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The Roles of Cytochrome *c* in Membranes of *Methylophilus methylotrophus*

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The soluble and membrane-bound cytochromes *c* of methylotrophic bacteria were investigated in order to determine their relationships with each other, and their roles in electron transport. The proportions of soluble cytochromes c_H and c_L in various methylotrophs were not markedly dependent on the type of methylotroph nor on conditions of growth. Between 30 and 50% of the cytochrome *c* of *Methylophilus methylotrophus* was bound tightly to membranes; 8% of this was cytochrome c_H, 37% was cytochrome c_L and 55% was the cytochrome *c* component of the oxidase, cytochrome *co*. These cytochromes were purified and characterized. It was concluded that cytochrome c_H has no role in the membrane. By contrast, the membrane cytochrome c_L has a separate role from that of soluble cytochrome c_L; the membrane-bound cytochrome c_L may play a role analogous to that of mitochondrial cytochrome c_1 in mediating between cytochrome *b* and the terminal oxidase during the oxidation of NADH.

INTRODUCTION

All methylotrophic bacteria growing on methanol contain at least two different soluble cytochromes *c*, plus a considerable amount of cytochrome *c* bound to membranes (Anthony, 1982; Vrdoljak & Froud, 1982; Beardmore-Gray & Anthony, 1984; Carver & Jones, 1984; Froud & Anthony, 1984).

The electron acceptor for methanol dehydrogenase in all bacteria tested is the soluble cytochrome c_L; this has a low isoelectric point, a high molecular weight and a relatively low midpoint redox potential (Beardmore-Gray *et al.*, 1982, 1983; Beardmore-Gray & Anthony, 1984). Cytochrome c_H, by contrast, has a high isoelectric point, a low molecular weight and a higher redox potential. This cytochrome does not accept electrons from methanol dehydrogenase but it is 50 times more active than cytochrome c_L as electron donor to the *o*-type oxidase of *Methylophilus methylotrophus* (Froud & Anthony, 1984). This oxidase, the sole oxidase during carbon-excess growth, is a complex containing equal amounts of *b*-type and *c*-type cytochromes. It was suggested by Carver & Jones (1983), who first described the partially purified oxidase, that the membrane-bound cytochrome *c* component is identical to the soluble cytochrome c_L. This suggestion is consistent with the previous demonstration that membranes of O_2-limited bacteria contained two cytochromes *c* which, on the basis of their midpoint redox potentials, appeared to be membrane-bound cytochromes c_H and c_L (Cross & Anthony, 1980). Recent discussions of the oxidase concluded, however, that the membrane-bound cytochrome *c* component of the oxidase is not identical to either of the soluble cytochromes *c* of these bacteria (Froud & Anthony, 1984); and also that there is only one species of membrane-bound cytochrome *c* in *M. methylotrophus* (Carver & Jones, 1984). These observations raise the question of the number of membrane-bound cytochromes *c* in this organism, and their relationship with the soluble cytochromes; and the present paper describes an investigation into these questions.

Because of the possible importance of both of the soluble cytochromes *c* in the oxidation of methanol as described above, the effect of growth conditions on the relative proportions of these two cytochromes has also been investigated in a number of different methylotrophs.

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METHODS

Except for those described below, the methods used were as described by Froud & Anthony (1984).

Organisms and growth conditions. Methylophilus methylotrophus (NCIB 10515), *Paracoccus denitrificans* (NCIB 8944) and *Pseudomonas* AM1 (NCIB 9133) were obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, UK. *Hyphomicrobium* X was obtained from M. Attwood (Department of Microbiology, University of Sheffield, UK). *M. methylotrophus* was grown as described by Froud & Anthony (1984) and Cross & Anthony (1980); all the other organisms were grown aerobically in batch or continuous culture at 30 °C using similar methods, but with the modifications as described below. The growth medium was essentially that of MacLennan *et al.* (1971), except for the replacement of the KH_2PO_4 by 20 mM- or 6 mM-H_3PO_4, respectively, for batch or continuous culture (adjusted to pH 7·2 with 1 M-KOH/1 M-NaOH). For batch growth, carbon sources were added to a final concentration of 0·2% (w/v) except methanol (0·5%, w/v). For NH_3-limited continuous culture the final concentrations were 250 mM-methanol and 5·4 mM-$(NH_4)_2SO_4$; for carbon limitation the concentrations were 125 mM-methanol and 27 mM-$(NH_4)_2SO_4$.

