Journal of General Microbiology (1988), **134**, 369–377. *Printed in Great Britain*

The Interaction between Methanol Dehydrogenase and Cytochrome *c* in the Acidophilic Methylotroph *Acetobacter methanolicus*

By E. JAMES ELLIOTT AND CHRISTOPHER ANTHONY*

Department of Biochemistry, University of Southampton, Southampton SO9 3TU, UK

(*Received 10 July 1987; revised 13 August 1987*)

Acetobacter methanolicus contains only *c*- and *b*-type cytochromes. One of the *b*-type cytochromes is probably an *o*-type oxidase. During growth on methanol at pH 4 the soluble cytochromes *c* are induced fivefold compared with growth on glycerol. *A. methanolicus* contains a methanol dehydrogenase (MDH) that is more stable to low pH values than other quinoprotein MDHs but is similar in other respects. Its electron acceptor is an autoreducible cytochrome c_L which differs from others in this class in being reduced at pH 4 by MDH in the presence of methanol and in being autoreduced at relatively low pH (pH 7·0). MDH-stimulated autoreduction occurs at the pH of the periplasm (pH 4·0) as predicted if the process of autoreduction is of physiological importance, and the rate is fast enough not to be rate-limiting in electron transport from methanol to the electron transport chain.

INTRODUCTION

Acetobacter methanolicus has recently been classified as a new species of acetic acid bacteria (Uhlig *et al.*, 1986). It is a facultative methylotroph, able to grow at pH 4·0 (its pH optimum) on conventional substrates such as glycerol and glucose, and also on methanol by way of the ribulose monophosphate pathway (Steudel *et al.*, 1980). It is the only acidophilic methylotroph (except for yeasts) that has been described in any detail but all that is known about the oxidation of methanol is that extracts contain a dye-linked methanol dehydrogenase and at least one *c*-type cytochrome (Steudel & Babel, 1982; Loffhagen & Babel, 1984).

Methanol dehydrogenase (MDH) is a quinoprotein whose electron acceptor is an unusual cytochrome *c* called cytochrome c_L. This cytochrome occurs only in methylotrophs; it has a low isoelectric point and its molecular mass is about twice that of typical soluble *c*-type cytochromes. Both this cytochrome and MDH are periplasmic in location and operate at the pH value of the growth medium. The pH at which MDH and cytochrome c_L react is of especial interest because it has been proposed that the autoreduction of the cytochrome that occurs at high pH is involved in the mechanism of electron transport (O'Keefe & Anthony, 1980*a*; Beardmore-Gray *et al.*, 1983; Anthony, 1982, 1986). In neutrophilic methylotrophs autoreduction of cytochrome *c* occurs at high rates at about pH 10 in the absence of MDH. In the presence of MDH, the autoreduction occurs at pH 7 which is the pH value of the periplasm during normal growth. For this reason it has been concluded that MDH-stimulated autoreduction of cytochrome *c* is an important part of the mechanism of electron transfer between methanol and cytochrome *c*. If this is the case, then it would be expected that the process of autoreduction would be observable in all types of methylotroph and that the proteins of an acidophilic methylotroph would be markedly different, with respect to their response to pH, from those in conventional methylotrophs. The present paper confirms that this is indeed the case and also shows that rate of autoreduction is not likely to be the rate limiting step in electron transport from methanol to cytochrome c_L.

Abbreviations: MDH, methanol dehydrogenase.

0001-4293 © 1988 SGM

METHODS

Organism and growth media. Acetobacter methanolicus strain MB58 is the type strain of the species, deposited as IMET 10945 in the culture collection of the Institute of Microbiology and Experimental Therapy of the Academy of Science of the GDR. It was the kind gift of Professor W. Babel (Institut für Biotechnologie, Leipzig, GDR). Stock cultures were maintained on minimal agar slopes containing 1% methanol. The defined medium of Uhlig *et al.* (1986) was used, containing methanol (1%), glucose (0·5%) or glycerol (0·5%).

Batch cultures (500 ml) of bacteria were grown in 2 l baffled flasks at 30 °C on an orbital shaker to the end of the exponential phase, harvested by centrifugation, washed and resuspended in 50 mM-sodium acetate buffer (pH 4·0). Continuous cultures of carbon-limited bacteria for purification of soluble proteins and for isolation of membrane fractions were grown at pH 4·2 in a 3 l vessel with a 2 l working volume at a dilution rate of 0·1–0·15 h^{-1}. They were harvested in a Sharples Supercentrifuge, washed and suspended in the buffer appropriate for the particular purification procedure (see below). Purity of cultures was checked as described by Uhlig *et al.* (1986).

Disruption and fractionation of bacteria. Bacteria [1 g wet wt (ml buffer)$^{-1}$] were disrupted in an MSE Soniprep 150 ultrasonic disintegrator for four cycles of 2 min sonication followed by 2 min cooling. Whole bacteria and debris were removed by centrifugation (40000 ***g*** for 30 min) and the supernatant was centrifuged at 130000 ***g*** for 2 h to yield a soluble fraction and membrane pellet which was suspended in 50 mM-MOPS/KOH buffer (pH 7) (20–30 mg protein ml^{-1}) and stored in liquid nitrogen.

