Respiration-linked proton translocation in the obligate methylotroph *Methylophilus methylotrophus*

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The stoichiometries of respiration-linked proton translocation in *Methylophilus methylotrophus* were determined by using both the oxygen-pulse and initial-rate methods. The latter has also been used to determine $\rightarrow$ charge/O quotients (measured as $\rightarrow$ K⁺/O quotients) in order to ascertain whether the $\rightarrow$ H⁺/O quotients might be underestimated by H⁺/anion symport. The results suggest that 6H⁺/O are translocated during NADH oxidation, and that 2H⁺/O are translocated during the oxidation of methanol to formaldehyde. There was no evidence for underestimation of the $\rightarrow$ H⁺/O quotients due to H⁺/anion symport, except by the movement of formic acid during formate oxidation. By comparing these results with the known growth efficiencies of this organism, an $\rightarrow$ H⁺/ATP quotient of close to 2g-ions of H⁺/mol of ATP can be calculated. It is proposed that the respiratory chain in *Methylophilus methylotrophus* is arranged such that there are three sites of energy conservation for NADH oxidation, each translocating 2H⁺ and each linked to the synthesis of one molecule of ATP. Only the third site of energy conservation is involved in methanol oxidation.

Methylophilus methylotrophus is an obligate methylotroph that uses methanol as the sole source of carbon and energy. This organism is of commercial importance in single-cell protein production. Carbon assimilation occurs via the ribulose monophosphate pathway (Beardmore & Quayle, 1978), and complete oxidation of formaldehyde to CO₂ is thought to occur via the dissimilatory ribulose monophosphate cycle, rather than via linear oxidation involving formate. This organism contains cytochromes of the *a*, *b* and *c* types (Cross & Anthony, 1978), but nothing is known about the efficiency of energy conservation. The stoichiometries of respiration-linked proton translocation have been determined for a number of methylotrophs (Tonge *et al.*, 1977; Hammond & Higgins, 1978; O'Keefe & Anthony, 1978; Keevil & Anthony, 1979). Both in methylotrophs and in other bacteria, however, these stoichiometries have been measured only by the oxygen-pulse method (Mitchell & Moyle, 1967), which requires complete starvation of bacterial suspensions in order to obtain unambiguous $\rightarrow$ H⁺/O quotients (g-ions of H⁺/g-atom of O) for the oxidation of added substrates. This is frequently difficult to attain and, even when it is possible

to starve cells totally, prolonged incubation in the absence of carbon substrate may damage the cytoplasmic membrane and/or the respiratory system. Furthermore, the oxygen-pulse method for evaluation of $\rightarrow$ H⁺/O quotients in mitochondria has been criticised on the grounds that extrapolation procedures are inadequate to correct for a rapid phase of decay of the Δ pH due to proton symport on the phosphate carrier (Brand *et al.*, 1976a). Interestingly, Cox & Haddock (1978) showed that a mutant of *Escherichia coli*, which was unable to transport phosphate, exhibited the same $\rightarrow$ H⁺/O quotient as the wild-type organism. However, it is possible that rapid movements of protons, in symport with other endogenous anions, may lead to underestimation of $\rightarrow$ H⁺/O quotients in bacteria.

In order to determine the stoichiometry of respiration-linked proton translocation in *M. methylotrophus*, we have measured $\rightarrow$ H⁺/O quotients by the oxygen-pulse method, and both $\rightarrow$ H⁺/O and $\rightarrow$ charge/O quotients by an initial-rate method, previously applied to mitochondria (Brand *et al.*, 1976b; Reynafarje & Lehninger, 1978). The $\rightarrow$ charge/O quotient (measured as the $\rightarrow$ K⁺/O quotient) should be unaffected by movements of protons in symport with anions, and hence may be used to determine whether the $\rightarrow$ H⁺/O quotients are underestimated by such secondary proton movements.

Abbreviations used: HQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Materials and methods

Chemicals

Valinomycin, HQNO, antimycin A, rotenone, carbonic anhydrase and alcohol dehydrogenase were obtained from Sigma (London) Chemical Co., Poole, Dorset BH17 7NH, U.K. Duroquinol was obtained from ICN Pharmaceuticals Inc., K & K Labs. Division, Plainview, NY 11803, U.S.A., and FCCP was from Boehringer Corp. (London), Lewes, East Sussex BN7 1LG, U.K. Nigericin was the generous gift of Dr. R. L. Hamil, Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, IN 46206, U.S.A. All other chemicals were of the highest purity commercially available.

Formaldehyde was prepared from paraformaldehyde by the method of Strom *et al.* (1974), and was assayed by using yeast alcohol dehydrogenase (Bernt & Bergmeyer, 1974).