Preparation of soluble and membrane fractions. The only modification to the methods described by Froud & Anthony (1984) was that bacteria, other than *M. methylotrophus*, were sonicated for 5 × 2 min periods.

Purification of the 'membrane-bound' cytochromes c of M. methylotrophus. The membranes were washed with a solution of 1 M-KCl to remove loosely associated cytochrome *c*, and solubilized by the method described previously (Froud & Anthony, 1984); about 90% of the cytochrome *c* was solubilized by this procedure. The oxidase, cytochrome *co*, was purified as described previously using ion-exchange (DEAE- and CM-cellulose) and gel permeation chromatography.

The cytochrome *c* that was adsorbed by the DEAE-cellulose (designated 'membrane' cytochrome c_L) was eluted using a linear gradient of NaCl; it eluted as a broad peak in approximately 180 mM-NaCl (step A). The cytochrome was concentrated by ultrafiltration and applied to an upward flow column of Fractogel TSK HW55(s) (78 × 2·4 cm) equilibrated with 100 mM-Tris/HCl (pH 8·0) containing 0·5% (v/v) Triton X-100; it eluted as two cytochrome *c* fractions differing in their molecular weights.

The cytochrome *c* that was adsorbed by the CM-cellulose during purification of the oxidase (designated 'membrane' cytochrome c_H) was eluted with a linear salt gradient; it eluted in approximately 150 mM-NaCl (step B). The cytochrome was concentrated by ultrafiltration and applied to an upward flow Sephadex G-75 column (76 × 1·6 cm) equilibrated with 12 mM-MOPS buffer (pH 7·0) containing 0·5% (v/v) Triton X-100. The purified cytochrome appeared as a single protein band following SDS-PAGE, and peak integration showed that it was at least 97% pure.

Measurement of the proportions of the cytochromes c in methylotrophs. Soluble cell extracts (from 2–4 g wet bacteria) were applied to a DEAE-cellulose column (9 × 3 cm) equilibrated with 20 mM-Tris/HCl (pH 8·0). Soluble cytochromes *c* of high isoelectric point (designated cytochrome c_H) were not adsorbed, whereas soluble cytochromes *c* of low isoelectric point (cytochrome c_L) were adsorbed and were then eluted by raising the buffer concentration to 500 mM. The fractions were assayed spectrophotometrically for cytochrome *c* content.

The membrane cytochromes *c* of *M. methylotrophus* were assayed in a similar manner after separation from one another by ion-exchange chromatography (steps A and B, above).

Measurement of methanol-dependent reduction of cytochrome c by methanol dehydrogenase. This was performed exactly as described by Beardmore-Gray *et al.* (1983).

RESULTS

The results in Table 1 show that growth conditions had relatively little effect on the relative proportions of soluble cytochromes c_H and c_L in a number of methylotrophic bacteria. These bacteria differed with respect to their carbon assimilation pathways and terminal oxidases but these characteristics had little influence on the relative proportions of soluble cytochromes *c*. The bacteria in Table 1 differ from the facultative autotroph *Paracoccus denitrificans*, which has no cytochrome c_H, but which has a cytochrome c_L that interacts with methanol dehydrogenase and that is only induced during growth on methanol (van Verseveld & Stouthamer, 1978; Beardmore-Gray *et al.*, 1983). It is not immediately obvious why soluble cytochrome c_L, the electron acceptor for methanol dehydrogenase, is always present during heterotrophic growth in those bacteria listed in Table 1; it is possible however, that when bound tightly to membranes this cytochrome functions in the electron transport chain for the oxidation of NADH (see Discussion).

The results in Table 2 show that between 30% and 50% of the total cytochrome *c* in O_2-limited *M. methylotrophus* was bound to membranes, the remainder being released during extraction of

Table 1. *Proportions of the soluble cytochromes c in five methylotrophic bacteria*

Experimental details are given in Methods. The data from Beardmore-Gray (1982), S. J. Froud (unpublished) and Cross (1980) are the means of determinations performed in duplicate (at least).