Measurement of protein, MDH and cytochromes. Protein was assayed by the Lowry method using bovine serum albumin (fraction V) as standard. MDH was assayed spectrophotometrically using a method based on that of Anthony & Zatman (1967) but modified as described by Bamforth & Quayle (1978). For rapid detection of MDH in column eluates this method was scaled down for use in 96 well 'micro-ELISA' plates. The polarographic assay of MDH was as described by Froud & Anthony (1984) except that the reaction was started by addition of phenazine ethosulphate. Cytochrome contents of whole bacteria, membranes and soluble fractions were estimated from reduced-*minus*-oxidized difference spectra as described by Cross & Anthony (1980*b*) except that the extinction coefficient for CO-binding *b*-type cytochromes published by Wood (1984) was used. Haem was measured as the pyridine haemochrome by the method of Fuhrhop & Smith (1975).

Absorption spectra were recorded using a Shimadzu UV-3000 dual wavelength/double beam spectrophotometer (V. A. Howe, UK) with attachments for measurements at 77 K. Reduced-*minus*-oxidized difference spectra were recorded using samples that had been reduced with a few grains of sodium dithionite and oxidized with a few crystals of either potassium ferricyanide or ammonium persulphate.

Electrophoresis. SDS-PAGE was routinely done at pH 8·3 using the SDS-Tris/glycine system, and protein stained with Coomassie Brilliant Blue, as described by Weber & Osborn (1975), with final acrylamide concentrations of 10–15% (w/v). Gels were stained for haem proteins with 3,3′,5,5′-tetramethylbenzidine (TMBZ) by the method of Thomas *et al.* (1976). Molecular masses were determined as described by O'Keeffe & Anthony (1980*b*); commercial standard kits were used (BDH 44262 2I, Sigma Dalton Mark VII and Pharmacia PMW kits). The proportions of each protein in electrophoresed samples was determined by peak integration using a Chromoscan-3 gel scanner (Joyce-Loebel). Analytical isoelectric focusing was done as described in the Pharmacia handbook *'Isoelectric Focusing'*.

Measurement of midpoint redox potentials, and reduction and autoreduction of cytochrome c. Redox potentials were measured by the method of O'Keeffe & Anthony (1980*b*).

Autoreduction was measured by the methods of O'Keeffe and Anthony (1980*b*) and Beardmore-Gray *et al.* (1983). Cytochrome (0·5–10 nmol in 100 µl) at pH 3·5 was added to 300 µl 40% (v/v) glycerol in buffer (pH 3·5). Autoreduction was initiated by addition of concentrated buffer (100 µl) to give a final concentration of 20–50 mM. Buffers were as described below. The reduction of cytochrome by MDH was essentially similar to the measurement of autoreduction except that MDH (0·1–1·0 nmol) and not glycerol was used.

The methanol-dependent reduction of cytochrome *c* by MDH, with concomitant production of formaldehyde was as described by Beardmore-Gray *et al.* (1983).

Cytochromes and MDH from Methylobacterium AM1 and Methylophilus methylotrophus. Cytochrome *c* from *Methylobacterium* AM1 (previously *Pseudomonas* AM1), and the antiserum raised against it were a kind gift from Dr D. N. Nunn of this department. The cytochrome was purified as described by O'Keeffe & Anthony (1980*b*) and the MDH as described by O'Keeffe & Anthony (1980*a*). Cytochrome c_L from *Methylophilus methylotrophus* and the antiserum raised against it were the kind gift of Dr T. C. Wilkinson of this department; the cytochrome was prepared as described by Cross & Anthony (1980*a*). MDH from *Methylophilus methylotrophus* was a gift of Helen Ashworth of this department and was purified by the method of Beardmore-Gray *et al.* (1983). Western blotting for immunodetection was done as described by Nunn & Lidstrom (1986).

Purification of MDH from A. methanolicus. This method was developed for rapid purification of the relatively small amounts of MDH used in this work. The soluble fraction (30 ml) from methanol-limited *A. methanolicus* was prepared using 50 mM-MES/KOH buffer (pH 6·0) and applied to a column of fast flow S-Sepharose (7·3 × 2·2 cm)

equilibrated in the same buffer, attached to a Pharmacia FPLC system at a flow rate of 3 ml min^{-1}. MDH was adsorbed but most proteins, including the *c*-type cytochromes, were not. A gradient of 50–150 mM NaCl in 50 mM MES/KOH buffer (pH 6·0) was applied and the pure MDH eluted at 100 mM-NaCl. Only two other minor protein peaks were eluted with this gradient. The MDH purified by this rapid method was identical in all respects to that purified by previous methods (O'Keeffe & Anthony, 1980*a*; Beardmore-Gray *et al.*, 1983). Its stability was determined by incubating samples of pure enzyme (0·8 mg) in 50 mM-buffers (pH 2·2–6·5), portions being removed for assay in the spectrophotometric assay over a period of 24 h. The following buffers were used: glycine/NaOH; sodium acetate; sodium citrate; MES/KOH; MOPS/KOH and Tris/HCl. The molecular mass of MDH by gel filtration was determined as described by Ford *et al.* (1985).