Growth of bacteria

M. methylotrophus (originally called *Pseudomonas methylotrophica*; N.C.I.B. 10515; Byrom & Ousby, 1975) was obtained from I.C.I. Agricultural Division, Billingham, Cleveland, U.K. Stock cultures were maintained in freeze-dried ampoules, and on methanol/salts agar plates containing, in 1 litre: K_2HPO_4 , 1.9 g; $NaH_2PO_4 \cdot 2H_2O$, 1.56 g; $(NH_4)_2SO_4$, 1.8 g; $MgSO_4 \cdot 7H_2O$, 20 mg; $FeCl_3 \cdot 6H_2O$, 9.7 mg; Fisons trace-element solution [Cu, 5 p.p.m.; Mn, 24–25 p.p.m.; Zn, 22–23 p.p.m.; Ca, 720 p.p.m. (Fisons, Loughborough, Leics., U.K.)], 1 ml; Oxoid purified agar (L28; Oxoid, London SE1 9HF, U.K.), 15 g; methanol, 5 ml; at pH 7.2. Bacteria were grown in methanol (0.25%, v/v)-limited continuous culture, in a 1 litre laboratory fermentor (L. H. Engineering Co., Stoke Poges, Bucks., U.K.) at 40°C and at a dilution rate of $0.18 h^{-1}$. The medium contained, in 1 litre: $MgSO_4 \cdot 7H_2O$, 0.27 g; K_2SO_4 , 0.035 g; K_2HPO_4 , 0.112 g; 1.1 M- H_3PO_4 , 3.75 ml; Fisons trace-element solution, 6 ml. Iron was pumped into the fermentor vessel separately as $FeSO_4$ solution (0.16 g of $FeSO_4 \cdot 7H_2O + 2$ ml of conc. H_2SO_4 per litre) to give a concentration in the fermentor of 0.8 p.p.m. The nitrogen source was 10% (w/v) NH_3 , which was added automatically to maintain the pH at 7.0 ± 0.1 . Bacteria were harvested from the fermentor vessel, and used within 3 h.

Measurement of respiration-linked proton translocation by the oxygen-pulse method

The oxygen-pulse technique used was essentially that of Mitchell & Moyle (1967). The pH was measured by using a Beckman 4500 pH meter, and was recorded on a Servoscribe 2s potentiometric recorder. The reaction vessel contained, in a 5 ml reaction mixture, 140 mM-KCl, 1.5 mM-glycylgly-

cine, 25–35 mg dry wt. of washed bacterial suspension, 1 µg of valinomycin/mg dry wt. of bacteria, 250 µg of carbonic anhydrase (Scholes & Mitchell, 1970), plus substrates and inhibitors as required. Bacterial suspensions were maintained at 40°C and allowed to attain anaerobiosis by oxidation of endogenous substrates. Valinomycin was added after anaerobiosis, and the suspensions were allowed to equilibrate for 30 min. Substrates and inhibitors, when required, were added and the pH was adjusted to 6.2 by small additions of anaerobic KOH or HCl. Oxygen was introduced in 5–50 µl volumes of air-saturated 140 mM-KCl, ten different volumes being used for each determination. It was assumed that 10 µl of air-saturated KCl at 40°C contains 3.80 ng-atoms of O. The resulting acidifications of the external medium was calibrated by 1–10 µl additions of anaerobic 10 mM-KOH. Acidification was complete within 3–4 s of adding oxygen and ΔpH was estimated by extrapolating back to the time taken for half the pH rise to occur (Mitchell & Moyle, 1965).

Measurement of respiration rates and use of inhibitors

Respiration rates were measured by using a Rank oxygen electrode (Rank Bros., Bottisham, Cambs., U.K.). Bacterial suspensions were incubated in the same mixture as for measurement of respiration-linked proton translocation by the oxygen-pulse method, and aerated by shaking briefly before use. Inhibitors, when used, were added 2 min before addition of substrate. HQNO was dissolved in dilute KOH solution and assayed by A_{345} (ϵ_{345} 9450 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$); antimycin A and rotenone were dissolved in dimethyl formamide.

Measurement of respiration-linked proton translocation and charge translocation, by the initial-rate method

Respiration-linked proton translocation was measured by a method, previously applied to mitochondria (Brand *et al.*, 1976b), in which initial rates of respiration and acidification were measured after the addition of substrate to aerobic suspensions of bacteria. Bacterial suspensions were incubated in the same mixture as for the oxygen-pulse method, and aerated by shaking briefly before use. These suspensions had very low endogenous substrate-oxidation rates [1.4 ± 0.1 (10) ng-atoms of O/min per mg], which were markedly stimulated on addition of substrates.

Respiration-linked charge translocation was measured by following K^+ movements in the presence of valinomycin (Reynafarje & Lehninger, 1978), with a K^+ -selective electrode and sulphate reference electrode (Chemetric, Milborne Port, Sherborne, Dorset DT9 5ER, U.K.). Conditions

were as for measurement of $\rightarrow H^+/O$ quotients, except that the KCl concentration was lowered to 10 mM. LiCl (140 mM) was present to maintain the ionic strength at a reasonable value, and the buffer concentration was increased to 10 mM-glycylglycine. K^+ movements were calibrated by 5–25 μ l additions of 100 mM-KCl.