Organism	Growth substrate*	Growth condition	Assimilation pathway†	Cytochrome oxidase type†	Cytochrome c_H (%)	Cytochrome c_L (%)
Pseudomonas AM1	Methanol[a]	Batch	Serine	aa_3	70	30
	Methanol[a]	Nitrogen-limited	Serine	aa_3	69	31
	Methanol[a]	Carbon-limited	Serine	aa_3	76	24
	Succinate[a]	Batch	Heterotrophic	aa_3	70	30
	Formate[a]	Batch	Serine	aa_3	65	35
Methylophilus methylotrophus	Methanol[b]	Oxygen-limited	Ribulose monophosphate	o	66	34
	Methanol[a]	Oxygen-limited	Ribulose monophosphate	o	77	23
	Methanol[a]	Nitrogen-limited	Ribulose monophosphate	o	46	54
	Methanol[a]	Carbon-limited	Ribulose monophosphate	aa_3/o	40	60
	Methanol[c]	Carbon-limited	Ribulose monophosphate	aa_3/o	54	46
	Methanol[d,e]	Carbon-limited	Ribulose monophosphate	aa_3/o	68	32
Hyphomicrobium X	Methanol[a]	Batch	Serine	aa_3/o	75	25
	Acetate[a]	Batch	Heterotrophic	aa_3/o	51	49
Methylomonas J	Methanol[f]	Batch	Ribulose monophosphate	ND	73	27
	Methylamine[f]	Batch	Ribulose monophosphate	ND	52	48

* References: *a*, Beardmore-Gray (1982); *b*, S. J. Froud (unpublished); *c*, Cross (1980); *d*, Jones *et al.* (1982); *e*, Carver & Jones (1984); *f*, Ohta & Tobari (1981).
† See Anthony (1982).
ND, Not determined.

Table 2. *The cytochrome c composition of Methylophilus methylotrophus when grown under conditions of oxygen-limitation*

The soluble cytochromes include the material washed from the membranes with 1 M-KCl prior to their solubilization for determination of membrane-bound cytochromes c_H and c_L. The average amounts of cytochrome *c* in the cell extracts were: 823 pmol (mg soluble protein)$^{-1}$ (soluble fraction) and 1270 pmol (mg membrane protein)$^{-1}$ (membrane fraction). The ratio of total soluble protein to membrane protein recovered after disruption of the bacteria was 4:1. In the calculation of these data it is assumed that the proportions of the cytochromes *c* measured upon separation (i.e. the data of the first two columns) were the same as the proportions of each cytochrome in the initial cell extract.

Cytochrome *c*	Percentage in soluble fraction	Percentage in solubilized membrane fraction	Amount in whole bacteria [pmol (mg bacterial protein)$^{-1}$]	Percentage of total cytochrome *c*	Percentage of cytochrome c_H	Percentage of cytochrome c_L
Cytochrome c_H (soluble)	66	–	437 ($\pm$73, n = 2)	48	94	–
Cytochrome c_H (membrane bound)	–	8	29 ($\pm$3, n = 2)	3	6	–
Cytochrome c_L (soluble)	34	–	222 ($\pm$8, n = 2)	24	–	73
Cytochrome c_L (membrane bound)	–	37	81 ($\pm$38, n = 2)	9	–	27
Cytochrome *c* (from cytochrome oxidase *co*)	–	55	145 ($\pm$32, n = 2)	16	–	–