To calculate the rate of MDH-induced autoreduction occurring *in vivo* it was assumed that 25% of the dry weight of bacteria is soluble protein, that MDH is 10% of the soluble protein (measured) and that the periplasm is 20% by volume of the bacteria (see Beardmore-Gray *et al.*, 1983). If the periplasm is less than 20% then the calculated values would be proportionately higher.

Purification of cytochrome c_H from A. methanolicus. The soluble fraction (290 ml) was prepared from 160 g wet wt of methanol-limited bacteria suspended in 20 mM-Tris/HCl (pH 8·0). The pH was adjusted to 6·0 with HCl and protamine sulphate (1·5%, w/v, pH 5·5) added to remove nucleic acids. After removal of precipitate by centrifugation, the process was repeated until no more precipitate was formed. The pH of the supernatant was adjusted to 8·0 with NaOH, dialysed against 10120 mM-Tris/HCl (pH 8·0), applied to a column of DEAE-cellulose (Whatman DE-52; 11 × 6 cm) equilibrated with the same buffer, and washed with two column volumes of the same buffer. MDH and then cytochrome c_H were eluted with the same buffer containing 30 mM-NaCl; cytochrome c_L remained bound to the column. The cytochrome fractions were pooled and concentrated under nitrogen on an Amicon YM2 membrane before gel filtration on an upward flow column (86 × 2·2 cm) of Sephadex G-50 equilibrated with 20 mM-MOPS/KOH buffer, pH 7·0. This procedure was repeated to remove traces of higher molecular mass contaminants revealed by SDS-PAGE and the pure cytochrome was stored at −20 °C.

Purification of cytochrome c_L from A. methanolicus. The soluble fraction (550 ml) prepared from 100 g wet wt of bacteria suspended in 20 mM-Tris/HCl (pH 8·0) was acid-treated (pH 4·0) to precipitate nucleic acids and some proteins as described previously (O'Keeffe & Anthony 1980*b*). The supernatant was applied to a column of DEAE-cellulose (8·2 × 6·0 cm; Whatman DE-52) equilibrated with 20 mM-Tris/HCl at pH 8·0. MDH and cytochrome c_H were eluted from the column with the starting buffer containing 30 mM-NaCl and cytochrome c_L was eluted with 85 mM-NaCl in the starting buffer. The pooled cytochrome c_L fractions were concentrated by ultrafiltration (as described above) before gel filtration on an upward flow column of Sephadex G-75 (7·6 × 3·2 cm) equilibrated with 20 mM-MOPS/KOH buffer (pH 7·0). After concentration, the suspending buffer was changed to 20 mM-Tris/HCl by passage down a pre-packed PD-10 column (Pharmacia) and subjected to anion-exchange chromatography on a Pharmacia FPLC Mono-Q column (1 ml) equilibrated with 20 mM-Tris/HCl at pH 8·0 using a linear gradient of 0–500 mM-NaCl (17·5 mM ml^{-1}). Reduced cytochrome c_L was eluted at 120 mM-NaCl and the oxidized form at 25 mM-NaCl. The pure cytochrome was desalted into 20 mM-MOPS/KOH buffer, pH 7·0, or into water, and stored at −20 °C.

The amino acid composition of the cytochrome was determined by Dr R. P. Ambler (University of Edinburgh, UK) as previously described (Ambler *et al.*, 1984).

RESULTS

MDH of A. methanolicus MB58

After about tenfold purification as described in Methods, MDH usually showed a single band on SDS-PAGE, indicating that, as with other MDHs it constituted about 10% of the soluble bacterial protein. When very high sample loadings were used during SDS-PAGE a second protein band (molecular mass 7–10 kDa) was occasionally seen to constitute 5% of the total protein present. A similar small protein has been observed in highly purified MDHs from many different types of methylotroph but the significance of this observation is uncertain. The MDH was stable at −20 °C, losing less than 10% of activity over 3 months; substrate or substrate analogues were not essential for stability.

As might be expected for a periplasmic enzyme from an acidophile, the MDH was exceptionally stable at low pH values; it was stable for 24 h at room temperature at pH 2·8 but it was unstable at pH 2·2.

The MDH from *A. methanolicus* was similar in most respects to MDHs previously described (see Anthony, 1982, 1986 for reviews). Its molecular mass was 115 kDa as determined by gel filtration and 62 kDa when measured by SDS-PAGE indicating that it is a typical dimeric

Table 1. *Properties of soluble cytochromes from A. methanolicus*

The procedures for measurement of these parameters are given in Methods. For comparison with values measured for proteins from *Methylobacterium* AM1 see O'Keeffe & Anthony (1980*b*) and for those from *Methylophilus methylotrophus* see Cross & Anthony (1980*a*).