All numerical results are given as means $\pm$ S.E.M., with the numbers of determinations in parentheses.

Results

Proton translocation linked to endogenous respiration in *Methylophilus methylotrophus*

Addition of air-saturated KCl to anaerobic suspensions of bacteria resulted in rapid acidification of the medium. The extent of the Δ pH was maximal at pH 6.2 (Fig. 1a), and in the presence of 1 μ g of valinomycin/mg dry wt. of bacteria (Fig. 1b). The decay of the Δ pH showed first-order kinetics for at least 1 min, but after this period the rate of decay slowed and the pH often failed to reach the original baseline. The $t_{1/2}$ (half-time) of the first-order region of decay was 104 ± 10 s (6) in the absence of valinomycin, decreasing to 54 ± 7 s (7) in the presence of 1 μ g of valinomycin/mg dry wt. of bacteria. The extent of the Δ pH was less when KCNS was used in place of valinomycin to collapse the $\Delta\Psi$ component of the protonmotive force; this was probably due to the rapid rate of decay of the Δ pH [$t_{1/2} = 10.9 \pm 2.2$ s (8)] observed in the presence of thiocyanate (120 mM). The rate of decay of the Δ pH was increased by low concentrations of the uncoupling agent FCCP (10 nM-FCCP caused a 50% decrease in the $t_{1/2}$ of decay); 1 μ M-FCCP totally abolished the response to subsequent additions of oxygen. Bacterial suspensions incubated with nigericin (1 μ g/mg dry wt. of bacteria) for 15 min, in the presence or absence of valinomycin, showed no pH response on addition of oxygen.

Under optimum conditions, the $\rightarrow H^+/O$ quotient for the oxidation of endogenous substrates was found to be 5.92 ± 0.17 (9) g-ions of H^+ /g-atom of O. This value was not significantly affected when bacterial suspensions were prepared by washing under anaerobic conditions [$\rightarrow H^+/O = 6.04 \pm 0.18$ (5)], in order to remove any permeant anions which might otherwise cause underestimation of the $\rightarrow H^+/O$ quotient (Brand *et al.*, 1976a). *N*-Ethylmaleimide, a thiol-blocking reagent which increases the $\rightarrow H^+/O$ quotient in mitochondria (Mitchell, 1972), possibly by inhibiting H^+ /phosphate symport (or OH^- /phosphate antiport) (Brand *et al.*, 1976a) caused the $\rightarrow H^+/O$ quotient for the oxidation of endogenous substrates to decrease significantly at concentrations between 10 and 100 nmol/mg dry wt. of bacteria (in the presence of 100 nmol of *N*-

ethylmaleimide/mg dry wt. of bacteria $\rightarrow H^+/O = 1.6$).

Respiration of exogenous substrates and sensitivity to inhibitors of electron transport

Bacterial suspensions were able to oxidize metha-

Fig. 1. Effect of pH and valinomycin concentration on the $\rightarrow H^+/O$ quotient measured by the oxygen-pulse method

$\rightarrow H^+/O$ quotients for the oxidation of endogenous substrates were determined as described in the Materials and methods section, except in (a) the pH was varied, and in (b) the concentration of valinomycin was varied.

nol, formaldehyde and formate, but not succinate, pyruvate, malate or lactate. Respiration was not sensitive to the NADH dehydrogenase inhibitor rotenone, but it was affected by the mid-chain inhibitors antimycin A and HQNO, which exhibited similar differential effects on the oxidation of formate, methanol and formaldehyde. Fig. 2 shows that oxidation of the NAD^+ -linked substrate, formate, was inhibited by 78% by $75\mu\text{M}$ -HQNO, whereas the oxidation of formaldehyde and methanol was inhibited by only 59% and 26% respectively by this concentration of inhibitor. The intermediate inhibition of formaldehyde oxidation, between that of formate and methanol oxidation, suggests that there may be two routes for formaldehyde oxidation, via methanol dehydrogenase, and via the NAD(P)H -yielding ribulose monophosphate cycle. Duroquinol is believed to donate reducing equivalents to the respiratory chain at the quinone level (Lawford & Garland, 1973), and was used here in the absence of a suitable flavin-linked physiological substrate. Duroquinol oxidation was very sensitive to inhibition by HQNO, 50% inhibition occurring at about $3\mu\text{M}$ -HQNO.

Stoichiometry of proton translocation linked to oxidation of exogenous substrates, measured by the oxygen-pulse method

Although preferable for these experiments, it did not prove possible to starve bacteria of all their endogenous substrates by shaking in 140mM -KCl/

Fig. 2. Effect of HQNO on the oxidation of formate, methanol, formaldehyde and duroquinol

Respiration rates were measured as described in the Materials and methods section. Substrates (all 1mM): ■, formate; □, formaldehyde; ▲, methanol; △, duroquinol.