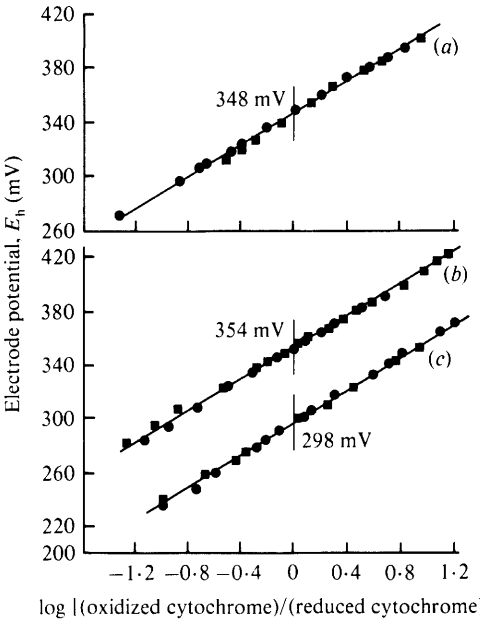

Fig. 1. Potentiometric titrations of the pure cytochromes *c* from *M. methylotrophus* at pH 7·0. (*a*) The cytochrome *c* component of the *o*-type cytochrome oxidase (cytochrome *co*); (*b*) soluble cytochrome c_H; (*c*) soluble cytochrome c_L. ●, Reductive titration with ascorbate; ■, oxidative titration with ferricyanide. The lines on the figure are theoretical curves for single-electron transfer reactions. The buffer was 25 mM-MOPS/NaOH (pH 7·0). Methods were those of Cross & Anthony (1980).

the bacteria or during washing membranes with 1 M-KCl. These proportions are similar to those measured in this organism when grown under a variety of other growth conditions (Cross & Anthony, 1980; Jones *et al.*, 1982; Burton *et al.*, 1983).

No cytochrome *c* having a molecular weight of less than 17000 was detected by SDS-PAGE of extracts of membranes; this result is consistent with the low concentration of the small cytochrome c_H measured in membranes (Table 2). The small amount of cytochrome c_H that was present was purified (after solubilization) to homogeneity (more than 97% pure), and shown to be identical to soluble cytochrome c_H in a number of characteristics: its molecular weight was 8600 ($\pm$ 140, n = 5); its α-absorption maximum was 551·25 nm; it was not an electron acceptor for methanol dehydrogenase; and its rate of oxidation by pure cytochrome *co* (the *o*-type oxidase) was faster than that of cytochrome c_L. The demonstration that less than 6% of the cytochrome c_H was tightly bound to membranes in O_2-limited *M. methylotrophus*, and that this cytochrome was identical to the soluble cytochrome c_H, together with the observation that membranes of methanol-limited bacteria also contained negligible amounts of this cytochrome (Jones *et al.*, 1982; Carver & Jones, 1984), suggest that it always functions solely as a soluble cytochrome in the periplasm or bound loosely to the periplasmic side of the bacterial membrane. This would be consistent with the demonstration that the cytochrome *c* that is released into the culture medium during growth of *M. methylotrophus* is predominantly the small cytochrome c_H (Cross & Anthony, 1980; Beardmore-Gray, 1982) which is also the cytochrome *c* most readily removed from whole bacteria by treatment with EDTA (Carver & Jones, 1984).

Although the amount of membrane-bound cytochrome c_H can probably be considered to be negligible, this was not the case for the membrane-bound cytochrome c_L which was shown to constitute 37% of the membrane-bound cytochrome *c* (Table 2). This was partially purified and shown to be identical to the soluble cytochrome c_L with respect to a number of characteristics: it was isolated as two components having molecular weights of 20900 ($\pm$ 50, n = 2) and 17100 ($\pm$ 360, n = 3); the α-absorption maximum was 550·0 nm; it was an electron acceptor from methanol dehydrogenase (the rate being similar to that measured with soluble cytochrome c_L);

and the rate of its oxidation by pure cytochrome *co* (the *o*-type oxidase) was very low. The partially pure cytochrome c_L from membranes differed from the cytochrome *c* component of the pure oxidase which has a molecular weight of 23800, and which was not an electron acceptor for methanol dehydrogenase (Froud & Anthony, 1984). During SDS-PAGE on the same gels, the electrophoretic mobility of this oxidase cytochrome *c* component was always clearly different from that of the cytochrome c_L (both soluble and solubilized forms; these had identical electrophoretic mobilities).

As shown in Table 2, 55% of the membrane-bound cytochrome *c* was the cytochrome *c* component of the *o*-type oxidase, cytochrome *co*. Although when this cytochrome *c* was separated from the cytochrome *b* component of the oxidase it had a low isoelectric point, it was not adsorbed to DEAE-cellulose during its purification and so at first sight appeared to be a membrane-bound cytochrome c_H (apparently having a high isoelectric point) (Carver & Jones, 1983; Froud & Anthony, 1984). The midpoint redox potential of the cytochrome *c* component of the oxidase was determined by potentiometry, the titration being presented in Fig. 1 together with those of cytochromes c_H and c_L for comparison. The value for the oxidase cytochrome *c* (348 mV) was completely different from that measured for cytochrome c_L (298 mV) but almost identical to that of the cytochrome c_H (354 mV) from which it differs in all other respects (see above).