Property	Cytochrome c_H	Cytochrome c_L
Relative amounts	30–40%	60–70%
Molecular mass (kDa)	9	21; 19
Isoelectric point (pI)	5·8	4·9; 4·7
Midpoint redox potential (mV) (pH 7)	224 (see text)	324
Midpoint redox potential (mV) (pH 4)	374	365
Absorption maxima (α and γ) of ferrocytochrome	551·3; 416·9	550·2; 415·6
Extinction coefficients (α and γ) of ferrocytochrome (mM^{-1} cm^{-1})	32·9; 160	23·3; 134
Ratio of α absorbance to absorbance at 280 nm	1·16	0·89

MDH. The isoelectric point of the MDH was 7·8 as measured by isoelectric focusing, its pH optimum in the dye-linked assay was 9·5 and its specific activity was 4·4–5·8 μmol methanol oxidized min^{-1} (mg protein)$^{-1}$. Ammonia was required as activator (as expected) but up to 20% of maximum activity was observed in the absence of ammonia which is unusual (Anthony, 1986). The spectrum of pure MDH was typical of partially reduced MDHs having a peak at 345 nm and shoulder at 400 nm due to the PQQ prosthetic group, and protein absorption at 280 nm and 290 nm (shoulder). The ratio of absorbance at 280 nm to that at 345 nm was 7·1.

Cytochromes of A. methanolicus

When *A. methanolicus* was grown on methanol, glycerol or glucose and harvested before the end of the exponential phase of growth, only *b*- and *c*-type cytochromes were observed. Cytochromes *a* and *d* were never observed in whole bacteria or in isolated membranes. The only cytochrome able to react rapidly with carbon monoxide was a cytochrome *b*, which was presumably the *o*-type oxidase previously reported in this organism. The concentration of cytochrome *b* in membranes was similar after growth on methanol, glucose and glycerol [about 700–860 pmol (mg protein)$^{-1}$]. About half of this was able to react with carbon monoxide and was thus presumably the cytochrome *o*.

The concentration of cytochrome *c* on membranes was similar to that of total cytochrome *b* [550–780 pmol cytochrome (mg membrane protein)$^{-1}$] under all growth conditions. The concentration of soluble cytochrome *c* was markedly affected by the growth substrate. During growth on glycerol it was similar to that of total cytochrome *b*, but during growth on glucose and methanol it increased threefold and 13-fold respectively. The concentrations [nmol soluble cytochrome *c* (mg soluble protein)$^{-1}$] were 0·14 on glycerol, 0·39 on glucose and 1·84 on methanol.

Soluble c-type cytochromes of A. methanolicus

A characteristic of all Gram-negative methylotrophs growing on methane or methanol is the presence of high concentrations of two types of soluble (periplasmic) *c*-type cytochromes. Cytochrome c_H has a higher isoelectric point and is similar in all respects to the small cytochrome *c* that mediates between the cytochrome bc_1 complex and the terminal oxidase of mitochondria and many other bacteria. Cytochrome c_L has a lower isoelectric point; it is specific to methylotrophs and is the physiological electron acceptor for MDH.

Cytochrome c_H of A. methanolicus. This cytochrome constituted 30–40% of the total soluble cytochrome *c* of *A. methanolicus* after growth on methanol. Its molecular mass was 9 kDa and its isoelectric point was 5·8. A low isoelectric point for the small cytochrome *c* has also been observed in *Paracoccus denitrificans* (Beardmore-Gray *et al.*, 1983; Husain & Davidson, 1986) and *Methylomonas* J (Ohta & Tobari, 1981). The properties of this cytochrome were typical for small *c*-type cytochromes (Table 1), the only feature of note being the high extinction coefficient for the α-band of the ferrocytochrome. No splitting of the α-band occurred at low temperature;

in this respect it is similar to cytochrome c_L (see below) and to both cytochromes from *Methylobacterium* AM1 (O'Keeffe & Anthony, 1980*b*), but different from most other soluble *c*-type cytochromes (Lemberg & Barrett, 1973). The midpoint redox potential of this cytochrome at pH 4·2 was 374 mV. It was not possible to obtain an equilibrium titration at pH 7·0 although attempts were made, using a range of concentrations of different mediators; after about 25% of the cytochrome had become reduced, the measured potential decreased slowly without further addition of reductant. During this change in potential it was noted that the potential at which 50% reduction occurred was 225 mV.

