °C), washed with Tris/HCl buffer (1 mM, pH 7.1) containing KCl (140 mM) and resuspended in the same buffer to a density of 5 to 10 mg dry wt ml⁻¹. For subsequent use, organisms were usually starved by incubating suspensions for 2 to 6 h at 30 °C on a rotary shaker at 210 rev. min⁻¹.

Determination of $\rightarrow H^+/O$ ratios. The ratios were measured as described by Drozd (1976), using the following substrate concentrations: methane, 30 μM ; methanol, 0.5 to 2 mM; formaldehyde, 2 mM; sodium formate, 0.5 to 10 mM; ascorbate (1.5 mM)/*N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD), 4.0 mM. Incubation mixtures contained carbonate dehydratase (EC. 4.2.1.1; 30 μg ml⁻¹), and potassium thiocyanate (150 mM) was added at least 10 min before making measurements to neutralize any electrical charge across the membrane and to maximize the pH difference. The scale was calibrated by adding nitrogen-sparged 0.01 M-KOH.

Determination of respiration rates. Respiration rates were measured at 30 °C in a Rank oxygen electrode (Rank Bros, Bottisham, Cambridgeshire) using substrate concentrations as described for the determination of $\rightarrow H^+/O$ ratios. Incubation mixtures (total volume 3 ml) contained, in addition to substrate: Tris/HCl buffer (1 mM, pH 7.1); KCl (140 mM); and organism suspension (15 to 30 mg dry wt).

Chemicals. Methane was purchased from British Oxygen Co., methanol, paraformaldehyde and formate from BDH. Formaldehyde was prepared from paraformaldehyde as described by Strøm, Ferenci & Quayle (1974). Carbonyl cyanide *m*-chlorophenylhydrazone and carbonate dehydratase were obtained from Sigma.

RESULTS

Typical small acidifications of the external medium (proton pulses) were observed when air-saturated potassium chloride (140 mM) was added to suspensions of *M. trichosporium* incubated with methane, ascorbate/TMPD or known intermediates in methane oxidation. The magnitude of these pulses was independent of the pH of the Tris/HCl buffer over the pH range 6.6 to 7.8 and the $t_{\frac{1}{2}}$ of decay of the pulses was in the range 40 to 80 s. The $\rightarrow H^+/O$ ratios obtained using starved suspensions of *M. trichosporium* are shown in Table 1. Separate determinations using an oxygen electrode (see Methods) showed that these preparations had negligible endogenous respiration rates but, with the substrates tested in Table 1, values of 29 to 37 μmol O₂ consumed (g dry wt)⁻¹ min⁻¹ were recorded. The use of unstarved organisms did not result in appreciable deviation from these $\rightarrow H^+/O$ ratios and the presence of carbonate dehydratase in the assay obviated any need to correct for proton production in the formate oxidase reaction (Garland, Downie & Haddock, 1975). A wide range of sodium formate concentrations was used to ensure that the formate dehydrogenase was saturated with substrate. The rates of decay of pulses were unaltered by sodium formate. There was, therefore, no evidence for any rapid Na⁺:H⁺ antiport mechanisms (West & Mitchell, 1974) which might have interfered with extrapolation of pulse heights.

The addition of ethanolic carbonyl cyanide *m*-chlorophenylhydrazone uncoupling agent (5 μM final concn) at pulse maxima caused their rapid collapse and thereafter it was not possible to evoke pulses by further additions of air-saturated KCl (140 mM).

DISCUSSION

The effects of carbonyl cyanide *m*-chlorophenylhydrazone are consistent with predictions of the chemiosmotic hypothesis since this is an uncoupling agent that would increase proton conduction across membranes. Thus these results suggest that the minute pH changes measured are genuine reflexions of metabolically-linked proton extrusion. A rapid uptake of hydroxyl ions by the bacteria would also explain the results, but in terms of the chemiosmotic theory this would not satisfy the postulated mechanisms for proton-driven ATP

Table 1. $\rightarrow H^+/O$ ratios for washed suspensions of *Methylosinus trichosporium*

Ratios were determined using starved organisms as described in Methods. Average values are quoted together with the standard error of the mean and the number of determinations (in parentheses). Measurements were made on four different batches of organisms for each substrate.

Added substrate	$\rightarrow H^+/O$
Methane	1.9 ± 0.13 (4)
Methanol	1.8 ± 0.12 (5)
Formaldehyde	1.9 ± 0.05 (7)
Formate	1.7 ± 0.15 (9)
Ascorbate/TMPD	1.5 ± 0.05 (5)

synthesis. The $\rightarrow H^+/O$ ratios suggest a probable P/O ratio of 1 for all substrates tested on the assumption that 1 mol ATP is synthesized per 2 g-equiv. H^+ translocated. The value for ascorbate/TMPD is entirely consistent with entry of electrons into a conventional respiratory chain at the level of cytochrome *c*. Methanol and formaldehyde oxidation in *M. trichosporium* and other methylotrophs is closely linked to cytochrome *c* reduction (Tonge *et al.*, 1975; Higgins *et al.*, 1977; Anthony, 1975*b*; Widdowson & Anthony, 1975), and therefore a P/O ratio of 1 would be expected for the activity of proton-translocating loop 3 (Mitchell, 1970). These results are similar to those obtained for thiosulphate oxidation in *Thiobacillus neapolitanus* (Drozd, 1974) and hydroxylamine oxidation in *Nitrosomonas europaea* (Drozd, 1976), where substrate oxidation is closely linked to cytochrome *c* reduction (Aleem & Lees, 1963; Aleem, 1970; Suzuki, 1974).

A P/O ratio of 1 for formate is unexpected since its oxidation is known to be NADH-linked (Quayle, 1972). This suggests two of the three proton translocating loops normally associated with NADH oxidation are missing or that NADH oxidation via a conventional electron transport pathway does not occur. There is some evidence for the latter hypothesis (Higgins *et al.*, 1977; Tonge, Drozd & Higgins, 1976; Tonge *et al.*, 1977). During oxidation of methane to carbon dioxide the only source of NADH is the formate dehydrogenase reaction (Quayle, 1972). Some of this NADH is required for general biosynthetic reactions and in *M. trichosporium* (a serine pathway organism) a large proportion is required to fix carbon dioxide. Therefore, only a small amount of NADH is available for energy transduction and there may have been little selective advantage during evolution in developing more than one coupling site. During the oxidation of methane, oxygen is consumed in an oxygenase reaction (Tonge *et al.*, 1977) and therefore the apparent P/O ratio of 1 for methane suggests a true P/O ratio of 2. However, it is possible that the $\rightarrow H^+/O$ ratio is an overestimate due to the difficulty of measuring proton pulses using a limited quantity of gaseous substrate whose further oxidation can lead indirectly to endogenous cytochrome *c* reduction. A scheme for electron supply to the methane monooxygenase of *M. trichosporium*, in which electrons derived from the oxidation of methanol and formaldehyde are recycled into the monooxygenase reaction, has been proposed (Tonge *et al.*, 1975; Higgins *et al.*, 1977). It is possible that there is a further coupling site available during this recycling process but this would not be necessary to account for observed growth yields on methane or methanol which suggest that as much ATP is probably produced during the oxidation of 1 mol methane as from the oxidation of 1 mol methanol.

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