DISCUSSION

The observations in the present paper show that the membrane-bound cytochrome c_H of O_2-limited *M. methylotrophus* was identical to the soluble cytochrome c_H and, being present in only very small amounts, should not be considered to be a separate integral membrane cytochrome. This agrees with the conclusion published recently by Carver & Jones (1984) that cytochrome c_H is almost exlusively a soluble cytochrome in this organism when grown under conditions of carbon-limitation.

Carver & Jones (1984) concluded that only one *c*-type cytochrome of any importance was present in membranes of *M. methylotrophus* and that this was the cytochrome c_L. Both of these conclusions conflict with those of Cross & Anthony (1980), whose redox potential measurements showed that membranes always contained two types of cytochrome *c* which they identified as cytochrome c_H (about 70%) and cytochrome c_L (about 30%). The results in the present paper resolve this apparent conflict. Fig. 1 shows that the membrane-bound cytochrome *c*, which has a high midpoint potential and was therefore previously assumed to be cytochrome c_H, was in fact the cytochrome *c* component of the integral membrane oxidase, cytochrome *co*. This cytochrome differs from the other membrane-bound cytochrome *c* which appears to be identical with the soluble cytochrome c_L.

Prior to solubilization of membrane cytochromes, the membranes were always washed with 1 M-KCl; this would suggest, therefore, that the binding of cytochrome c_L to the membrane is stronger than would be expected for a typical periplasmic protein. Furthermore, the results presented above show that the cytochrome c_L that is bound tightly to the membrane is unlikely to be more important than the soluble cytochrome c_L in mediating electron transport between methanol dehydrogenase and the terminal oxidase. These observations perhaps suggest, therefore, that the membrane-bound cytochrome c_L plays a role in the electron transport chain for oxidation of NADH by way of cytochrome *b*; this role is played in mitochondria and *Pa. denitrificans* by a distinct membrane-bound cytochrome c_1, a cytochrome for which (except in *Pa. denitrificans*) there is no evidence in methylotrophic bacteria. Such a role would explain why cytochrome c_L is present in all the methylotrophs reported in Table 1 in all growth conditions.

Although the soluble cytochrome c_H is the preferred electron donor to cytochrome *co* (Froud & Anthony, 1984), it is not known if it is also an electron donor to cytochrome aa_3. If it is, then it may be at a branch point, mediating between soluble cytochrome c_L and the terminal oxidases during methanol oxidation, and also between the cytochrome bc_L complex and the terminal oxidases during NADH oxidation. In this context it should be noted that the soluble

cytochromes *c* are able to interact with each other *in vitro*, and that all substrates are able to reduce all of the cytochrome *c* present in whole cells of *M. methylotrophus* (Cross & Anthony, 1980; Burton *et al.*, 1983).

This being the case, it is difficult to imagine how either of the oxidases could be completely specific for the cytochrome chains responsible for oxidation of methanol or of NADH. This is particularly so when it is considered how the nature of the terminal part of the electron transport chains of *M. methylotrophus* varies according to the growth conditions. During growth in methanol-excess conditions (O_2- or NH_3-limited continuous culture, or exponential batch culture) the *o*-type oxidase (cytochrome *co*) is the sole oxidase; by contrast, during growth with methanol as limiting carbon source (in continuous culture or late phases of batch culture) both cytochrome *co* and cytochrome aa_3 are present (Cross, 1980; Cross & Anthony, 1980; Dawson & Jones, 1981). These observations rule out the possibility discussed by Dawson & Jones (1981) that *M. methylotrophus* contains two respiratory chains with functionally distinct pools of cytochrome *c* such that NADH and methanol are oxidized via different cytochrome oxidases. These observations also argue against the conclusion of Carver & Jones (1984) that the cytochrome *co* complex may be specifically involved in the oxidation of NADH and various flavin-linked substrates.

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