1.5mM -glycylglycine at $\text{pH } 7.0$ for several hours. When bacteria were sufficiently starved to give no pH response to oxygen pulses, it was not possible to restore the response by adding substrate. Bacteria harvested from methanol-limited continuous culture, however, had very low endogenous substrate oxidation rates, which were markedly stimulated on addition of substrate.

Figs. 3(a)–3(d) show the effect of formate, methanol, formaldehyde and duroquinol on the whole-cell respiration rate and $\rightarrow\text{H}^+/\text{O}$ quotient. The rate of decay of the ΔpH which resulted from an oxygen pulse in the presence of these substrates was in all cases similar to that observed in the presence of endogenous substrates alone. Formate is not thought to be involved in the main route of formaldehyde oxidation in this organism, but it is readily oxidized by whole cells via NADH ($K_m = 0.4\text{mM}$; $V_{\text{max}} = 36\text{ng-atoms of O/min per mg}$). The $\rightarrow\text{H}^+/\text{O}$ quotient for formate oxidation (Fig. 3a) decreased as a function of increasing formate concentration, even beyond the region where there was a rapid increase in respiration rate with increasing concentration. There are several lines of evidence that indicate that the uptake of formate by this organism is linked to the entry of a proton, i.e. as the neutral species, formic acid. Firstly, small alkalinizations of the external medium were observed when anaerobic formate was added to an anaerobic suspension of bacteria. Secondly, formate added during the early stage of the decay of the ΔpH induced by an oxygen pulse caused the rapid collapse of the ΔpH . Thirdly, formate oxidation was inhibited by nigericin, which dissipates the ΔpH across the membrane, and by FCCP which dissipates both ΔpH and $\Delta\Psi$, but not by valinomycin, which dissipates only the $\Delta\Psi$ component of the protonmotive force. Garland *et al.* (1975) observed biphasic decays of the ΔpH in *E. coli* in the presence of formate, and attributed the initial rapid phase of decay to movement of formic acid across the membrane in response to the ΔpH established by proton translocation. If this initial phase of decay was more rapid in *M. methylotrophus* than in *E. coli*, it might not be observed in these experiments. Furthermore, should the extent of the rapid phase of decay vary with formate concentration, then the outcome would be a decrease in $\rightarrow\text{H}^+/\text{O}$ quotient with increasing formate concentration (Fig. 3a). Attempts to observe an initial rapid phase of decay, by decreasing the temperature of the assay, were unsuccessful.

Methanol oxidation in *M. methylotrophus* occurs via a methanol dehydrogenase similar to that observed in other methylotrophs (Ghosh & Quayle, 1978). The presence of methanol substantially increased the respiration rate ($K_m = 3.5\mu\text{M}$; $V_{\text{max}} = 49\text{ng-atoms of O/min per mg}$), but had little effect on proton translocation, where it caused only a

Fig. 3. Respiration rates and $\rightarrow H^+/O$ quotients (measured by the oxygen-pulse method) for the oxidation of formate, methanol, formaldehyde and duroquinol

Respiration rates and $\rightarrow H^+/O$ quotients were measured as described in the Materials and methods section for (a) formate, (b) methanol, (c) formaldehyde and (d) duroquinol. $\blacktriangle$, Respiration rate; $\bullet$, $\rightarrow H^+/O$ quotient.

slight decrease in the $\rightarrow H^+/O$ quotient below the endogenous value (Fig. 3b). However, since the bacteria could not be fully starved of endogenous substrates, it is not possible completely to rule out interference in the measurement of the $\rightarrow H^+/O$ quotient for methanol because of endogenous substrate oxidation. In the presence of $75\mu M$ -HQNO, proton translocation driven by the oxidation of endogenous substrates was slow, and very small pulses were obtained. However, after the addition of methanol (1–10 mM), pulses became rapid and increased in extent to give an $\rightarrow H^+/O$ quotient of 1.59 ± 0.11 (5). There was some increase in the rate of decay of ΔpH in the presence of HQNO (Slater, 1967), making extrapolation of pulses difficult, but

this could by no means totally account for the low $\rightarrow H^+/O$ quotient that was observed for methanol oxidation (similar concentrations of antimycin A also caused the rapid collapse of the ΔpH after an oxygen pulse). It is evident, therefore, that a substantial increase in respiration rate on addition of substrate does not necessarily imply that proton translocation, during an oxygen pulse, will be linked solely to oxidation of the added substrate, with no interference from endogenous substrates. In the anaerobic incubation period between pulses, reduced redox carriers (principally NADH) will accumulate slowly, owing to reduction by endogenous substrates. This pool of reductant will then be rapidly oxidized when an oxygen pulse is introduced. Added

substrates must compete with the rapid oxidation of the reductant pool, rather than with the slow endogenous respiration measured continuously under aerobic conditions. Experimentally, it was possible to deplete the reductant pool by frequently introducing large oxygen pulses, so that endogenous respiration was insufficient to refill the pool between pulses. In the absence of added substrates, there was little pH response to added oxygen after the first two pulses (Fig. 4a). In the presence of methanol, however, repeated oxygen pulses yielded $\rightarrow\text{H}^+/\text{O}$ quotients in the range 2–3 after the reductant pool

