Biochimica et Biophysica Acta, 1099 (1992) 1–15

BBABIO 43546

Review

The *c*-type cytochromes of methylotrophic bacteria

Christopher Anthony

Department of Biochemistry, University of Southampton, Southampton (U.K.)

(Received 16 July 1991)

Key words: Cytochrome *c*; Methylotrophic bacterium; Electron transport

Contents

Abbreviations: MDH: methanol dehydrogenase; MNDH: methylamine dehydrogenase; MMO: methane mono-oxygenase.

Correspondence: C. Anthony, Department of Biochemistry, University of Southampton, Southampton, SO9 3TU, U.K.

I. Introduction

Methylotrophic bacteria are known to contain high concentrations of *c*-type cytochromes and this review will provide a survey of the structure and functions of

TABLE I

The methylotrophs discussed in this review

All grow at pH 7.0 except for the acidophilic *Acetobacter methanolicus* which grows at pH 4.0. The pathways and general biochemistry of these bacteria is fully described in Ref. 1. RuMP, ribulose monophosphate pathway; SerP, serine pathway; RuBP, ribulose bisphosphate pathway.

Organism	Type	C_1-Substrate	Assimilation pathway	Assimilation substrates
Methylococcus	obligate	methane	RuMP	HCHO
Methylophilus	obligate	methanol, methylamine	RuMP	HCHO
Organism 4025	obligate	methanol, methylamine	RuMP	HCHO
Acetobacter	facultative	methanol	RuMP	HCHO
Methylobacterium	facultative	methanol, methylamine	SerP	$HCHO + CO_2$
Hyphomicrobium	facultative	methanol, methylamine	SerP	$HCHO + CO_2$
Paracoccus	facultative	methanol, methylamine	RuBP	CO_2

the various types, concentrating on those having some special significance in these bacteria.

Methylotrophic bacteria grow at the expense of reduced carbon compounds containing one or more carbon atoms but containing no carbon–carbon bonds; some are obligate methylotrophs and are unable to grow on any other substrates whereas facultative methylotrophs may be typical heterotrophs that are also able to grow on these special compounds. Methylotrophic bacteria that grow on methane are also called methanotrophs and these usually grow on no other substrate except (in some strains) methanol. Most other methylotrophs grow on methanol or methylated amines (or both); other C_1 substrates, used by relatively few methylotrophs, include formate, formamide, CO, dimethylsulphide or trimethylsulphonium compounds (see Ref. 1 for a comprehensive account of the biochemistry of methylotrophs).

There are two main aspects of the metabolism of methylotrophs that have bioenergetic relevance; these are the special systems for oxidising their exceptionally reduced substrates, and the special biosynthetic pathways involved in assimilating them. There are four different assimilation pathways and these differ with respect to the amount of NADH and ATP required (or produced) in them. Table I lists the bacteria whose metabolism is mainly discussed in this work to illustrate the range of different types of bacteria used.

Fig. 1 summarises the pathway for oxidation of the main substrates used by methylotrophs; it illustrates the important point that these substrates are more reduced than typical multicarbon substrates, and they must first be oxidised to formaldehyde which is at the oxidation level of cell material (CH_2O); formaldehyde is then either assimilated or oxidised to CO_2. Methane is oxidised to methanol by methane monooxygenase, which requires NADH as reductant [2]; these bacteria will be reductant-limited rather than ATP-limited which is the case with most other bacteria [2]. Every molecule of growth substrate must be oxidised by way of methanol dehydrogenase (MDH) or methylamine dehydrogenase (MNDH), including those that are eventually assimilated into cell material. These considerations lead to an important general conclusion: that the electron transport chain from NADH is relatively less important and that between 50 and 90% of the oxygen consumed by the terminal oxidases during methanol or methylamine oxidation is for the oxidation of MDH or MNDH [1,2]. These dehydrogenases are both NAD^+-independent quinoproteins; the prosthetic group of MDH is PQQ and that of MNDH is tryptophyl-tryptophan [3]. These unusual dehydrogenases are

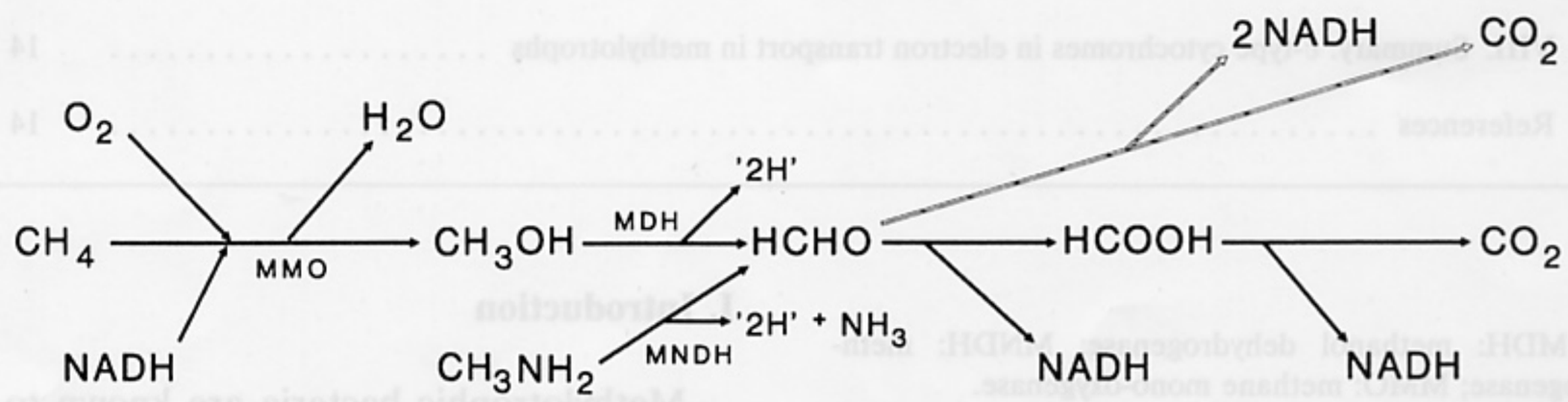

Fig. 1. The oxidation of C_1-compounds by methylotrophic bacteria. Methane is oxidised to methanol by methane monooxygenase (MMO); methanol is oxidised by a quinoprotein methanol dehydrogenase (MDH) and methylamine is oxidised by a second type of quinoprotein, methylamine dehydrogenase (MNDH); formaldehyde is oxidised either by way of formate or by a cyclic mechanism [1,2].

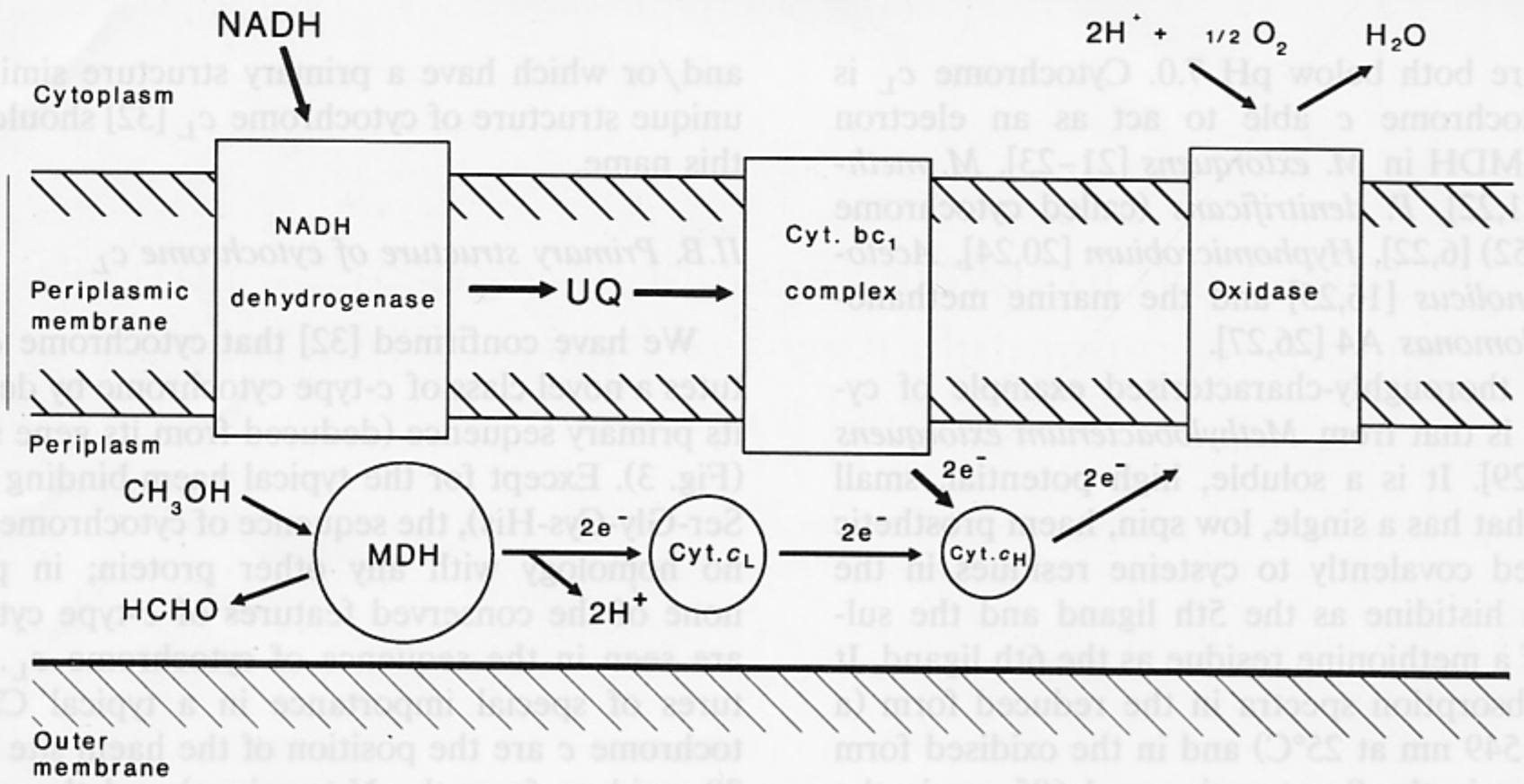

Fig. 2. The electron transport chain for methanol oxidation. The sizes of MDH, cytochromes, periplasmic membrane and periplasm are more or less to scale if the distance between the membranes is 70 nm [5] (but see Ref. 15). Reaction of MDH with cytochrome c_L (in two successive steps) releases two protons into the periplasm, and two protons (per two electrons) are consumed on the cytoplasmic side of the oxidase, thus establishing a protonmotive force.

the basis of the unusual electron transport chains in methylotrophic bacteria [1,2,4]. They are unique in bacteria that are not chemolithotrophs; the 'methanol oxidase' and 'methylamine oxidase' systems bypass the low redox potential part of the electron transport chain that usually involves ubiquinone and the cytochrome bc_1 complex.