Cytochrome c_L. This cytochrome comprised 60–70% of the total soluble cytochrome *c* after growth on methanol. As found in other methylotrophs, there were two forms, differing in their molecular masses; it is assumed that the higher (21 kDa) is the native form and that the other (19 kDa) is derived from it. The isoelectric point of the larger form was 4·9 and that of the smaller was 4·7. The properties of the cytochrome c_L were typical of other cytochromes of this type (Table 1). The amino acid composition was not markedly different from that of other examples of cytochrome c_L (Beardmore-Gray *et al.*, 1982): Asp (20), Thr (21), Ser (15), Glu (16), Pro (14), Gly (17), Ala (18), Val (9), Met (4), Ile (6), Leu (15), Tyr (3), Phe (10), His (8), Lys (11), Arg (5). The values in parentheses are the nearest integer; tryptophan and cysteine were not determined. The pure cytochrome did not cross react with antisera raised against pure cytochromes c_L from *Methylobacterium* AM1 or *Methylophilus methylotrophus*.

It has previously been shown that the periplasmic cytochromes *c* of *Methylobacterium* AM1 have two ionizing groups that affect their redox potentials. The p*K* values for these are 3·5 and 5·5 in the oxidized forms, and 4·5 and 6·5 in the reduced forms. It has been proposed that the higher of the p*K* values is likely to be due to the rear (inner) haem propionate in the haem cleft, and the lower to the front (outer) propionate in its more hydrophilic environment (O'Keeffe & Anthony, 1980*b*). As described above for cytochrome c_H, it was impossible to determine midpoint potentials of the cytochrome c_L of *A. methanolicus* above pH 7·5 but the values obtained for the range pH 3·5–7·5 were as follows: 3·45 (371 mV); 4·3 (361 mV); 4·6 (362 mV); 5·15 (370 mV); 6·05 (348 mV); 6·5 (340 mV); 6·75 (330 mV); 7·0 (324 mV); 7·5 (302 mV). The apparent E_0' value was 365 mV. The simplest interpretation of these data is that there is a single ionizing group affecting redox potentials in this cytochrome, its p*K* being 6·2 in the oxidized form and 7·7 in the reduced form (for the analytical method, see O'Keeffe & Anthony, 1980*b*). It is not possible to say whether or not this dissociation is due to one of the haem propionates.

Autoreduction of cytochrome c_L

Neither of the soluble cytochromes *c* of *A. methanolicus* was autoreduced at the high pH values (around pH 10) that led to rapid autoreduction of the cytochromes of *Methylobacterium* AM1 or *Methylophilus methylotrophus*. Furthermore, both cytochromes were rapidly oxidized by air when the pH of the reduced forms was raised above pH 8·0. It is thus possible that the cytochromes were autoreduced at high pH values but that the reduced cytochrome was immediately re-oxidized.

Cytochrome c_L became autoreduced on raising the pH from pH 3, at which it is fully oxidized, to pH 7, the rate of autoreduction being faster at the higher pH values (Fig. 1); the process was reversed by lowering the pH. The process was first order with respect to oxidized cytochrome *c* and so it was concluded that it is the same intramolecular autoreduction previously described for the cytochromes of *Methylobacterium* AM1 and *Methylophilus methylotrophus* (O'Keeffe & Anthony, 1980*b*; Beardmore-Gray *et al.*, 1983). As found with these cytochromes, the presence or absence of oxygen was irrelevant to the rate of auroreduction but glycerol stimulated the rate two- to fivefold. The autoreduction sometimes occurred in two phases; both were first order with respect to oxidized cytochrome *c*. The first order rate constant for the fast phase was about twice that for the second slower phase (at pH 4·0) but up to five times faster at pH 5·5. The extent of the first, fast phase was variable. It was always seen when the cytochrome had been stored in the oxidized state (below pH 4 or above pH 8), and it was more extensive when the cytochrome had been frozen and thawed a number of times. Complete reduction never occurred by way of the fast phase but it did so in some samples by way of the slow phase. When samples showing both