Fig. 4. Response of an anaerobic suspension of *Methylophilus methylotrophus* to frequent, large oxygen pulses in the presence or absence of methanol

The bacterial suspensions (2–3 mg dry wt. of bacteria/ml) were incubated as for the measurement of $\rightarrow\text{H}^+/\text{O}$ quotients by the oxygen-pulse method, as described in the Materials and methods section (a) with no added substrate, and (b) plus 1 mM-methanol. The arrows indicate the addition of 50 μl of air-saturated KCl (19 ng-atoms of O).

had been depleted by the first two pulses (Fig. 4b). An estimate of 2 nmol of NADH/mg dry wt. of bacteria can be made for the size of the pool. No pH change was observed on addition of anaerobic methanol to an anaerobic suspension of bacteria, thus suggesting that methanol is taken up as the neutral molecule. In contrast with formate oxidation, methanol oxidation was slightly stimulated by FCCP, possibly owing to alleviation of respiratory control.

Formaldehyde oxidation in this organism may occur via two different pathways. This view is supported by the intermediate inhibition of formaldehyde oxidation by HQNO and by the known ability of methanol dehydrogenase to oxidize formaldehyde (Anthony, 1975). As discussed above for methanol, measurement of the $\rightarrow\text{H}^+/\text{O}$ quotient for formaldehyde oxidation might also be subject to interference by endogenous substrate oxidation. The effect of formaldehyde on the respiration rate ($K_m = 40 \mu\text{M}$; $V_{\text{max}} = 100 \text{ ng-atoms of O/min per mg}$) and $\rightarrow\text{H}^+/\text{O}$ quotient is shown in Fig. 3(c) (concentrations of formaldehyde above 1 mM were found to be inhibitory). HQNO (75 μM) eliminated the NAD^+ -linked component of formaldehyde oxidation, and thus allowed measurement of the $\rightarrow\text{H}^+/\text{O}$ quotient for formaldehyde oxidation via methanol dehydrogenase; a value of 2.08 ± 0.13 (5) was observed under these conditions, although it should be noted that the inhibitor again caused an increase in the rate of decay of ΔpH . Small acidifications were observed on addition of anaerobic formaldehyde to an anaerobic suspension of bacteria. The source of these acidifications is unknown, but they were also observed in *Pseudomonas* AM1 by O'Keefe & Anthony (1978). FCCP caused a slight increase in the rate of formaldehyde oxidation, again possibly owing to alleviation of respiratory control.

The sensitivity of duroquinol oxidation to HQNO is consistent with the idea that duroquinol donates reducing equivalents to the respiratory chain at the level of the endogenous quinone. The high rate of duroquinol oxidation (Fig. 3d; $K_m = 0.15 \text{ mM}$; $V_{\text{max}} = 190 \text{ ng-atoms of O/min per mg}$) compared with that of the physiological substrates suggests that duroquinol oxidation should compete effectively with oxidation of the endogenous reductant pool, during an oxygen pulse. At saturating concentrations of duroquinol an $\rightarrow\text{H}^+/\text{O}$ quotient of 3.65 ± 0.10 (11) was observed.

Stoichiometry of respiration-linked proton translocation, measured by the initial-rate method

Fig. 5 shows typical traces of the changes in pH and oxygen concentration which were obtained when methanol was added to an aerobic suspension of bacteria. The pH trace was linear for about 10 s,

Fig. 5. Measurement of $\rightarrow H^+/O$ quotients by the initial-rate method

The traces of pH and oxygen concentrations were obtained as described in the Materials and methods section. The arrows indicate the addition of methanol (final concn. 1 mM).

and the oxygen-uptake rate also became linear after an initial lag of 2–3 s (the linear region being used for the determination of the $\rightarrow H^+/O$ quotient). Values for the $\rightarrow H^+/O$ quotient were constant over a wide range of oxygen-uptake rates, thus suggesting that the response times of the electrodes were not limiting under these conditions. There is no possibility of interference by endogenous substrate oxidation in the initial-rate method, since the reductant pools are oxidized before addition of substrate. Table 1 shows the $\rightarrow H^+/O$ quotients for formate, methanol, formaldehyde and duroquinol oxidation, measured by the initial-rate method. The value of approx. 3.5 for formate oxidation is probably underestimated, owing to movement of formic acid across the membrane, as discussed in the previous section. The $\rightarrow H^+/O$ quotient of approx. 2.0 for methanol oxidation indicates that there is net appearance of $2H^+$ on the outside of the membrane during oxidation of one molecule of methanol to formaldehyde. This value was not significantly affected by the presence of 100 nmol of *N*-ethylmaleimide/mg dry wt. of bacteria [$\rightarrow H^+/O = 1.95 \pm 0.24$ (11)]. The $\rightarrow H^+/O$ quotient of approx. 3.4 for formaldehyde oxidation is consistent with the view that two routes are available for the oxidation of this substrate, i.e. via NADH oxidase and via methanol oxidase. The $\rightarrow H^+/O$ quotient of approx. 3.5 for duroquinol oxidation indicates that up to $4H^+$ may be translocated during the passage of $2e^-$ from the quinone region of the respiratory chain to oxygen.