The main reasons that *c*-type cytochromes are important in methylotrophs relate to these special systems, especially that for methanol oxidation (Fig. 2). Except for the membrane-bound oxidase the whole chain is soluble, within the periplasm [2,5,6]. The electron acceptor for MDH is a specific cytochrome *c*, called cytochrome c_L, which passes electrons indirectly to the oxidase by way of a typical high-potential Class I *c*-type cytochrome (see Section V for a discussion of this class of cytochromes). This electron transport chain has been confirmed in vitro by reconstitution using the pure soluble dehydrogenases and cytochromes, and solubilised oxidases from *Methylophilus methylotrophus* [7], organism 4025 [8] and *Methylobacterium extorquens* [9]. Reaction of MDH with cytochrome c_L releases protons into the periplasm, and protons are consumed by the oxidase reaction on the cytoplasmic side of the membrane, thus establishing a protonmotive force which drives ATP synthesis, giving a P/O ratio of one or less [4,10–14].

In addition to the cytochromes indicated in Fig. 2, there are other periplasmic cytochromes *c* that have no known function; these are usually present at low concentrations but they may be induced in some growth conditions or in some mutants that lack the normal cytochromes. The *c*-type cytochromes found in the periplasmic membrane include the cytochrome *c* that forms part of the cytochrome bc_1 complex, and the cytochrome *c* component of the *o*-type oxidase (cytochrome *co*) that is found in some methylotrophs.

This review will provide a complete account of the biochemistry of the specific cytochrome c_L. This will be followed by a less detailed survey of the structure and function of other periplasmic *c*-type cytochromes, including their roles in methylamine oxidation. The membrane *c*-type cytochromes will then be briefly discussed and the review will conclude with a brief overall summary of the roles of *c*-type cytochromes in electron transport.

II. Cytochrome c_L, the electron acceptor for methanol dehydrogenase

II-A. Introduction

All methylotrophic bacteria that have been investigated contain at least two soluble, periplasmic, *c*-type cytochromes [2,6,16–20]. They were originally labelled according to their isoelectric points, cytochrome c_H having the higher isoelectric point and cytochrome c_L the lower [17]. Although applicable to the cytochromes from *Methylobacterium*, *Methylophilus* and *Hyphomicrobium*, because their isoelectric points differ by about 4 pH units, the distinction is not so marked for the cytochromes from *Methylomonas* and *Paracoccus*. Their cytochromes *c* with the lower isoelectric points correspond to the cytochromes c_L from other methylotrophs in their function, but the differences in p*I* are not so great and the p*I* values for the cytochromes

c_H and c_L are both below pH 7.0. Cytochrome c_L is the only cytochrome *c* able to act as an electron acceptor for MDH in *M. extorquens* [21–23], *M. methylotrophus* [21,22], *P. denitrificans* (called cytochrome *c*-551$_i$ or *c*-552) [6,22], *Hyphomicrobium* [20,24], *Acetobacter methanolicus* [16,25] and the marine methanotroph *Methylomonas* A4 [26,27].

The most thoroughly-characterised example of cytochrome c_L is that from *Methylobacterium extorquens* AM1 [17,28,29]. It is a soluble, high potential, small cytochrome that has a single, low spin, haem prosthetic group, bonded covalently to cysteine residues in the protein, with histidine as the 5th ligand and the sulphur atom of a methionine residue as the 6th ligand. It has typical absorption spectra in the reduced form (a maximum at 549 nm at 25°C) and in the oxidised form (about 410 nm in the Soret region and 695 nm in the near infra red). In these features cytochrome c_L is similar to typical Class I *c*-type cytochromes [30,31]. It differs in being larger (17–21 kDa) and (usually) in having a low isoelectric point (3.5–4.5); these properties reflect the fact that the primary sequence of the cytochrome is completely different from any other *c*-type cytochrome (see below).

The cytochromes c_L from other methylotrophs are very similar to those from *Methylobacterium* and their properties are summarised in Table II. The nomenclature of some of these cytochromes is confusing and the names (e.g., cytochrome *c*-551) convey little; the difference between an α-band at 550 and 552 nm is small and unreliable as a means of identification. I propose that all *c*-type cytochromes that react with MDH and/or which have a primary structure similar to the unique structure of cytochrome c_L [32] should be given this name.

II.B. Primary structure of cytochrome c_L

We have confirmed [32] that cytochrome c_L constitutes a novel class of *c*-type cytochrome by determining its primary sequence (deduced from its gene sequence) (Fig. 3). Except for the typical haem-binding site (Cys-Ser-Gly-Cys-His), the sequence of cytochrome c_L shows no homology with any other protein; in particular, none of the conserved features of *c*-type cytochromes are seen in the sequence of cytochrome c_L. Key features of special importance in a typical Class I cytochrome *c* are the position of the haem site (less than 20 residues from the N-terminus) and the position of the sixth ligand methionine which is usually about 60 residues towards the C-terminal from the haem-binding histidine [30,31,39,40]. In cytochrome c_L there are about 60 residues between the N-terminal and the haem binding site and all three methionines in this cytochrome are within 50 residues of this histidine; furthermore, the sequences around the methionines bear no relation to those around the methionines of other *c*-type cytochromes. The position of many lysine residues in Class I *c*-type cytochromes are highly conserved and arranged around the haem pocket; these are of particular importance in binding to the cytochrome bc_1 complex and cytochrome aa_3 [31]. That there is no homologous arrangement of lysines in cytochrome c_L is not surprising because this cytochrome

TABLE II

Properties of cytochrome c_L

For the purpose of this table, cytochromes are considered to be cytochrome c_L if reaction with MDH has been demonstrated. The *Methylobacterium* species referred to here is *M. extorquens* AM1 (previously called Pseudomonas AM1), the *Methylophilus* species is *M. methylotrophus*, the *Paracoccus* species is *P. denitrificans* and the *Acetobacter* species is *A. methanolicus*. [a] this cytochrome often produces a 17 or 19 kDa form which remains active with MDH; [b] although having a low M_r, this cytochrome is the only one showing any activity with MDH and it has some similarity in its N-terminal sequence. Other methylotrophs having 2 *c*-type cytochromes, one of which might be cytochrome c_L but which have not been tested with MDH or compared in terms of sequence are summarised in Ref. 20.

Organism	Cytochrome synonyms	Molecular mass (kDa)	p*I*	E_{m7} (mV)	Absorbance of ferrocytochrome				Reaction with MDH	References
					Absorbance maxima (nm)		Ext. coeffs. (mM cm^{-1})			
					α	γ	α	γ		
Methylobacterium		18.74	4.2	256	549	416	21.8	64.5	+	17, 22, 28, 32, 33
Methylophilus		21 [a]	4.2	310	550	416	26.9	139	+	18, 22, 29
Hyphomicrobium X		19.5	4.3	270	550	414	21.6	n.d.	+	20, 24, 34
Paracoccus	*c*-551$_i$, *c*-552	22	3.5	190	551.6	416	23.1	138	+	6, 35–37
Acetobacter		21	4.9	324	550	415	23.3	134	+	16, 25
Organism 4025		18	3.8	284	550	417	n.d.	n.d.	+	38
Methylomonas A4	*c*-552 [b]	14	4.7	n.d.	552	417	24.0	n.d.	+	26, 27

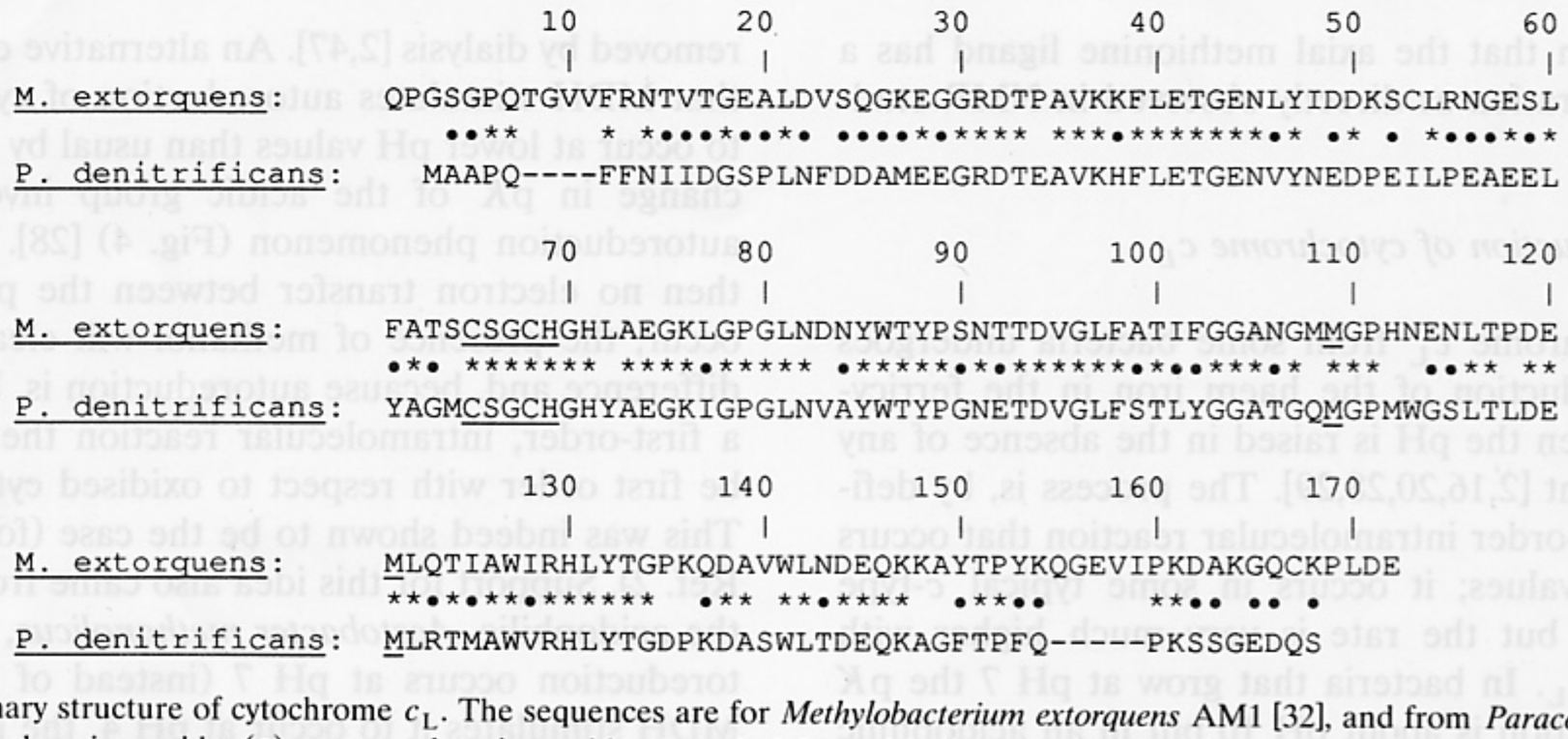

Fig. 3. The primary structure of cytochrome c_L. The sequences are for *Methylobacterium extorquens* AM1 [32], and from *Paracoccus denitrificans* [42]. (*) identical amino acids; (•) conserved amino acids. A typical haem-binding site (CXXCH) is underlined and conserved methionine residues that might be the 6th ligand are also underlined. These sequences do not include the typical N-terminal signal peptides shown to be present and typical of proteins that must be exported into the periplasm.

is not a substrate for the oxidase; its function is to mediate between MDH and the typical Class I cytochrome c_H [4,23,41].