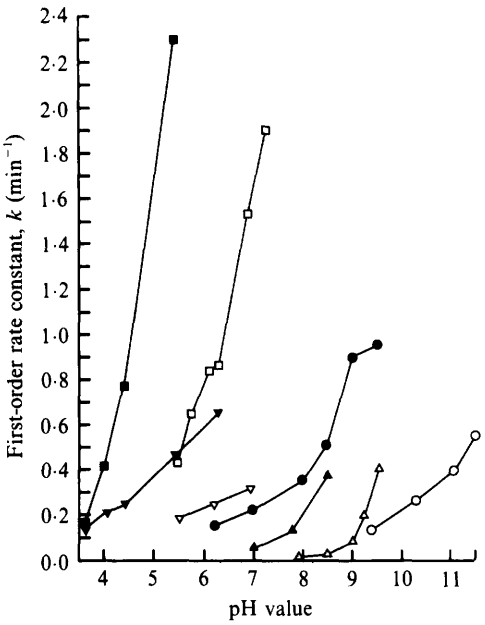


Fig. 1. Autoreduction of the cytochrome c_L from three methylotrophs. Autoreduction was measured by the method of O'Keeffe & Anthony (1980*a*). Results from *Methylophilus methylotrophus* were taken from Beardmore-Gray *et al.* (1983), and those for *Methylobacterium* AM1 from O'Keeffe & Anthony (1980*a*). Measurements were made in the absence of glycerol when using the proteins from *A. methanolicus*, but those made with cytochrome *c* alone (no MDH) from *Methylophilus methylotrophus* and *Methylobacterium* AM1 were made in the presence of 30% (v/v) glycerol. *A. methanolicus* (fast phase): □, cytochrome *c* (1·3 μM); ■, cytochrome plus MDH (0·13 μM). *A. methanolicus* (slow phase): ▽, cytochrome *c* (1·3 μM); ▼, cytochrome *c* plus MDH (0·13 μM). *Methylophilus methylotrophus*: △, cytochrome *c* (1 μM); ▲, cytochrome *c* plus MDH (1 μM). *Methylobacterium* AM1: ○, cytochrome *c* (0·7 μM); ●, cytochrome *c* plus MDH (1·13 μM).

phases of autoreduction were stored under oxygen in the oxidized state the proportion of autoreduction occurring by the fast phase increased; the same samples when stored anaerobically were unaltered. All results were consistent with the proposal that the fast phase occurs when the protein is modified in some way by its prior treatment. It should be noted that the rate constant for the slow phase was very similar to those measured for the cytochromes of *Methylobacterium* AM1 and *Methylophilus methylotrophus*.

The most important conclusion from these experiments is that the cytochrome c_L of *A. methanolicus* is autoreducible but that the pH range over which this occurs is about 3 pH units lower than the pH at which it occurs in other methylotrophs. This is the same as the difference in pH optima for growth between these organisms.

Effect of MDH on the autoreduction of cytochrome c_L of A. methanolicus

It has been proposed that autoreduction is an intramolecular process, the mechanism of which involves electron donation by the dissociated species of a weakly acidic group close to the haem iron (O'Keeffe & Anthony, 1980*a*; Beardmore-Gray *et al.*, 1982, 1983). When MDH is added to the cytochrome the same intracellular autoreduction is stimulated to occur at a lower, more physiological, pH value (pH 7·0) than in the absence of MDH. It is assumed that MDH acts by lowering the pK for dissociation of the group which donates the electron for autoreduction.

Using the proteins from *A. methanolicus* it was shown that MDH stimulated autoreduction of cytochrome c_L; both phases of autoreduction were stimulated. As was found with autoreduction in the absence of MDH, there was considerable variability with respect to 'methanol-induced'

autoreduction. The process was pseudo-first-order with respect to oxidized cytochrome *c*, being independent of the concentration of cytochrome at a constant concentration of MDH, and dependent on the concentration of MDH. This is exactly as shown previously for other methylotrophs (see above). Also as previously observed, the MDH-induced autoreduction occurred at about 3 pH units below that for autoreduction in the absence of MDH.

Methanol : cytochrome c oxidoreductase activity of MDH from A. methanolicus

Methanol-dependent reduction of cytochrome *c* was demonstrated by the method of Beardmore-Gray *et al.* (1983) using the pure proteins from *A. methanolicus*, and mammalian cytochrome *c* as the terminal electron acceptor as described in Methods. This cytochrome was not reduced by MDH until catalytic quantities of cytochrome c_L were added. As found with this system from other methylotrophs, there was some reduction of the cytochrome by endogenous substrate on the MDH; the nature of this is unknown. When cytochrome reduction due to this endogenous reductant was complete methanol was added and the rate of electron transfer from this substrate by way of MDH and cytochrome c_L measured.

At pH 4·0 the apparent K_m for cytochrome c_L was 14 μM and the specific activity (at saturating cytochrome concentration) was 18·75 nmol cytochrome reduced min^{-1} (nmol MDH)$^{-1}$. Rates of methanol-dependent cytochrome reduction at pH 7 were 50–75% of the rates at pH 4. There was no activity at pH 9. Rates were identical in the presence or absence of ammonia (the activator for the dye-linked enzyme assay) or oxygen.

In the assay at pH 4 and pH 7 no formaldehyde was produced during oxidation of endogenous reductant. In the presence of methanol the stoichiometry observed was 2 mol cytochrome reduced per mol of formaldehyde produced. As found with other methylotrophs, formaldehyde was also able to act as substrate when included instead of methanol.

Cytochrome c_L was the specific acceptor for MDH, cytochrome c_H being unable to replace it. Cytochrome c_H was able to replace mammalian cytochrome *c* as terminal electron acceptor, demonstrating that rapid electron transfer between the two methylotrophic cytochromes *c* is able to occur. Cytochrome c_L from *A. methanolicus* could be replaced by this cytochrome from *Methylobacterium* AM1 but not by cytochrome c_L from *Methylophilus methylotrophus*. Conversely, the cytochrome c_L from *A. methanolicus* was able to act as electron acceptor from MDH of *Methylobacterium* AM1 but not from that of *Methylophilus methylotrophus*. In these experiments, which were done at pH 4·0, formaldehyde was shown to be produced concomitantly with reduction of cytochrome.