Stoichiometry of respiration-linked charge translocation measured by the initial-rate method

Unfortunately it was not possible to determine

Table 1. $\rightarrow H^+/O$ and $\rightarrow K^+/O$ quotients for the oxidation of formate, methanol, formaldehyde and duroquinol, measured by the initial-rate method

$\rightarrow H^+/O$ and $\rightarrow K^+/O$ quotients were measured in separate experiments by the initial-rate method, in the presence of 140 mM-KCl or 10 mM-KCl plus 140 mM-LiCl respectively, as described in the Materials and methods section. The final concentration of substrate was in all cases 1 mM, except for measurement of the $\rightarrow K^+/O$ quotient for formate oxidation, when the formate concentration was between and 10 mM (see the text). Abbreviations: N.D., not determined (see the text).

Respiratory substrate	$\rightarrow H^+/O$ quotient (g-ions of H^+ +/g-atom of O)	$\rightarrow K^+/O$ quotient (g-ions of K^+ +/g-atom of O)
Formate	3.46 ± 0.28 (10)	6.27 ± 0.14 (39)
Methanol	1.99 ± 0.19 (9)	1.78 ± 0.09 (8)
Formaldehyde	3.42 ± 0.34 (8)	3.12 ± 0.16 (11)
Duroquinol	3.48 ± 0.13 (8)	N.D.

$\rightarrow$ charge/O quotients (measured as $\rightarrow K^+/O$ quotients) by the oxygen-pulse method, as consistent movements of neither K^+ nor H^+ could be obtained at the very low K^+ concentration (less than 1 mM) that was required by the K^+ -selective electrode in this procedure. The magnitude of the ion movements measured in the initial-rate method is considerably greater than in the oxygen-pulse method, thus allowing a higher K^+ concentration to be used. $\rightarrow K^+/O$ quotients for methanol oxidation were found to be constant at K^+ concentrations of 10–20 mM; at concentrations of K^+ below 10 mM, the $\rightarrow K^+/O$ quotient was significantly decreased (20 mM was the highest concentration of K^+ that could be used to allow adequate sensitivity of the electrode for this procedure). Table 1 shows the $\rightarrow K^+/O$ quotients that were obtained for the oxidation of formate, methanol and formaldehyde in the presence of 10 mM-KCl (140 mM-LiCl being present to maintain the ionic strength). The $\rightarrow K^+/O$ quotient for formate oxidation was close to 6 g-ions of K^+ /g-atom of O and was independent of the formate concentration between 1 and 10 mM. This result confirms that $6H^+/O$ are translocated during the oxidation of NADH, and that the $\rightarrow H^+/O$ quotient for formate oxidation (measured both by the initial-rate method and, at higher formate concentrations, by the oxygen-pulse method) was underestimated, owing to the movement of formic acid across the membrane. The $\rightarrow K^+/O$ quotients for the oxidation of methanol and formaldehyde were similar in magnitude to the $\rightarrow H^+/O$ quotients reported in the previous section, i.e. approx. 1.8 and 3.1 respectively. The $\rightarrow K^+/O$ quotient for methanol oxidation was not significantly affected by the presence of 100 nmol of *N*-ethylmaleimide/mg dry wt. of bacteria [$\rightarrow K^+/O =$

2.05 ± 0.18 (8)]. Unfortunately, it was not possible to measure $\rightarrow K^+/O$ quotients for duroquinol oxidation, since the organic solvent used (dimethylformamide) appeared to interfere with the K^+ -selective electrode, causing a lag in the response.

Discussion

Oxidation of one molecule of NADH by *M. methylotrophus* is associated with the translocation of $6H^+$ across the membrane. This conclusion is supported by oxygen-pulse experiments which yielded $\rightarrow H^+/O$ quotients of approx. 6, both for the oxidation of endogenous substrate (NADH) and for the oxidation of low concentrations of formate (via NAD^+ -linked formate dehydrogenase). Furthermore, the $\rightarrow K^+/O$ quotient of approx. 6 for formate oxidation is numerically equal to the true number of protons translocated during oxidation of one molecule of formate and is unaffected by movement of the neutral species formic acid.