II-C. The haem environment of cytochrome c_L, and its reaction with CO

The cytochrome c_L of many methylotrophs is able to react with CO. Such a reaction has been used to indicate an oxidase function [43], but there is no evidence that this cytochrome functions as an oxidase [2]. The slow, incomplete reaction with CO probably reflects the structure around the haem pocket that allows a more readily-dissociable iron-methionine bond [17,44]. That the haem environment is slightly unusual is indicated by the unusual response of the midpoint redox potential to changing pH values; there are two ionizing groups affecting redox potentials, the pK values being 3.5 and 5.5 in the oxidised form and 4.5 and 6.5 in the reduced form [17]. If these dissociations arise from the haem, then the higher of the pK values is likely to be due to the inner haem propionate in the hydrophobic environment of the haem cleft, and the lower pK values due to the outer propionate in its more hydrophilic environment. The suggestion that the haem environment may be unusual is supported by the

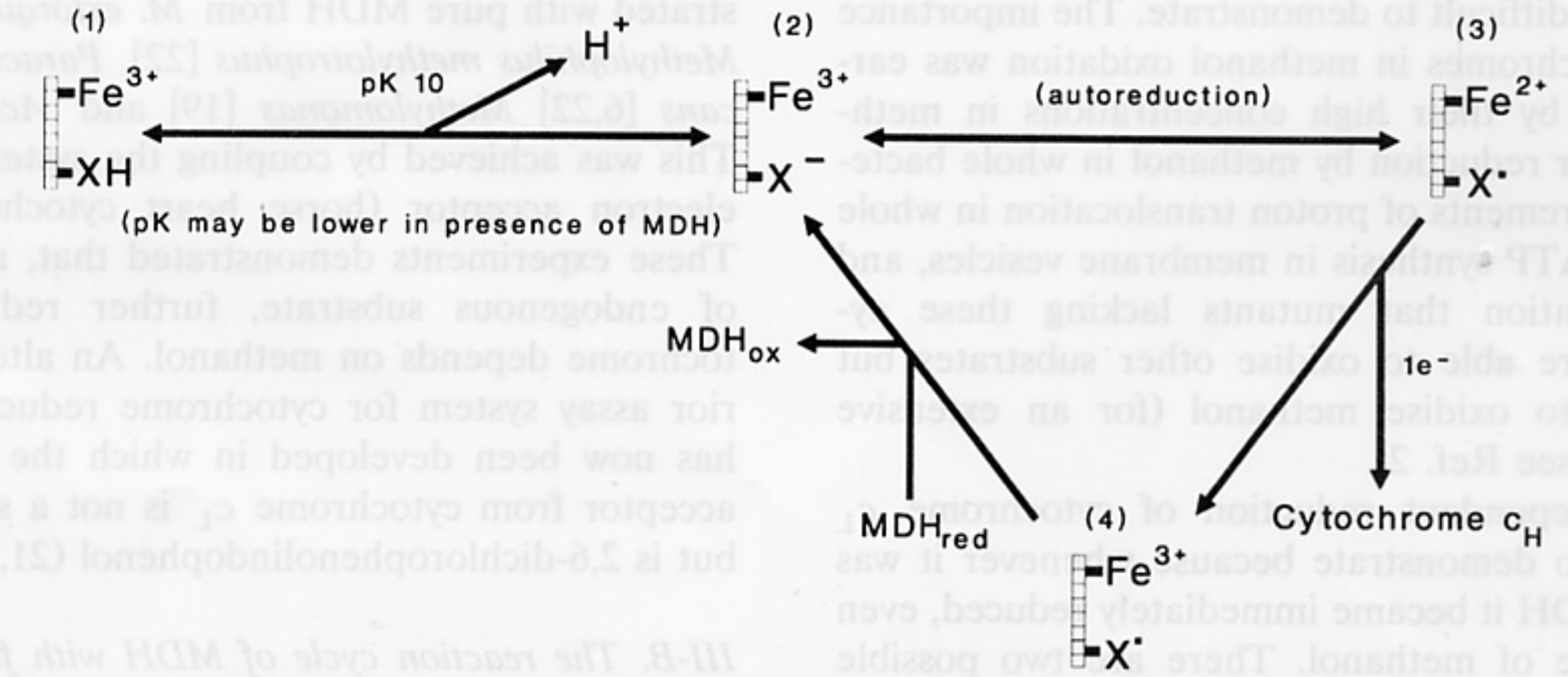

Fig. 4. A speculative mechanism for involvement of autoreduction of cytochrome c_L in the reaction with MDH. This figure is based on Refs. 28 and 29. In this scheme the electron donor for autoreduction is a weakly acidic group (XH) on the cytochrome that dissociates to give a negatively-charged species able to donate an electron to the haem. Species (1) is the undissociated ferricytochrome which dissociates on binding MDH (or at high pH) to species (2). Species (3) is the the radical complex of ferrous iron, isoelectronic with the ferric species (2). Species (4) is the ferric form of the radical and can only be produced in the presence of electron acceptor. Species (4) is reduced to (3) by reduced MDH. There is now considerable doubt that the autoreduction process is involved in electron transfer processes (i.e. species 4 may not be produced) (see text).

demonstration that the axial methionine ligand has a novel configuration as directly observed in NMR studies [45,46].

II-D. Autoreduction of cytochrome c_L

The cytochrome c_L from some bacteria undergoes rapid autoreduction of the haem iron in the ferricytochrome when the pH is raised in the absence of any reducing agent [2,16,20,28,29]. The process is, by definition, a first-order intramolecular reaction that occurs at high pH values; it occurs in some typical *c*-type cytochromes, but the rate is very much higher with cytochrome c_L. In bacteria that grow at pH 7 the pK for autoreduction is about pH 10 but in an acidophilic methylotroph, *A. methanolicus*, the pK is about pH 7 [16]. It has been proposed that the mechanism involves dissociation of a weakly acidic group which dissociates on raising the pH to give a negatively charged species able to donate an electron to the haem, the free radical produced by this process being stabilized by sharing an electron with the haem iron (Fig. 4) [2,28]. As discussed below this mechanism may be involved in the MDH–cytochrome interaction. Whether or not this is the case, the phenomenon of autoreduction in this cytochrome is an intriguing characteristic.

III. Reaction of MDH with its cytochrome electron acceptor

III-A. Introduction

It was known for some years that MDH interacts with the electron transport chain at the level of the *c*-type cytochromes, but a direct, methanol-dependent interaction between MDH and a *c*-type cytochrome was extremely difficult to demonstrate. The importance of *c*-type cytochromes in methanol oxidation was earlier indicated by their high concentrations in methylotrophs, their reduction by methanol in whole bacteria, the measurements of proton translocation in whole bacteria and ATP synthesis in membrane vesicles, and the demonstration that mutants lacking these cytochromes were able to oxidise other substrates but were unable to oxidise methanol (for an extensive review of this see Ref. 2).

Methanol-dependent reduction of cytochrome c_L was difficult to demonstrate because whenever it was mixed with MDH it became immediately reduced, even in the absence of methanol. There are two possible explanations for this. The first is that the endogenous reductant on MDH provides electrons for reduction of the cytochrome in the absence of methanol. The presence of this endogenous reductant is well-known but not understood; each molecule of MDH has about 90 molecules of unidentified reductant which cannot be removed by dialysis [2,47]. An alternative explanation is that MDH stimulates autoreduction of cytochrome c_L to occur at lower pH values than usual by stimulating a change in pK of the acidic group involved in the autoreduction phenomenon (Fig. 4) [28]. If this is so, then no electron transfer between the proteins need occur, the presence of methanol will clearly make no difference and, because autoreduction is, by definition, a first-order, intramolecular reaction the kinetics will be first order with respect to oxidised cytochrome c_L. This was indeed shown to be the case (for review, see Ref. 2). Support for this idea also came from work with the acidophilic *Acetobacter methanolicus*, in which autoreduction occurs at pH 7 (instead of pH 10), and MDH stimulates it to occur at pH 4, the growth pH of the organism [16]. Against this explanation, however, is the fact that first-order kinetics are difficult unequivocally to demonstrate, and that MDH-stimulated reduction of cytochrome c_L can be demonstrated with proteins of *Hyphomicrobium* at a rate that is considerably greater than the rate of autoreduction of the cytochrome c_L of this organism [20]. Furthermore, these authors have shown that in *Hyphomicrobium* cyclopropanol-inactivated MDH does not reduce ferricytochrome c_L in the absence of methanol, suggesting that electron transfer is required, and not merely binding of MDH to stimulate autoreduction. On balance, therefore, it is appearing more probable that MDH-catalysed reduction of cytochrome c_L in the absence of methanol may be due to the endogenous reductant.

Methanol-dependent reduction of *c*-type cytochromes was eventually demonstrated using anaerobically-prepared crude extracts of *Hyphomicrobium* X [48] and of *M. extorquens* [28]; and methanol-dependent reduction of the pure cytochrome with concomitant formaldehyde production has now been demonstrated with pure MDH from *M. extorquens* AM1 [22], *Methylophilus methylotrophus* [22], *Paracoccus denitrificans* [6,22] *Methylomonas* [19] and *Acetobacter* [25]. This was achieved by coupling the system to a second electron acceptor (horse heart cytochrome *c*) [22]. These experiments demonstrated that, after oxidation of endogenous substrate, further reduction of cytochrome depends on methanol. An alternative, superior assay system for cytochrome reduction by MDH has now been developed in which the final electron acceptor from cytochrome c_L is not a second protein but is 2,6-dichlorophenolindophenol (21,25].