It has been suggested previously (Beardmore-Gray & Anthony, 1984) that in the absence of added (terminal) electron acceptor no electron transfer should occur from methanol to cytochrome c_L and hence no formaldehyde should be produced. This is because the proposed mechanism for involvement of autoreduction in this electron transfer had suggested that the electron acceptor for MDH is the free radical form of the cytochrome c_L produced by oxidation of the autoreduced species. In the absence of added (terminal) electron acceptor this species would not be produced. In order to test this, the usual terminal electron acceptor (mammalian cytochrome *c*) was replaced by a large excess of cytochrome c_L, and formaldehyde was measured. It was found that a high proportion of this excess cytochrome c_L was reduced and that this occurred concomitantly with production of formaldehyde. Contrary to what was originally suggested, this does not 'disprove' the proposed mechanism. This result is what would be expected if, after MDH-stimulated autoreduction, electron transfer occurred from the autoreduced cytochrome c_L to free oxidized cytochrome c_L.

DISCUSSION

The results described above show that *A. methanolicus* is not markedly different from other methylotrophic bacteria despite the fact that its pH optimum for growth is 3 pH units lower than that for other methylotrophic bacteria. It contains a typical small cytochrome (cytochrome c_H) that differs from that in many methylotrophs, but is similar to that in *Paracoccus denitrificans* in having a low isoelectric point. It contains a typical MDH which differs from others in its slightly

lower isoelectric point, its stability at lower pH values and the pH at which it interacts with cytochrome *c*. As in other methylotrophs, the specific electron acceptor from MDH is a large *c*-type cytochrome (cytochrome c_L). The main difference in *A. methanolicus* related to its special mode of growth that was observed is the nature of the response to pH of the MDH and cytochrome c_L, both of which occur in the periplasm and therefore operate in an environment 3 pH units lower than in other methylotrophs.

The cytochrome c_L is autoreduced by increasing the pH of suspending buffer, but the pH range over which this occurs is 3 pH units lower than for other cytochromes c_L. This is consistent with the previous suggestion that the autoreduction phenomenon does constitute part of the electron transport process from MDH to cytochrome c_L. This conclusion is further supported by the observation that the pH range for autoreduction is lowered even further, by interaction with MDH, to the pH occurring in the periplasm (pH 4·0). As expected, the oxidation of methanol to formaldehyde, catalysed by MDH and cytochrome c_L also occurs at the pH of the periplasm.

It was clearly of interest to determine whether the rate of autoreduction occurring in the presence of MDH is sufficiently high for this process to be part of the mechanism of MDH as measured by its methanol:cytochrome *c* oxidoreductase activity.

As seen in Fig. 1, at pH 4·0 the pseudo-first-order rate constant for the slow phase of MDH-induced autoreduction of cytochrome *c* was 0·21 min^{-1} (equivalent to a second-order rate constant of 1·6 $\mu M\ min^{-1}$ at the concentration of MDH used in the experiment). Using this rate constant it can be calculated that the rate of autoreduction in the periplasm containing 370 μM-MDH and 750 μM-cytochrome c_L would be about 1200 nmol cytochrome *c* reduced min^{-1} (nmol MDH)$^{-1}$. This is almost sufficient to account for the respiration rate measured *in vivo* and it is about 60 times faster than the very low overall rate of reduction by methanol of cytochrome *c*, catalysed by MDH that is measured *in vitro*. These results thus demonstrate that autoreduction is not rate-limiting in the MDH reaction mechanism and that the low rates measured must be due to the rate of electron transfer between reduced methanol dehydrogenase and the oxidized form of the cytochrome *c*.

This work was supported by SERC by way of a project grant and a CASE studentship with ICI (industrial collaborator, Dr S. J. Collins).

REFERENCES

AMBLER, R. R., DANIEL, M., MELIS, K. & STOUT, C. D. (1984). The amino acid sequence of the dihaem cytochrome c_4 of the bacteria *Azotobacter vinelandii*. *Biochemical Journal* **22**, 217–227.

ANTHONY, C. (1982). *The Biochemistry of Methylotrophs*. London: Academic Press.

ANTHONY, C. (1986). The bacterial oxidation of methane and methanol. *Advances in Microbial Physiology* **27**, 113–210.

ANTHONY, C. & ZATMAN, L. J. (1967). Purification and properties of the alcohol dehydrogenase of *Pseudomonas* sp. M27. *Biochemical Journal* **104**, 953–959.

BAMFORTH, C. W. & QUAYLE, J. R. (1978). The dye-linked alcohol dehydrogenase of *Rhodopseudomonas acidophila*. Comparison with dye-linked methanol dehydrogenases. *Biochemical Journal* **169**, 677–686.