Methanol dehydrogenase appears to donate electrons to the respiratory chain at the level of cytochrome *c*, as proposed by Widdowson & Anthony (1975) for *Pseudomonas* AM1, since methanol oxidation was relatively insensitive to the mid-chain inhibitor HQNO and exhibited an $\rightarrow H^+/O$ quotient approaching 2 when measured by the oxygen-pulse method in the presence of HQNO. $\rightarrow H^+/O$ and $\rightarrow K^+/O$ quotients measured by the initial-rate method were also close to 2. The inhibition of formaldehyde oxidation by HQNO was intermediate between that of methanol and formate oxidation, in agreement with the proposal that there are two alternative routes for the oxidation of this substrate. The major physiological route is presumably via the dissimilatory ribulose monophosphate cycle, since low amounts of formate dehydrogenase and high amounts of 6-phosphogluconate dehydrogenase are present in this organism (Beardsmore & Quayle, 1978); NAD(P)H is generated via glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Oxidation of formaldehyde via methanol dehydrogenase is also possible,

since formaldehyde is mainly present in the hydrated form in solution, but linear oxidation of formaldehyde via methanol dehydrogenase and formate dehydrogenase would clearly be associated with a lower efficiency of energy conservation than would oxidation via the dissimilatory ribulose monophosphate cycle.

The oxidation of duroquinol yielded an $\rightarrow H^+/O$ quotient of approx. 3.5 by both the oxygen-pulse and initial-rate methods. The exact nature of the interaction of duroquinol with the endogenous quinone pool is unknown, but these values are consistent with the translocation of up to $4H^+$ when $2e^-$ pass from the quinone region of the respiratory chain to oxygen.

Scheme 1 is the simplest scheme for the respiratory system of *M. methylotrophus* which is consistent with all the results presented here. The scheme is similar to that proposed by Keevil & Anthony (1979) for carbon-limited cultures of *Pseudomonas* AM1. It is proposed that there are three energy-conserving sites, each translocating $2H^+$ per electron pair transferred (i.e. the $\rightarrow H^+/\text{site}$ quotient is 2). Site I is associated with NADH-ubiquinone oxidoreductase, whereas sites II and III are associated with ubiquinol oxidase and encompass cytochromes *b* and *c* plus cytochrome oxidases *o* and *aa₃*. Sites II and III may be fused into a protonmotive quinone cycle (Mitchell, 1975), which translocates four protons during the passage of $2e^-$ from ubiquinone to cytochrome *c*, net H^+ translocation being completed by transmembrane electron flow between cytochrome *c* and oxygen. The existence of energy coupling at site III is as expected for a bacterium which contains a high-potential membrane-bound cytochrome *c* linked to cytochrome oxidase *aa₃* and/or *o* (Jones, 1977).

The oxygen-pulse method did not prove a particularly useful technique for investigation of respiration-linked proton translocation in this organism. Despite the presence of a low endogenous substrate-oxidation rate, which was substantially increased after the addition of substrate, there was clearly interference by endogenous substrate oxidation during the measurement of $\rightarrow H^+/O$ quotients

Scheme 1. Proposed scheme for respiration and proton translocation in *Methylophilus methylotrophus*. The hatched bar indicates the site of inhibition by HQNO. Abbreviations: Q, ubiquinone; *b*, *c*, *aa₃*, *o*, cytochromes *b*, *c*, *aa₃*, *o*.

for the oxidation of exogenous substrates. In contrast, the initial-rate method proved a very useful technique, since it allowed the measurement of both $\rightarrow\text{H}^+/\text{O}$ and $\rightarrow\text{charge}/\text{O}$ ($\rightarrow\text{K}^+/\text{O}$) quotients. The similarity of the $\rightarrow\text{H}^+/\text{O}$ (measured at high K^+ concentration) and $\rightarrow\text{K}^+/\text{O}$ quotients, except in the

case of formate oxidation, where $\rightarrow\text{H}^+/\text{O}$ quotients are underestimated owing to movements of formic acid, gives strong support to the argument that $\rightarrow\text{H}^+/\text{O}$ quotients in this organism are not underestimated due to rapid secondary proton movements in symport with endogenous anions (as is possibly the case with mitochondria). It is unlikely that the $\rightarrow\text{K}^+/\text{O}$ quotients were affected by K^+ /anion symport (Russell & Rosenberg, 1979), since, in the presence of 10 mM-KCl, the magnitude of K^+ movements involved in these experiments would be insufficient to establish a K^+ gradient large enough to act as a driving force for K^+ /anion symport; neither the ΔpH nor $\Delta\Psi$ (which is anyway minimized in the presence of K^+ /valinomycin) is able to drive the movement of the neutral K^+ /anion species. *N*-Ethylmaleimide, at similar concentrations to those used to inhibit phosphate transport in mitochondria, did not cause an increase either in