III-B. The reaction cycle of MDH with ferricytochrome c_L as electron acceptor

Despite the unequivocal evidence of the specific, direct, reaction of cytochrome c_L with MDH, considerable discussion of the significance of these results has occurred because the rates measured were too slow to

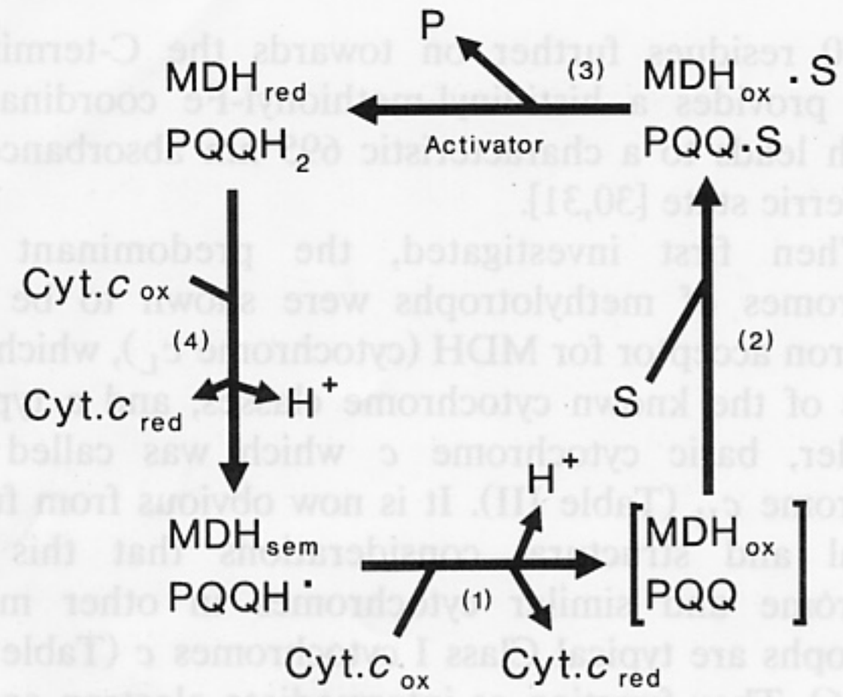

Fig. 5. The reaction of MDH and cytochrome c_L. This figure is based on the work of Dijkstra, Frank and Duine [34]. Cyt c_{ox} and Cyt c_{red} are the oxidised and reduced forms of cytochrome c_L. The substrates used in this work were fully-reduced MDH (MDH_{red}, containing fully-reduced quinol, $PQQH_2$), and MDH as it is normally isolated (MDH_{sem}, containing half-reduced PQQ, $PQQH^{\cdot}$). The oxidation of methanol (S) to formaldehyde (P) during this cycle releases two protons. It is obvious from consideration of this cycle that the cytochrome must be released after reduction in step (1) and bound again to the MDH for step (4). In this diagram it is implied that substrate is bound to the prosthetic group (PQQ.S) in the enzyme-substrate complex (MDH_{ox}.S); the evidence for this is not at present substantial (see Ref. 50).

account for the rate of respiration in bacteria [22,34]. Some explanation for this has been presented by Dijkstra, Frank and Duine [34] who have analysed the separate steps in the methanol; cytochrome c_L oxidoreductase reaction using steady-state and stopped-flow kinetic techniques, and studies of isotope effects using deuterated methanol (see Fig. 5). They showed that ferricytochrome c_L is an excellent oxidant of reduced MDH at pH 7, but the substrate oxidation step is very slow, and there was little activation by ammonia (the activator in the dye-linked MDH assay). By contrast, at pH 9 the cytochrome is such a poor oxidant that the activation of the substrate oxidation step that does now occur, becomes irrelevant. It was concluded that the relatively slow overall rate is because of the need for an activator for the substrate oxidation step. It was suggested that this might be the low M_r, oxygen-labile component described previously [49], but this was not tested in this system.

III-C. Analysis of the MDH / cytochrome c_L interaction by chemical modification of the proteins

It has been suggested previously that the interaction of MDH with cytochrome c_L is likely to be electrostatic in nature, involving ionic interactions between lysine or arginine residues on MDH and carboxyl residues on the cytochrome [51,52]. It was speculated that the lysine residues that form such a well-defined pattern in predictions of the secondary structure of the β-subunit of the MDH of *M. extorquens* might be involved in 'docking' with carboxyls of cytochrome c_L which might also be involved in interaction with lysines of the binding domain of the typical Class I cytochrome, cytochrome c_H [51,52]. That ionic interactions are involved has now been demonstrated using the two proteins from *M. extorquens*, *M. methylotrophus* and *Acetobacter methanolicus*, the reaction being strongly inhibited by low concentrations of salts. The extent of inhibition was directly related to the square root of the ionic strength of the medium, NaCl acting by decreasing the affinity of the cytochrome for MDH [21,25].

That lysine residues on MDH are involved in 'docking' with carboxyl groups on the cytochrome was indicated by chemically modifying MDH. The modified MDH retained activity in the dye-linked assay systems showing that the active site for reaction with substrate had not been altered. It was shown that reagents which change the charge on lysines led to inactive MDH, whereas those that modified MDH with retention of charge had relatively little affect. The inhibition by reagents specific for arginine residues suggests that these may also be involved. When cytochrome c_L was modified with lysine-modifying reagents its activity was retained, but those reagents that modified carboxyl groups led to greatly diminished activity [21,25].

III-D. Analysis of the MDH / cytochrome c_L interaction by cross-linking studies

Although, initially, our attention was drawn to the potential importance of lysines in the interaction by the high proportion of lysine residues in the β-subunit of the MDH of *M. extorquens* [51], it appears that these lysines are unlikely to be involved in 'docking' with cytochrome c_L because the small β-subunit from the acidophilic methylotroph *A. methanolicus* does not have those lysines thought to be important in the 'docking' process [25]. It now appears that the lysines on the larger α-subunit are involved. The evidence for this comes from cross-linking the two proteins using 'zero-length' cross-linking agents recently described by Grabarek and Gergely [53]. When carboxyl groups on the cytochrome are modified and then attacked with the unmodified MDH, lysine residues on the MDH displace the reagent from the cytochrome and form isopeptide bonds with its carboxyl groups. Using proteins from three different methylotrophs these experiments demonstrated in every case, that carboxyl groups on cytochrome c_L interact only with lysine residues on the larger α-subunit of MDH [21,25].

As illustrated in Fig. 2, MDH, cytochrome c_L, cytochrome c_H and the membrane oxidase form a complete electron transport chain, catalysing the oxidation

of methanol by molecular oxygen. The three periplasmic proteins of this chain may either operate as separate entities, forming short-lived bimolecular complexes during which electron transfer occurs, or they might form stable complexes of more than two proteins in a 'wire' system (see Refs. 5 and 54). In such a system electrons would flow from MDH through cytochrome c_L to cytochrome c_H, the site of entry of electrons into cytochrome c_L being different from the site of exit. If cytochrome c_L has only a single site for electron transfer, dissociation from MDH must occur prior to reaction with the typical Class I cytochrome *c*, and it should thus be possible to demonstrate cross-linking between the two cytochromes. This, indeed, has been demonstrated in conditions where the carboxyl groups of cytochrome c_L are modified and lysine residues on the second cytochrome are involved in forming the isopeptide bond [21]. By contrast, no cross-linking occurred when the carboxyls of the Class I cytochrome were modified for the reaction. Furthermore, it proved impossible to cross-link the three proteins in a ternary complex together; MDH and cytochrome c_H competed for the cytochrome c_L. These results all suggest that cytochrome c_L has a single site by which carboxyl groups are involved in 'docking' with lysyl groups on MDH and cytochrome c_H, and by which electron transfer occurs [21].

IV. The Class I *c*-type cytochromes of methylotrophs, including cytochrome c_H

In 1982, Ambler recognised four sequence classes of *c*-type cytochrome [39,40]. Class I includes the classical soluble cytochromes *c* of mitochondria and bacteria, with the haem-attachment site towards the N-terminus, and the 6th ligand provided by a methionine ligand 45–70 residues further on towards the C-terminus. This provides a histidinyl-methionyl-Fe coordination which leads to a characteristic 695 nm absorbance in the ferric state [30,31].

When first investigated, the predominant cytochromes of methylotrophs were shown to be the electron acceptor for MDH (cytochrome c_L), which fits none of the known cytochrome classes, and a typical smaller, basic cytochrome *c* which was called cytochrome c_H (Table III). It is now obvious from functional and structural considerations that this cytochrome and similar cytochromes in other methylotrophs are typical Class I cytochromes *c* (Table III, Fig. 6). They function as intermediate electron acceptors between cytochrome c_L and the oxidase as indicated in Fig. 2. Although they fulfil the same function, they do not all fall into the same structural subclasses, however; for example, the best known in terms of structure is the cytochrome *c*-550 of *P. denitrificans* which falls into Class IA (also called cytochrome c_2 [39,40]), whereas the cytochrome c_H of *Methylophilus* is Class IB which is a major category that Ambler suggests should be called cytochrome c_8 [39,40]. From the similarity of structure of the Class I cytochromes it is probable that they all have similar three-dimensional structures and will interact in similar fashions with protein electron donors and acceptors. For an outstanding review of all aspects of the structure and function of *c*-type cytochromes, the encyclopaedias of Moore and Pettigrew [30,31] should be consulted.

By analogy with the mitochondrial Class I cytochrome *c*, it is reasonable to assume that these cytochromes fulfil the same function in bacteria; that of mediating electron transfer between the membrane complexes, cytochrome bc_1 and the oxidase. However, a more general description of their function is that they

TABLE III

Properties of Class I c-type cytochromes of methylotrophs (cytochrome c_H)

This table includes Class I *c*-type cytochromes which mediate between cytochrome c_L and the oxidase (often called cytochrome c_H in methylotrophs). M_r values are from SDS-PAGE. The sequence classes given here are those suggested by Ambler [39,40]. Class IB cytochromes *c* are similar to typical mitochondrial cytochrome *c*, Class ID is a class of smaller cytochromes *c*, and Class IA cytochromes *c* have extra loops in their sequences compared with the Class IB cytochromes *c*. [a] It was suggested that cytochrome *c*-554 is equivalent to cytochrome c_H but its N-terminal sequence does not support this. n.d., not determined.