BEARDMORE-GRAY, M. & ANTHONY, C. (1984). The methanol:cytochrome *c* oxidoreductase activity of methylotrophs. In *Microbial Growth on C-1 Compounds*, pp. 97–105. Edited by R. L. Crawford & R. S. Hanson, Washington, DC: American Society for Microbiology.

BEARDMORE-GRAY, M., O'KEEFFE, D. T. & ANTHONY, C. (1982). The autoreducible cytochromes of the methylotrophs *Methylophilus methylotrophus* and *Pseudomonas* AM1. *Biochemical Journal* **207**, 161–165.

BEARDMORE-GRAY, M., O'KEEFFE, D. T. & ANTHONY, C. (1983). The methanol:cytochrome *c* oxidoreductase activity of methylotrophs. *Journal of General Microbiology* **129**, 923–933.

CROSS, A. R. & ANTHONY, C. (1980*a*). The purification and properties of the soluble cytochromes *c* of the obligate methylotroph *Methylophilus methylotrophus*. *Biochemical Journal* **192**, 421–427.

CROSS, A. R. & ANTHONY, C. (1980*b*). The electron transport chains of the obligate methylotroph *Methylophilus methylotrophus*. *Biochemical Journal* **192**, 429–439.

FORD, S., PAGE, M. D. & ANTHONY, C. (1985). The role of methanol dehydrogenase modifier protein and aldehyde dehydrogenase in the growth of *Pseudomonas* AM1 on 1,2-propanediol. *Journal of General Microbiology* **131**, 2173–2182.

FROUD, S. J. & ANTHONY, C. (1984). The purification and characterization of the *o*-type oxidase of *Methylophilus methylotrophus* and its reconstitution into a 'methanol oxidase' electron transport chain. *Journal of General Microbiology* **130**, 2201–2212.

FUHRHOP, J.-H. & SMITH, K. M. (1975). In *Porphyrins and Metalloporphyrins*, p. 804. Edited by K. M. Smith, Amsterdam: Elsevier.

HUSAIN, M. & DAVIDSON, V. L. (1986). Characterisation of two inducible periplasmic *c*-type cytochromes

from *Paracoccus denitrificans*. *Journal of Biological Chemistry* **261**, 8577–8580.

LEMBERG, R. & BARRETT, J. (1973). *Cytochromes*. London: Academic Press.

LOFFHAGEN, N. & BABEL, W. (1984). Localisation of primary alcohol-oxidising enzymes in the facultative methylotroph *Acetobacter* sp. MB70. *Zeitschrift für allgemeine Mikrobiologie* **24**, 143–149.

NUNN, D. N. & LIDSTROM, M. E. (1986). Isolation and complementation analysis of 10 methanol oxidation mutant classes and identification of the methanol dehydrogenase structural gene of *Methylobacterium* sp. strain AM1. *Journal of Bacteriology* **166**, 581–590.

OHTA, S. & TOBARI, J. (1981). Two cytochromes *c* of *Methylomonas* J. *Journal of Biochemistry* **90**, 215–224.

O'KEEFFE, D. T. & ANTHONY, C. (1980*a*). The interaction between methanol dehydrogenase and autoreducible cytochromes *c* of the facultative methylotroph *Pseudomonas* AM1. *Biochemical Journal* **190**, 481–484.

O'KEEFFE, D. T. & ANTHONY, C. (1980*b*). The two cytochromes *c* in the facultative methylotroph *Pseudomonas* AM1. *Biochemical Journal* **192**, 411–419.

STEUDEL, K. & BABEL, K. (1982). Das Cytochromkomplement des methylotrophen Essigsaurebakteriums MB58 in Abhangigkeit von den Wachstumsbedingungen. *Zeitschrift für allgemeine Mikrobiologie* **22**, 379–388.

STEUDEL, K., MIETHE, D. & BABEL, W. (1980). Bakterium MB58, ein methylotrophes "Essigsaurebakterium". *Zeitschrift für allgemeine Mikrobiologie* **20**, 633–672.

THOMAS, P. E., RYAN, D. & LEVIN, W. (1976). An improved staining procedure for the detection of the peroxidase activity of cytochrome P450 on SDS-polyacrylamide gels. *Analytical Biochemistry* **75**, 168–176.

UHLIG, H., KARBAUM, K. & STEUDEL, A. (1986). *Acetobacter methanolicus* sp. nov., an acidophilic facultatively methylotrophic bacterium. *International Journal of Systematic Bacteriology*. **36**, 317–322.

WEBER, K. & OSBORN, M. (1975). Proteins and sodium dodecyl sulphate; molecular weight determination on polyacrylamide gels and related procedures. In *The Proteins*, vol. 1, 3rd edn, pp. 179–223. Edited by H. Neurath & R. L. Hill. London: Academic Press.

WOOD, P. M. (1984). Bacterial proteins with CO-binding *b*- or *c*-type haem functions and absorption spectroscopy. *Biochimica et biophysica acta* **768**, 293–317.