only a soluble, energy-independent transhydrogenase; M. J. Dawson & C. W. Jones, unpublished work), and that the formula for cell biomass is $\text{C}_4\text{H}_8\text{O}_2\text{N}$ (Anthony, 1978), we can construct the following equation to describe the growth of this organism:

where MH_2 is reduced methanol dehydrogenase. The requirement for 17 mol of ATP to produce 102 g of biomass is deduced from the Y_{ATP} (102/6.0). NADH (0.5 mol) is required to reduce the 4 mol of methanol to the level of cell biomass, and each mol of methanol assimilated yields 1 mol of MH_2 , since assimilation occurs at the level of formaldehyde. By applying the $\rightarrow\text{H}^+/\text{O}$ quotients of approx. 2 and 6 determined in this study for the oxidation of methanol and NADH respectively, it is possible to calculate the amount of methanol that must be completely oxidized to CO_2 to produce the 0.5 mol of NADH and 17 mol of ATP required for assimilation, as a function of the $\rightarrow\text{H}^+/\text{ATP}$ quotient. Assuming that complete oxidation of methanol to CO_2 yields 1 mol of MH_2 and 2 mol of NADH, 0.25 mol of methanol must be fully oxidized to produce the 0.5 mol of NADH required. Omitting the NH_3 and H_2O for simplicity:

the $\rightarrow\text{H}^+/\text{O}$ quotient for the oxidation of endogenous substrates, measured by the oxygen-pulse method, or in the $\rightarrow\text{H}^+/\text{O}$ or $\rightarrow\text{K}^+/\text{O}$ quotient for methanol oxidation, measured by the initial-rate method. It is not known whether the phosphate transport system(s) of this organism is sensitive to inhibition by *N*-ethylmaleimide, but no evidence was obtained for an increased $\rightarrow\text{H}^+/\text{O}$ quotient caused by inhibition of H^+ /phosphate symport.

It is not possible to draw conclusions about the efficiency of ATP synthesis during the oxidation of NADH or methanol without knowledge of the $\rightarrow\text{H}^+/\text{ATP}$ quotient, and the latter is currently the subject of considerable controversy (see, for example Brand, 1977). However, it is possible to deduce the likely value of the $\rightarrow\text{H}^+/\text{ATP}$ quotient by using the proton translocation stoichiometries determined here and the known growth efficiencies of this organism. Under similar growth conditions to those employed in this study, *M. methylotrophus* exhibits a growth yield on methanol (Y_{methanol}) of 0.50 g of cells/g of methanol and a molar growth yield with respect to ATP (Y_{ATP}) of 6.0 g of cells/mol of ATP (Taylor, 1977; P. J. Senior & J. Windass, personal communication). Assuming that NAD(P)H is the reductant for biosynthesis, that NADH and NADPH are energetically equivalent (this organism contains

The 4.25 MH_2 on the right-hand side of eqn. (2) will yield $4.25 \times 2/(\rightarrow\text{H}^+/\text{ATP})$ mol of ATP on oxidation. Therefore $17 - [8.50/(\rightarrow\text{H}^+/\text{ATP})]$ mol of ATP must be produced by further complete oxidation of methanol in order to supply the energy requirement for assimilation. Complete oxidation of 1 mol of methanol yields a total of $(2 + 6 + 6)/(\rightarrow\text{H}^+/\text{ATP})$ mol of ATP, therefore:

$$\frac{17 - [8.50/(\rightarrow\text{H}^+/\text{ATP})]}{14/(\rightarrow\text{H}^+/\text{ATP})}$$

mol of methanol must be fully oxidized to supply the ATP requirement, in addition to the 0.25 mol of methanol which must be oxidized to supply the NADH requirement and the 4 mol of methanol required for assimilation; i.e., omitting CO_2 for simplicity:

Since Y_{methanol} is 0.50 g of cells/g of methanol, then 102 g of cells requires 6.38 mol of methanol, and hence the $\rightarrow\text{H}^+/\text{ATP}$ quotient can be calculated from the above equation to be 2.25 g-ions of H^+ /mol

of ATP. It seems likely, therefore, that this organism exhibits an ATP/O quotient of approx. 3 for NADH oxidation and of approx. 1 for methanol oxidation. It is surprising that only 1 ATP results from the oxidation of methanol to formaldehyde, since the E_0' for the methanol/formaldehyde half-reaction is -0.182 V (Ribbons *et al.*, 1970); the $\Delta E_0'$ of the methanol oxidase reaction (-0.992 V) thus provides sufficient energy for an ATP/O quotient of at least 2. The explanation put forward by Anthony (1978) that NAD(P)H supply, as well as ATP supply, may be a limiting factor for growth of methylotrophs, is unlikely to be the case for an organism utilizing the ribulose monophosphate pathway for assimilation, and producing two molecules of NAD(P)H during the complete oxidation of one molecule of formaldehyde to CO_2 .

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