Organism	Synonym	M_r	p*I*	E_{m7} (mV)	Absorbance of ferrocytochrome				Reaction with oxidase	Sequence class	References
					Absorbance maxima (nm)		Ext. coeffs. (mM cm^{-1})				
					α	γ	α	γ			
Methylobacterium		11	8.8	294	550.5	416.5	31	162	+	IB	17, 28, 29, 33
Methylophilus		8.5	8.9	373	551	416	27.1	128	+	ID	7, 18, 28, 39
Hyphomicrobium X		14	7.4	292	550.6	414	23.7	n.d.	n.d.	n.d.	20
Paracoccus	*c*-550	15	4.5	253	550	415	30.2	148	+	IA	6, 35–37, 58–62, 99
Acetobacter		9	5.8	224	551	417	32.9	160	+	n.d.	16, 25
Organism 4025		12.5	9.4	n.d.	550	416	n.d.	n.d.	+	n.d.	38
Methylomonas A4	*c*-554 [a]	8.5	5.6–6.4	n.d.	554	418	21.3	n.d.	n.d.	n.d.	26, 27

```
                    *     *        *   **                    **         *
Methylophilus:       ADAAAA-K ALAQKSGCLA CHSID----- -----AK-VL GP-AYKDVAA KYKGDKGA-EA K-----LIEK
Tuna:                GDVAKGKK TFVQK--CAQ CHTVE----- ---NGGKHKV GPNLWGLFGR KT-GQAEG-Y- SYTD------
Horse heart:         GDVEKGKK IFVQK--CAQ CHTVE----- ---KGGKHKT GPNLHGLFGR KT-GQAPG-F- TYTD------
Methylobacterium:  EGDAAAGEK AF-AP--CKA CHNFE----- ---KN---GV GPTLKGVVGA KA-GEGGADY- AFSD------
Paracoccus:       QDGDAAKGEK EF-NK--CLA CHMIQAPDGT DIIKGGK--T GPNLYGVVGR KI-ASEEG-F- KYGEGIL-E-

                    *  *                          *
Methylophilus:     V--KKGGSGV W--------- --------GN -----IP---M -PANSPQVKD E--DIKTVIE WILTL
Tuna:              -ANKSKGI-V WNENTLM--- -----EYLEN P-KKYIPGTKM IFA-GIKKKG ERQDLVAYLK SATS
Horse heart:       -ANKNKGI-T WKEETLM--- ------YLEN P-KKYIPGTKM IFA-GIKKKT EREDLIAYLK KATNE
Methylobacterium: -ALKKSGL-T WDQADLK--- -----QWLAD P-KKKVPGTKM VFP-GISDPK KVDDIIAYLK TKS
Paracoccus:        VAEKNPDL-T WTEADLIEYV TDPKP-WLVK MTDDKGAKTKM TFKMGKMQAD VVAFLAQNSP DAGGD GEAAA EGESN
```

Fig. 6. Primary sequences of methylotrophic Class I *c*-type cytochromes. The sequence of cytochrome c_H from *Methylobacterium* is of a preliminary sequence (personal communication, R.P. Ambler and M. Athelye). The cytochrome c_H of *Methylophilus methylotrophus* is from Ref. 39 and the cytochrome *c*-550 sequence of *Paracoccus denitrificans* is from Refs. 55 and 99. The sequences from Bonito (tuna) [56] and horse heart [57] are given for comparison with two typical Class IB mitochondrial *c*-type cytochromes. According to Ambler's categories [39,40], the *M. extorquens* cytochrome is in this same class, the *M. methylotrophus* cytochrome is typical of Class ID (or cytochrome c_8) containing very small cytochromes having several proline residues around the methionine ligand; and the *P. denitrificans* cytochrome is a typical Class IA (or cytochrome c_2), having extra loops compared with the Class IB cytochrome (see Refs. 30 and 31 for alternative nomenclature for such sub-classes). The sequences are aligned to indicate maximum identity (* indicates residues that are identical in all five cytochromes). The haem binding sites and 6th methionine ligands are underlined.

mediate electron transfer between periplasmic redox proteins, from membrane complexes to these proteins, and from periplasmic redox proteins to membrane complexes (see Refs. 54 and 64).

Besides the bacteria listed in Tables II and III, other methylotrophs have multiple soluble *c*-type cytochromes, one of which is likely to be cytochrome c_L and the other likely to be a Class I cytochrome *c* filling the function of cytochrome c_H; these have not been sufficiently well-characterised with respect to primary sequence or function to identify them with certainty; they include the obligate methanol-utilisers *Methylomonas* J [19] and *Methylomonas* YK56 [65], and the marine methanotroph *Methylomonas* A4 [26,27].

V. Other soluble *c*-type cytochromes of methylotrophs

Most methylotrophs whose cytochromes have been studied in any detail have been shown to have more than the two main cytochromes shown in Fig. 2 and summarised in Tables II and III. Some of the more interesting examples are listed in Table IV. Most of these are minor components of no known function or whose function is uncertain. Some are induced during

TABLE IV

Other soluble c-cytochromes of methylotrophs

The cytochrome *c*-553 of *P. denitrificans* is induced during growth on methanol and is thus also called cytochrome *c*-553$_i$ [35]. The peroxidase of *P. denitrificans* [64,66] was previously identified as the 45 kDa cytochrome induced during growth in oxygen-deficient conditions [6,37]. When the p*I* is recorded as 'low' this indicates that the cytochrome bound to an anion-exchange column. The 46 kDa heterodimer was produced only in the periplasm of the 'Oxford' strain of *P. denitrificans* [6]. Absorbance values are for the ferrocytochrome *c* at 16–25°C.

Organism	Cytochrome	Molecular mass (kDa)	p*I*	E_{m7} (mV)	α-max (nm)	Ext. coeff. (mM cm^{-1})	Reaction with CO	No. of haem	References
Methylobacterium	Cytochrome *c*-553	23	low	194	553	25.3	rapid	1	67
Methylophilus	Cytochrome c_{LM}	16.8	4.6	336	551	25.2	slow	n.d.	18
	Cytochrome *c*″	15	8.7	−60	550	51.3	+	1	45, 46
Paracoccus	Cytochrome *c*-553$_i$	30	3.8	148	553	22.2	rapid	1	6, 35, 36
	Cyt. *c* peroxidase	45	low	128	552, 555	n.d.	+	2	6, 37, 64, 66
	46 kDa heterodimer	46	< 3.8	205	553	64	+	5	6
	30 kDa subunit	30	n.d.	n.d.	552	n.d.	+	3	6
	16 kDa subunit	16	n.d.	188	552	n.d.	+	2	6
	Cytochrome *c*′	12	low	202	550	9.2	n.d.	1	100
Methylomonas A4	Cytochrome *c*-553	34	4.9	n.d.	553	25.0	+	2	26, 27
	Cytochrome *c*-551	16.5	4.8	n.d.	551	28.7	−	2	26, 27

growth on methanol whereas some are only produced in large amounts in mutants. They are all likely to be periplasmic and if able to interact with other cytochromes may be part of a 'network' of redox mediators in the periplasm. Examples of these cytochromes from the three best-known methanol-utilising bacteria are given below.

V-A. Cytochrome c-553 from Methylobacterium extorquens

This cytochrome, which has no known function, constitutes less than 5% of the soluble cytochromes of *M. extorquens* [67] (Table IV). It reacts rapidly and completely with CO but is not autoxidisable, and is not an electron acceptor from methanol dehydrogenase or methylamine dehydrogenase, nor an important donor to the oxidase. It is able to participate in electron transfer reactions with both cytochrome c_H and cytochrome c_L. At first sight it appears to be a slightly larger version of cytochrome c_L and it was thought to be a precursor of this cytochrome because a class of mutants (*moxD*) produced no MDH or cytochrome c_L but produced cytochrome *c*-553 in amounts equal to the amounts of cytochrome c_L usually produced (30% of total cytochrome *c*) [68,69]. It was suggested that the *moxD* mutant might be lacking a processing function that explained three aspects of its phenotype: the lack of MDH, lack of normal cytochrome c_L and the presence of the slightly larger acidic cytochrome *c*-553. It was therefore proposed that this cytochrome might be cytochrome c_L with its signal peptide still attached. That this is not the case is demonstrated by the lack of serological relationship between the two cytochromes, and the markedly different amino acid compositions. That the cytochromes bare no direct relationship is supported by the genetic evidence: cytochrome *c*-553 is still synthesized in a mutant in which the gene has been completely deleted [67]. Cytochrome *c*-553 of *M. extorquens* is not induced to higher levels during methylotrophic growth but it bears some resemblance to cytochrome *c*-553$_i$ of *P. denitrificans* (Table IV), which is induced on methanol and methylamine and is therefore likely to have some (unknown) function in methylotrophic growth.

V-B. Cytochrome c" of Methylophilus methylotrophus

This is a highly unusual small, basic, monohaem cytochrome with a relatively low midpoint potential (−60 mV at pH 7.6) [45,46] (Table IV). It constitutes less than 8% of the total cytochrome *c*, it has no known function and is unrelated to any other cytochrome. There is no haem binding site in the 44 sequenced residues given here (N-terminal):

DVTNAEKLVYKYTNIAHSANPMYEAPSITDGKIFFNRKFKTPSG...

The exceptional characteristic of cytochrome *c″* is that the haem iron is in a high spin state in the reduced form and low spin in the oxidised form;typical difference spectra are not therefore obtained with this cytochrome, and there is no 695 nm absorption band in the ferricytochrome. The iron in the low-spin ferricytochrome is coordinated to two histidine residues, this cytochrome being a unique example of a water-soluble protein which exhibits bis-His coordination with near-perpendicular ligand orientation. On reduction of the cytochrome the spin state changes to high spin and one of the histidine residues is released leaving a single His coordination [46].

V-C. Periplasmic cytochromes of Paracoccus denitrificans

Paracoccus denitrificans is important for the study of cytochromes in methanol oxidation because of the ease with which periplasmic proteins can be isolated; the periplasmic nature of the systems for methanol oxidation was first demonstrated with this organism by Alefounder and Ferguson [70] who also established the periplasmic location of the nitrite reductase in *Paracoccus denitrificans* [71]. It is also important because of its central role, together with *Methylobacterium extorquens*, in the study of the molecular biology of methylotrophs [42,55]. As described above it has a typical cytochrome c_L and Class I cytochrome *c*-550 together with a number of other well-characterised periplasmic cytochromes, some of which may play a role in methylotrophic metabolism; these are described below and summarised in Table IV. Figs. 7 and 8 summarise how some of these cytochromes are involved in electron transport in *P. denitrificans*.

V-C.1. Cytochrome c-553$_i$

The first complete analysis of the periplasmic cytochromes of *P. denitrificans* showed that besides the Class I cytochrome *c*-550 there were two *c*-type cytochromes induced during growth on methanol; these were designated cytochrome *c*-551$_i$ and cytochrome *c*-553$_i$ (the suffix denoting inducibility) [35] (see also Ref. 37). It has now been demonstrated that cytochrome *c*-551$_i$ is equivalent in structure and function to cytochrome c_L [6] (Table II, Fig. 3). The function of the second inducible cytochrome (*c*-553$_i$) is not known. Its main distinguishing feature is its rapid reaction with CO (100% reaction within 1 min) which makes it likely to be the cytochrome responsible for the reaction with CO observed previously by Van Verseveld and Stouthamer and referred to as cytochrome c_{CO} [72]. It may well be the same as cytochrome *c*-553 described in *M. extorquens* [67] (see above).

V-C.2. Heterodimeric, multihaem 46 kDa cytochrome c

This cytochrome has been described only in the 'Oxford strain' of *P. denitrificans* [6]. It constitutes about 25% of the total periplasmic cytochrome. Its spectrum is unusual in the high ratio of absorbance in the a band compared with the β band. It reacts rapidly and completely with CO and has no methionine ligands. Although stable in 1 M NaCl, the dimeric cytochrome can be dissociated into monomers of 30 kDa and 16 kDa by high pH (above pH 11) or by 3 M guanidinium chloride. The dissociated subunits can be reconstituted into the dimer, although the 30 kDa subunit is relatively unstable.

The 30 kDa subunit contains three haems and is a low-spin cytochrome in the oxidised form. It reacts rapidly and completely with CO. Like the cytochrome *c″* of *M. methylotrophus* (above), reduction to the ferrocytochrome changes the spin-state, the spectrum having a poorly defined α peak near 550 nm and no defined β peak. If incubated at pH 11 for more than 30 min, the 30 kDa subunit is converted to a typical low-spin cytochrome and becomes 'damaged' and unable to reconstitute the typical dimeric cytochrome. This subunit differs from cytochrome *c″* in being twice the size, having three times as many haem groups, and in being able to react with KCN at high pH.

The 16 kDa subunit is also an exceptional cytochrome. Like most *c*-type cytochromes its iron is in a low-spin state but it has no methionine ligand to the iron; it is small but appears to have two haem groups, and an unusually high α/β ratio (about 3), which corresponds to the exceptionally high ratio observed in the dimer. Its redox potential was about 20 mV lower than that measured for the dimer. During SDS-PAGE this subunit is able to form a disulphide bridge leading to the presence of a 15 kDa band; this is not produced during sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of mercaptoethanol.

V-C.3. The 150 kDa cytochrome c complex

This large complex has only been described in the 'Oxford strain' [6]. It is produced in small amounts and consists of a non-haem protein (85% of the total protein) plus four haemoproteins (28, 33, 41 and 47 kDa). The extinction coefficient at 552 nm is extraordinarily high (157 mM^{-1} cm^{-1}), consistent with the demonstration that there are 6–7 mol of haem *c* per mol of complex; most of the haem reacts rapidly with CO.

V-C.4. The cytochrome c peroxidase

When grown under oxygen-limiting conditions a 45 kDa cytochrome *c* is produced which contains two haem groups [6,37], and it has subsequently been suggested that this cytochrome is cytochrome *c* peroxidase [64,66]. Its spectrum corresponds to that of other cytochrome *c* peroxidases which also contain two haem *c* groups. Part of the peroxidase remains attached to membranes [66], and this perhaps explains why some cross-reactivity was observed between the 45 kDa protein and antibodies raised to the ubiquinol oxidase complex [37].

V-C.5. Cytochrome c′

In the study of the periplasmic location of cytochrome *c* peroxidase [66], small amounts of a 12 kDa cytochrome with the spectral features of cytochrome *c′* was observed in the periplasmic fraction; this protein has now been purified and characterised [100]. Cytochromes *c′* form the Class IIA group of *c*-type cytochromes; they have no generally-accepted function; they are polypeptides of 125–132 amino acid residues containing a single covalently-bound haem *c* near the C-terminus of the protein [40]. The iron is coordinated by the porphyrin and by a single extraplanar histidine, giving rise to a predominantly high-spin type of spectrum. Although showing the overall spectroscopic features of the cytochrome *c′* family, the *Paracoccus* cytochrome *c′* is unusual in having a red-shifted Soret band in the oxidised form (at 407 nm); the single absorption band at 550 nm in the reduced from is also rather high. It has an exceptionally high midpoint redox potential (202 mV) for this class (−5 mV to 102 mV). The amino acid composition showed the high alanine and proline content characteristic of the group. During gel filtration the cytochrome behaved as a dimer.

V-C.6. Nitrite reductase: cytochrome cd_1

P. denitrificans is able to grow anaerobically on methanol with nitrite or nitrate as alternative to oxygen [73], and this anaerobic metabolism is also likely to be similar to that in *Hyphomicrobium* growing anaerobically on methanol [1]. During this growth nitrite reductase reduces nitrite to nitric oxide [74–77]. The nitrite reductase is periplasmic and contains a covalently-bound haem *c* and non-covalently-bound haem *d* [5,76]. The electron donor to the nitrite reductase is most likely to be cytochrome *c*-550 [78].

VI. Involvement of *c*-type cytochromes in methylamine oxidation

The physiological electron acceptor for methylamine dehydrogenase (MNDH) is usually the type I blue copper protein, induced during growth on methylamine, first discovered by Tobari and Harada in *Methylobacterium extorquens* AM1 and called amicyanin [79]. It is one of the two blue copper proteins, or cupredoxins, found in many methylotrophs [4].

Amicyanin is not detectable, however, in all methylotrophs growing on methylamine by way of meth-

ylamine dehydrogenase. In bacteria genuinely lacking all amicyanin then the obvious alternative is one of the periplasmic *c*-type cytochromes; these may be involved (instead of amicyanin) in the oxidation of methylamine in trimethylamine-grown *Methylophilus methylotrophus* [80], also in the closely related organism W3A1 [81], in copper-deficient *M. extorquens* [82] and perhaps in copper-deficient organism 4025 [83] (Fig. 7). In support of this conclusion is the observation that methylamine dehydrogenase is sometimes able to react directly with cytochrome c_H, but whether or not this has any physiological significance is not known. In at least one organism (*Thiobacillus versutus*) it has been shown, using the pure proteins, that the rate constants are sufficiently high for either amicyanin or cytochrome *c*-550 to act as electron acceptor from MNDH [84] (Fig. 7).

Organism 4025 provides an excellent example of an organism in which blue copper proteins completely replace soluble cytochromes [8,83] (Fig. 7). This obligate methylotroph usually produces so much amicyanin during growth on methylamine that it appears blue in colour [38,85]. When sufficient copper is present to achieve maximum growth, very large amounts of amicyanin and 'azurin' are produced; furthermore, complete electron transport chains could be reconstituted using methylamine dehydrogenase plus amicyanin and oxidase, with either cytochrome c_H or azurin as the intermediate electron carrier between amicyanin and the oxidase.

Neither of the two oxidases described in methylotrophs (cytochrome aa_3 or cytochrome *co*) is able to oxidise amicyanin although the second blue copper protein (azurin) may be oxidised [83]. As amicyanin is able to interact with azurin or periplasmic *c*-type cytochromes, some of which can act as electron donor to the oxidase, then the simplest electron transport chain will involve MNDH, amicyanin, a periplasic cytochrome *c* (or azurin) and a membrane oxidase (Fig. 7).

It had been suggested that the cytochrome *c*-551$_i$ of *P. denitrificans*, which is induced, together with cytochrome *c*-553$_i$ during growth on methanol or methylamine, might be involved in electron transport from methylamine [35,36,86]. However it has now been conclusively demonstrated that cytochrome *c*-551$_i$ is equivalent to cytochrome c_L, the electron acceptor from methanol dehydrogenase, and mutants lacking it grow perfectly well on methylamine [6,42,68,69]. Remarkably, mutants lacking the expected donor to the oxidase (cytochrome *c*-550) are still able to grow (more slowly) on methylamine, suggesting that the other periplasmic *c*-type cytochromes, or azurin, are able to take over some of the role of cytochrome *c*-550 [55].

In summary, the most likely electron transport chains for the oxidation of methylamine in methylotrophic bacteria are as shown in Fig. 7 in which amicyanin is usually the electron acceptor and in which electrons flow to the oxidase by way of the typical Class I cytochrome *c* (*c*-550 or c_H) or azurin. The proton translocation measured during respiration with methylamine in *M. extorquens* [11] is consistent with the proposal that it does not involve the mid-chain *b*-type cytochromes and that the protonmotive force is established as in methanol oxidation (Fig. 2), the yield of ATP being always one or less.

VII. The membrane-bound *c*-type cytochromes of methylotrophs

VII-A. The o-type oxidase of methylotrophs, cytochrome co

The bacterial *o*-type oxidases are defined loosely as oxidases having a CO-binding cytochrome *b* component, which for convenience is usually referred to as cytochrome *o* and which is assumed to be the oxygen-reactive site; they fall into two classes which differ fundamentally with respect to their structure and function [43]. These are the cytochrome *bo* and cytochrome *co* classes, which contain haem *b* and haem *c*, respectively, as their second prosthetic group. One of the first examples of the cytochrome *co* class to be purified and shown to be a cytochrome *c* oxidase was the cytochrome *co* from the obligate methylotroph *Methylophilus methylotrophus* [7]. It has two cytochrome *b* subunits (31.5 kDa) and two cytochrome *c* subunits (23.8 kDa). The cytochrome *c* component does not correspond to any of the soluble cytochromes. In *M. methylotrophus* this oxidase is the sole oxidase in carbon-excess conditions; in carbon-limited conditions the predominant oxidase is cytochrome aa_3 and the concentration of cytochrome *co* diminishes to about 10% of the concentration found in carbon-excess conditions [87]. In organism 4025 [8] and *Acetobacter methanolicus* [16,88] the sole oxidase is cytochrome *co*, whereas in *Methylobacterium* [9,82] and *Paracoccus* [58,60,72] the predominant oxidase during methylotrophic growth is cytochrome aa_3. The preferred electron donor to both types of oxidase is the Class I cytochrome *c* (cytochrome c_H) [7–9,58,88].

The cytochrome *co* of the acidophilic methylotroph, *A. methanolicus*, is unusual in being stable in high concentrations of salt over a wide range of pH values. During purification, however, it readily dissociates into its components and then is only able to oxidise rapidly those *c*-type cytochromes with which it is also able to reconstitute an active oxidase [88].

VII-B. The cytochrome bc_1 complex in methylotrophs

This complex has been studied extensively in only one methylotroph, *P. denitrificans*. Except for this or-

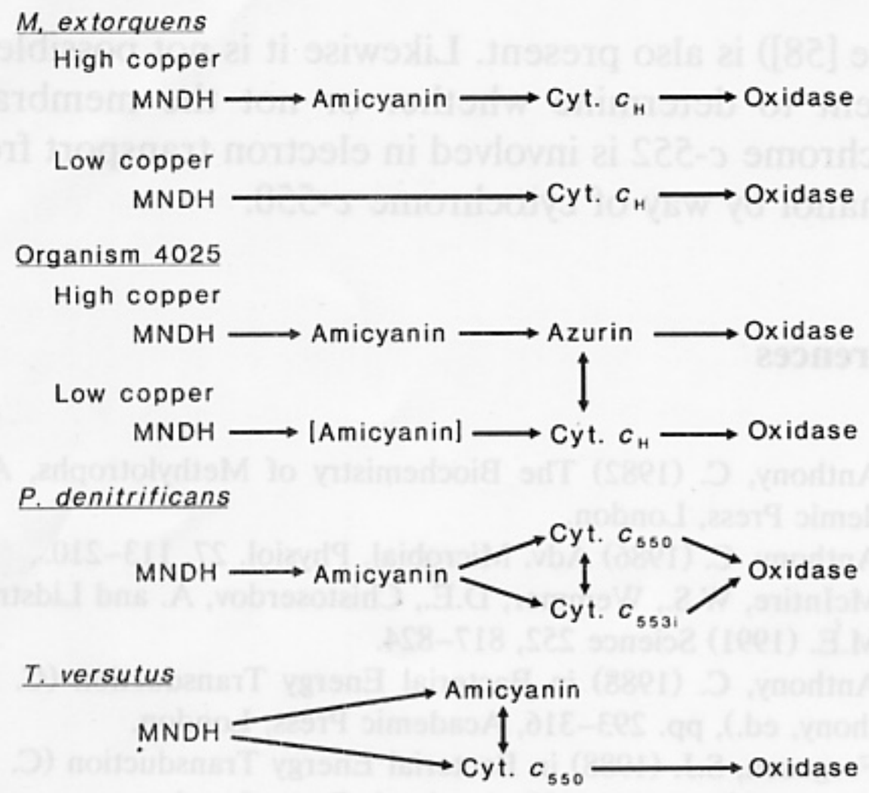

Fig. 7. Electron transport chains involved in methylamine oxidation. Azurin may replace the Class I *c*-type cytochromes in other bacteria as well as organism 4025. In many conditions in the bacteria shown here, more than one route of electron transport may operate. In *P. denitrificans* cytochrome *c*-553$_i$ may be able to replace cytochrome *c*-550 completely in a mutant lacking that cytochrome; and a membrane-bound cytochrome *c*-552 complexed with the oxidase (cytochrome aa_3) may also be involved.

ganism, no methylotroph has been reported to contain a membrane-bound cytochrome c_1. It plays no role in the oxidation of methanol or methylamine and func-

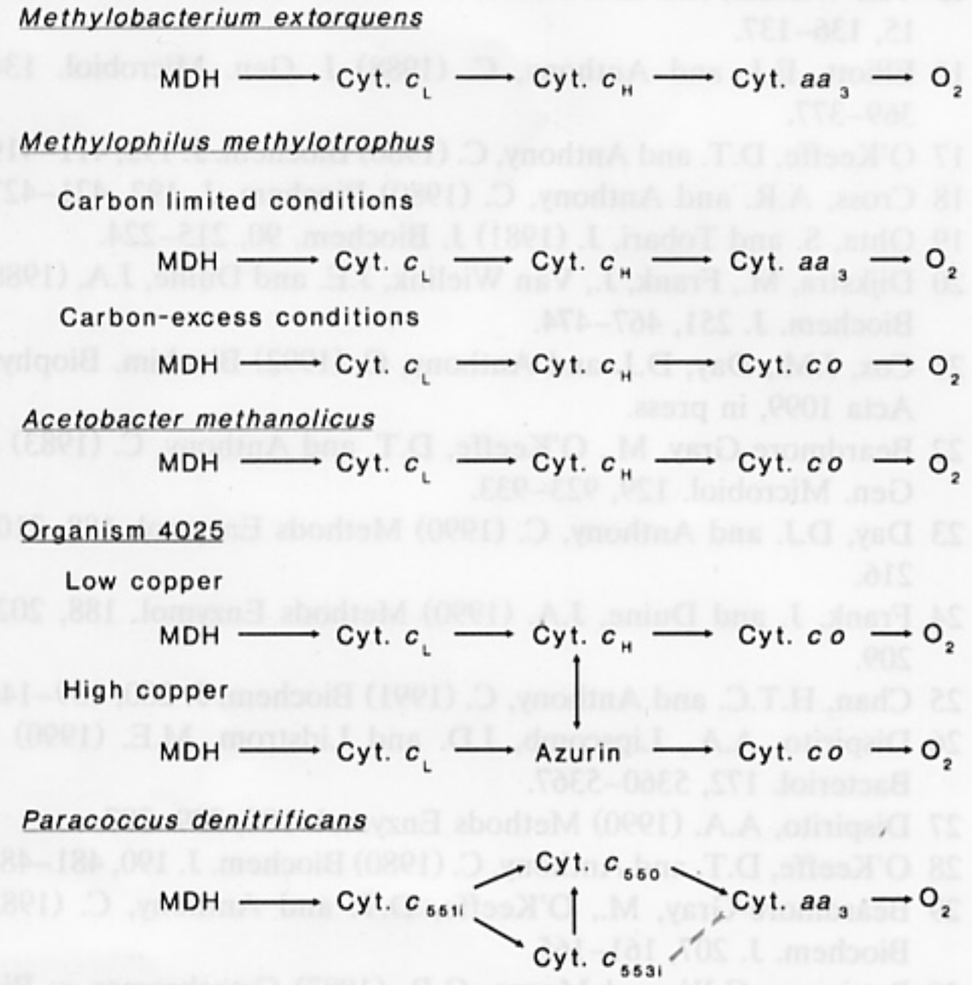

Fig. 8. Electron transport chains involved in methanol oxidation. In organism 4025 in many conditions cytochrome c_H and azurin are both present and in these conditions both these electron acceptors may operate in electron transport. In *P. denitrificans* cytochrome *c*-553$_i$ may be able to replace cytochrome *c*-550 completely in a mutant lacking that cytochrome; and a membrane-bound cytochrome *c*-552 complexed with the oxidase (cytochrome aa_3) may also be involved. Cytochrome *c*-550 is also the electron donor to the periplasmic cytochrome *c* peroxidase and nitrite reductase.

tions only in the oxidation of ubiquinol arising from the NAD^+-linked dehydrogenases. The complex contains only three subunits, one of which is the 62 kDa cytochrome c_1 [89]. This complex was also isolated in a ubiquinol oxidase 'supercomplex' together with cytochrome aa_3 and a novel membrane 22 kDa cytochrome *c*-552 [90]. It has been suggested by Trumpower [91] that electron transfer between the bc_1 and aa_3 complexes preferentially uses the membrane-bound cytochrome *c*-552, although at least partial redox equilibrium with the periplasmic cytochrome *c*-550 does occur. He suggested that the cytochrome *c*-552 appears to associate more tightly with the cytochrome aa_3 than with the cytochrome bc_1 and that a binary *c*-552/oxidase complex might serve as a common oxidant for the heterogeneous pool of *c*-type periplasmic cytochromes. The 22 kDa membrane cytochrome *c*-552 was also observed by other workers [37,86,92–94], and it was assumed that this cytochrome is the same as the soluble cytochrome *c*-552 [37]; indeed, the soluble cytochrome *c*-552 reacted with antibodies raised against the ubiquinol oxidase complex. Subsequent to this work it has been demonstrated that the soluble cytochrome *c*-552 is the electron acceptor for methanol dehydrogenase which is induced during growth on methanol and which is also called cytochrome *c*-551$_i$ (or cytochrome c_L) [6,42]. As this is absent except during growth on methanol and is a very soluble protein it is very unlikely that the two cytochromes are identical. That they are not identical is indicated by the observation that, on peptide sequencing [94], sequences were found that showed homology with typical *c*-type cytochromes.

Interpretation of results with bacterial electron transport chains is necessarily greatly influenced by conclusions drawn from studies of mitochondrial electron transport, one of the most important of which is that soluble cytochrome *c*-550 mediates electron transfer from the cytochrome bc_1 complex to cytochrome aa_3. That this is not necessarily the case in methylotrophs was indicated by the properties of a mutant of *M. extorquens* which lacked all *c*-type cytochromes but was able to grow normally on heterotrophic substrates but not on methanol or methylamine [44,95], and a similar result was observed with similar mutants of *P. denitrificans* [96]. This has been extended in a study of mutants of *P. denitrificans* specifically lacking cytochrome *c*-550 which are still able to respire by way of the two complexes which must therefore interact directly within the membrane [42,54,55,93].

In *Methylophilus methylotrophus* the only membrane cytochrome *c* (beside that in cytochrome *co*) was shown to be cytochrome c_L (37% of the total membrane cytochrome *c*) [97]. In this methylotroph, as in all others except *Paracoccus*, cytochrome c_L is produced in large amounts in all growth conditions. The mem-

brane cytochrome c_L could not be released with high concentrations of NaCl but was solubilised with Triton. After purification it was shown to be identical to the soluble cytochrome c_L (M_r, absorption spectrum, rate of reduction by MDH and rate of oxidation by the cytochrome *co*). It was suggested that the cytochrome c_L might be replacing cytochrome c_1 in this methylotroph. It should be noted that the suggestion that soluble cytochrome *c*-552 (cytochrome c_L) is part of the ubiquinol oxidase system in *Paracoccus* is only superficially similar to the suggestion that cytochrome c_L may be part of the quinol oxidase system in *Methylophilus* because in *Methylophilus* there is no cytochrome c_1 [97].

IX. Summary: *c*-type cytochromes in electron transport in methylotrophs

Figs. 7 and 8 summarise the electron transport chains in the methylotrophic bacteria discussed in this review.

It would be most convenient for reviewers of this subject if a single unambiguous scheme could be presented as a summary of the involvement of *c*-type cytochromes in electron transport from methanol and methylamine in methylotrophs. This is not possible for a number of reasons. One is that the periplasm contains a number of different redox mediators that are able to react with one another and with the oxidase system (directly or indirectly); hence a branched electron 'network' rather than a linear chain may be a better description. One approach to resolve which path might be more 'important' might appear to be the isolation of mutants lacking the various cytochromes. This, however, does not always eliminate alternatives. There is also the possibility that when one path is impossible, because of the absence of one of the intermediates, then a secondary path becomes more important. The evidence for the pathways summarised in Fig. 8 has been presented in the various sections above. In all cases cytochrome c_L (also called cytochrome *c*-551_i) is the electron acceptor for MDH, as confirmed by isolation of mutants specifically lacking this cytochrome [42,68,69]. Similarly, amicyanin is usually the electron acceptor for methylamine dehydrogenase and mutant studies have confirmed this for *Paracoccus* [98]. In vitro studies have demonstrated for many bacteria that the intermediate between cytochrome c_L or amicyanin and the oxidase is likely to be cytochrome c_H (cytochrome *c*-550), but mutants of *Paracoccus* lacking this cytochrome are still able to grow on methanol (but more slowly on methylamine) [42,55]. This is probably because *Paracoccus* also contains cytochrome *c*-553_i, induced during growth on methanol. Needless to say, it is impossible from these data to determine to what extent this cytochrome mediates electron transfer to the oxidase when cytochrome *c*-550 (its preferred substrate [58]) is also present. Likewise it is not possible at present to determine whether or not the membrane cytochrome *c*-552 is involved in electron transport from methanol by way of cytochrome *c*-550.

References

1 Anthony, C. (1982) The Biochemistry of Methylotrophs, Academic Press, London.
2 Anthony, C. (1986) Adv. Microbial. Physiol. 27, 113–210.
3 McIntire, W.S., Wemmer, D.E., Chistoserdov, A. and Lidstrom, M.E. (1991) Science 252, 817–824.
4 Anthony, C. (1988) in Bacterial Energy Transduction (C. Anthony, ed.), pp. 293–316, Academic Press, London.
5 Ferguson, S.J. (1988) in Bacterial Energy Transduction (C. Anthony, ed.), pp. 141–182, Academic Press, London.
6 Long, A.R. and Anthony, C. (1991) J. Gen. Microbiol. 137, 415–425.
7 Froud, S.J. and Anthony, C. (1984) J. Gen. Microbiol. 130, 2201–2212.
8 Auton, K.A. and Anthony, C. (1989) Biochem. J. 260, 75–79.
9 Mukai, K., Fukumori, Y. and Yamanaka, T. (1990) J. Biochem. 107, 714–717.
10 Keevil, C.W. and Anthony, C. (1979) Biochem. J. 182, 71–79.
11 O'Keeffe, D.T. and Anthony, C. (1978) Biochem. J. 170, 561–567.
12 Dawson, M.J. and Jones, C.W. (1981) Eur. J. Biochem. 118, 113–118.
13 Dawson, M.J. and Jones, C.W. (1981) Biochem. J. 194, 915–924.
14 Dawson, M.J. and Jones, C.W. (1982) Arch. Microbiol. 133, 55–61.
15 Van Wielink, J.E. and Duine, J.A. (1990) Trends Biochem. Sci. 15, 136–137.
16 Elliott, E.J. and Anthony, C. (1988) J. Gen. Microbiol. 134, 369–377.
17 O'Keeffe, D.T. and Anthony, C. (1980) Biochem. J. 192, 411–419.
18 Cross, A.R. and Anthony, C. (1980) Biochem. J. 192, 421–427.
19 Ohta, S. and Tobari, J. (1981) J. Biochem. 90, 215–224.
20 Dijkstra, M., Frank, J., Van Wielink, J.E. and Duine, J.A. (1988) Biochem. J. 251, 467–474.
21 Cox, J.M., Day, D.J. and Anthony, C. (1992) Biochim. Biophys. Acta 1099, in press.
22 Beardmore-Gray, M., O'Keeffe, D.T. and Anthony, C. (1983) J. Gen. Microbiol. 129, 923–933.
23 Day, D.J. and Anthony, C. (1990) Methods Enzymol. 188, 210–216.
24 Frank, J. and Duine, J.A. (1990) Methods Enzymol. 188, 202–209.
25 Chan, H.T.C. and Anthony, C. (1991) Biochem. J. 280, 139–146.
26 Dispirito, A.A., Lipscomb, J.D. and Lidstrom, M.E. (1990) J. Bacteriol. 172, 5360–5367.
27 Dispirito, A.A. (1990) Methods Enzymol. 188, 289–297.
28 O'Keeffe, D.T. and Anthony, C. (1980) Biochem. J. 190, 481–484.
29 Beardmore-Gray, M., O'Keeffe, D.T. and Anthony, C. (1982) Biochem. J. 207, 161–165.
30 Pettigrew, G.W. and Moore, G.R. (1987) Cytochromes *c*: Biological Aspects, Springer-Verlag, London.
31 Moore, G.R. and Pettigrew, G.W. (1990) Cytochromes *c*: Evolutionary, Structural and Physicochemical Aspects, Springer-Verlag, London.
32 Nunn, D.N. and Anthony, C. (1988) Biochem. J. 256, 673–676.
33 Day, D.J. and Anthony, C. (1990) Methods Enzymol. 188, 298–303.
34 Dijkstra, M., Frank, J. and Duine, J.A. (1989) Biochem. J. 257, 87–94.

35 Husain, M. and Davidson, V.L. (1986) J. Biol. Chem. 261, 8577–8580.
36 Gray, K.A., Knaff, D.B., Husain, M. and Davidson, V.L. (1986) FEBS Lett. 207, 239–242.
37 Bosma, G., Braster, M., Stouthamer, A.H. and Van Verseveld, H.W. (1987) Eur. J. Biochem. 165, 665–670.
38 Lawton, S.A. and Anthony, C. (1985) Biochem. J. 228, 719–726.
39 Ambler, R.P. (1991) Biochim. Biophys. Acta 1058, 42–47.
40 Ambler, R.P (1982) in From Cyclotrons to Cytochromes (Kaplan, N.O. and Robinson, A., eds.), pp. 263–280, Academic Press, New York.
41 Anthony, C. (1992) in Principles and Applications of Quinoproteins (Davidson, V.L., ed.), Marcel Dekker, New York.
42 Harms, N. and Van Spanning, R.J.M. (1991) J. Bioenerg. Biomembr. 23, 187–210.
43 Poole, R.K. (1988) in Bacterial Energy Transduction (C. Anthony, ed.), pp. 231–291, Academic Press, London.
44 Widdowson, D. and Anthony, C. (1975) Biochem. J. 152, 349–356
45 Santos, H. and Turner, D.L. (1988) Biochim. Biophys. Acta 954, 277–286.
46 Berry, M.J., George, S.J., Thomson, A.J., Santos, H. and Turner, D.L. (1990) Biochem. J. 270, 413–417.
47 Ghosh, R. and Quayle, J.R. (1981) Biochem. J. 199, 245–250.
48 Duine, J.A., Frank, J. and Ruiter, L.G. (1979) J. Gen. Microbiol. 115, 523–526.
49 Dijkstra, M., Frank, J. and Duine, J.A. (1988) FEBS Lett. 227, 198–202.
50 Anthony, C. (1992) in Principles and Applications of Quinoproteins (Davidson, V.L., ed.), Marcel Dekker, New York.
51 Nunn, D.N., Day, D.J. and Anthony, C. (1989) Biochem. J. 260, 857–862.
52 Anthony, C. (1990) FEMS Microbiol. Rev. 87, 209–214.
53 Grabarek, Z. and Gergely, J. (1990) Anal. Biochem. 185, 131–135.
54 Ferguson, S.J. and Page, M.D. (1990) FEMS Microbiol. Rev. 87, 227–233.
55 Van Spanning, R.J.M., Wansell, C., Harms, N., Oltmann, L.F. and Stouthamer, A.H. (1990) J. Bacteriol. 172, 986–996.
56 Nakayama, T., Titani, K. and Narita, K. (1971) J. Biochem. 70, 311–326.
57 Margoliash, E., Smith, E.L., Kriel, G. and Tuppy, H. (1961) Nature 192, 1121–1127.
58 Davidson, V.L. and Kumar, M.A. (1989) FEBS Lett. 245, 271–273.
59 Timkovich, R. and Dickerson, R.E. (1976) J. Biol. Chem. 251, 4033–4046.
60 Bolgiano, B., Smith, L. and Davies, H.C. (1988) Biochim. Biophys. Acta 933, 341–350.
61 Ambler, R.P., Meyer, T.E., Kamen, M.D., Schichman, S.A. and Sawer, L. (1981) J. Mol. Biol. 147, 351–356.
62 Veldink, G.A., Boelens, H., Maccarrone, M., Vanderlecq, F., Vliegenthart, J.F.G., Paz, M.A., Fluckiger, R. and Gallop, P.M. (1990) FEBS Lett. 270, 135–138.
63 Ambler, R.P., Dalton, H., Meyer, T.E., Bartsch, R.G. and Kamen, M.D. (1986) Biochem. J. 233, 333–337.
64 Pettigrew, G.W. (1991) Biochim. Biophys. Acta 1058, 25–27.
65 Tani, Y., Yoon, B.D. and Yamada, H. (1986) Agric. Biol. Chem. 50, 2545–2552.
66 Goodhew, C.F., Wilson, I.B.H., Hunter, D.J.B. and Pettigrew, G.W. (1990) Biochem. J. 271, 707–712.
67 Day, D.J., Nunn, D.N. and Anthony, C. (1990) J. Gen. Microbiol. 136, 181–188.
68 Nunn, D.N. and Lidstrom, M.E. (1986) J. Bacteriol. 166, 581–590.
69 Nunn, D.N. and Lidstrom, M.E. (1986) J. Bacteriol. 166, 591–597.
70 Alefounder, P.R. and Ferguson, S.J. (1981) Biochem. Biophys. Res. Commun. 98, 778–784.
71 Alefounder, P.R. and Ferguson, S.J. (1980) Biochem. J. 192, 231–240.
72 Van verseveld, H.W. and Stouthamer, A.H. (1978) Arch. Microbiol. 118, 13–20.
73 Bamforth, C.W. and Quayle, J.R. (1978) Arch. Microbiol. 119, 91–97.
74 Newton, M. (1969) Biochim. Biophys. Acta 185, 316–331.
75 Carr, G.J., Page, M.D. and Ferguson, S.J. (1989) Eur. J. Biochem. 179, 683–692.
76 Page, M.D. and Ferguson, S.J. (1989) Mol. Microbiol. 3, 653–661.
77 Stouthamer, A.H. (1991) J. Bioenerg. Biomembr. 23, 163–185.
78 Matchova, I. and Kucera, I. (1991) Biochim. Biophys. Acta 1058, 256–260.
79 Tobari, J. and Harada, Y. (1981) Biochem. Biophys. Res. Commun. 101, 502–508.
80 Burton, S.M., Byrom, D., Carver, M.A., Jones, G.D.D. and Jones, C.W. (1983) FEMS Microbiol. Lett. 17, 185–190.
81 Chandrasekar, R. and Klapper, M.H. (1986) J. Biol. Chem. 261, 3616–3619.
82 Fukumori, Y., Nakayama, K. and Yamanaka, T. (1985) J. Biochem. 98, 493–499.
83 Auton, K.A. and Anthony, C. (1989) J. Gen. Microbiol. 135, 1923–1931.
84 Van Wielink, J.E., Frank, J. and Duine, J.A. (1989) in PQQ and Quinoproteins (Jongejan, J.A. and Duine, J.A., eds.), pp. 269–278, Kluwer Academic Publishers, Dordrecht.
85 Lawton, S.A. and Anthony, C. (1985) J. Gen. Microbiol. 131, 2165–2171.
86 Gray, K.A., Davidson, V.L. and Knaff, D.B. (1988) J. Biol. Chem. 263, 13987–13990.
87 Cross, A.R. and Anthony, C. (1980) Biochem. J. 192, 429–439.
88 Chan, H.T.C. and Anthony, C. (1991) J. Gen. Microbiol. 137, 693–704.
89 Yang, X. and Trumpower, B.L. (1986) J. Biol. Chem. 261, 12282–12289.
90 Berry, E.A. and Trumpower, B.L. (1985) J. Biol. Chem. 260, 2458–2467.
91 Trumpower, B.L. (1991) J. Bioenerg. Biomembr. 23, 241–255.
92 Bosma, G., Braster, M., Stouthamer, A.K. and Van Verseveld, H.W. (1987) Eur. J. Biochem. 165, 657–665.
93 Bolgiano, B., Smith, L. and Davies, N.C. (1989) Biochim. Biophys. Acta 973, 227–234.
94 Steinrucke, P., Gerhus, E., Jetzek, M., Turba, A. and Ludwig, B. (1991) J. Bioenerg. Biomembr. 23, 227–239.
95 Anthony, C. (1975) Biochem. J. 146, 289–298.
96 Willison, J.C. and John, P. (1979) J. Gen. Microbiol. 115, 443–450.
97 Froud, S.J. and Anthony, C. (1984) J. Gen. Microbiol. 130, 3319–3325.
98 Van Spanning, R.J.M., Wansell, C., Reijnders, W.N.M., Oltmann, L.F. and Stouthamer, A.H. (1990) FEBS Lett. 275, 217–220.
99 Van Spanning, R.J.M., Wansell C., Harms, N., Oltmann, L.F. and Stouthamer, A.H. (1990) J. Bacteriol. 172, 3534.
100 Gilmour, R., Goodhew, C.F. and Pettigrew, G.W. (1991) Biochim. Biophys. Acta 1059, 233–238.