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The soluble cytochromes *c* of methanol-grown *Hyphomicrobium* X

Evidence against the involvement of autoreduction in electron-acceptor functioning of cytochrome c_L

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Hyphomicrobium X, grown on methanol with O_2 or nitrate as electron acceptor, contains two major soluble cytochromes *c*. These were isolated in electrophoretically homogeneous form. They are related to cytochromes *c* already described for other methylotrophic bacteria and designated cytochromes c_H and c_L (properties indicated in that order) in view of the following characteristics: absorption maxima of the reduced forms (414, 520 and 551 nm and 414, 520 and 550 nm); molar absorption coefficients of the α-bands (23700 $M^{-1} \cdot cm^{-1}$ and 21600 $M^{-1} \cdot cm^{-1}$); maxima of the α-bands (no splitting) at 77 K (547.6 nm and 548.5 nm); M_r values of the native proteins (15000 and 19500); pI values (7.4 and 7.5, and 4.3); midpoint potentials at pH 7.0 (+292 mV and +270 mV). Both were monomers containing 1 haem *c* group per protein molecule, the oxidized forms binding cyanide at high pH. Autoreduction also occurred at high pH but at a rate significantly lower than that reported for other ferricytochromes *c*. On the other hand, the reverse situation applies to the reduction of ferricytochrome c_L by reduced methanol dehydrogenase, the reduction occurring instantaneously at pH 7 but much more slowly at pH 9 (ferricytochrome c_H was reduced at a 7-fold lower rate, but the rates at pH 7 and 9 were similar). Insignificant reduction was observed with cyclopropanol-inactivated enzyme or with enzyme in the presence of EDTA. In view of the dissimilarities, it is concluded that different mechanisms operate in the autoreduction of ferricytochrome c_L and in its reduction by reduced methanol dehydrogenase.

INTRODUCTION

There is general agreement that methanol dehydrogenase (MDH) in methylotrophic bacteria donates its electrons to the respiratory chain at the level of cytochromes *c*. However, so far it has not been possible to prove this unequivocally in a direct experiment, that is to attain MDH-mediated electron transfer from methanol to cytochrome *c in vitro* at significant rates. Oxidized cytochromes *c* from *Hyphomicrobium* X are not electron acceptors in the assay for homologous MDH *in vitro*. An obvious explanation for this could be that a mediator or the components themselves become inactivated during preparation of the cell-free extract. This idea was confirmed, indeed, when it was found that electron transfer occurs in anaerobically prepared extracts and that this process became blocked as soon as O_2 was introduced into the system. Concomitantly, the normal assay with artificial electron acceptors became activator-dependent (ammonium salts), suggesting that aerobically isolated enzyme is an artifact [1].

Using the aerobically isolated components from *Methylobacterium* sp. strain AM1, Beardmore-Gray *et al.* [2] demonstrated cytochrome *c* reduction by MDH, albeit that the rates were much lower than observed with artificial electron acceptors and activator at pH 9. Two soluble cytochromes *c* were isolated, the one active as electron acceptor for the enzyme being designated as cytochrome c_L. High rates of autoreduction (100-fold that of horse heart cytochrome *c*) were measured at pH values above 9.0. However, comparable reduction rates could be achieved at pH 7.0 in the presence of MDH while methanol conversion took place under these conditions. It was concluded that both reduction mechanisms were similar, the presence of MDH allowing the autoreduction to occur at a lower pH.

In view of these controversial points, it was decided to characterize the cytochromes *c* from *Hyphomicrobium* X, and to study their autoreduction and reduction by MDH.

MATERIALS AND METHODS

Growth of the organism

Hyphomicrobium X was grown in batch culture at 30 °C on a mineral medium [3] supplemented with 0.4% (v/v) methanol. Growth under anaerobic conditions occurred with 5 g of KNO_3/l. Cells were harvested at the end of the exponential growth phase by centrifugation, washed with 0.05 M-potassium phosphate buffer, pH 7.0, and stored at −20 °C.

Purification of the cytochromes and methanol dehydrogenase

Frozen cells (88 g wt wt.) were suspended in 88 ml of 36 mM-Tris/39 mM-glycine buffer, pH 9.0, and the mixture was passed twice through a French pressure cell at 110 MPa. The suspension (viscosity was lowered by adding DNAase) was centrifuged (48000 *g* for 20 min at

Abbreviations used: PQQ, pyrroloquinoline quinone (2,7,9-tricarboxy-1*H*-pyrrolo[2,3-*f*]quinoline-4,5-dione); MDH, methanol dehydrogenase; Ches, 2-(*N*-cyclohexylamino)ethanesulphonic acid.

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4 °C), yielding the cell-free extract. This was applied to a DEAE-Sepharose (Pharmacia) column (30 cm × 4.5 cm) equilibrated with 36 mM-Tris/39 mM-glycine buffer, pH 9.0. The column was washed with this buffer until the red band of cytochrome c_H was completely eluted. The coloured fractions were pooled, dialysed against 0.02 M-potassium phosphate buffer, pH 7.0, and applied to a silica-gel column (10 cm × 2.4 cm) equilibrated with this buffer. After a washing of the column with the same buffer, the red band was eluted with 0.2 M-potassium phosphate buffer, pH 7.0, containing 10% (w/v) poly(ethylene glycol) 6000. The coloured fractions were pooled, and dialysed against 5 mM-sodium acetate buffer, pH 5.0, containing 5 mM-sodium ascorbate. The dialysed preparation was applied to a CM-Sepharose (Pharmacia) column (10.5 cm × 1.0 cm) equilibrated with 5 mM-sodium acetate buffer, pH 5.0. The cytochrome c_H was eluted by using a linear gradient of 0–0.2 M-NaCl in this buffer. Coloured fractions were pooled, dialysed against 0.05 M-Mops/KOH buffer, pH 7.0, concentrated by pressure filtration on an Amicon YM-2 membrane and stored at −20 °C.

MDH and cytochrome c_L remained adsorbed to the DEAE-Sepharose column under the conditions used for cytochrome c_H elution. MDH was eluted with 36 mM-Tris/21 mM-H_3PO_4 buffer, pH 6.5. Active fractions were pooled and further purification was performed as described previously [3].

Cytochrome c_L was eluted from the DEAE-Sepharose column with 36 mM-Tris/21 mM-H_3PO_4 buffer, pH 6.5, containing 0.1 M-NaCl. Coloured fractions were pooled, concentrated by pressure filtration on a Millipore membrane (M_r cut-off level 10^4), and the concentrate was applied to a Fractogel TSK HW-50S gel-filtration column (56.6 cm × 1.6 cm) in 0.05 M-potassium phosphate buffer, pH 7.0, containing 0.1 M-NaCl. Coloured fractions were pooled and dialysed against 10 mM-Tris/HCl buffer, pH 8.0, containing 5 mM-sodium ascorbate, and the dialysed preparation was applied to a DEAE-Sepharose column (9.5 cm × 1.0 cm) equilibrated with 10 mM-Tris/HCl buffer, pH 8.0. The red band was eluted with a concave gradient from 10 mM-Tris/HCl buffer, pH 8.0, to 125 mM-Tris/HCl buffer, pH 8.0, containing 125 mM-NaCl. Coloured fractions were pooled and dialysed against 0.05 M-Mops/KOH buffer, pH 7.0, and the dialysed preparation was concentrated by pressure filtration on a Millipore membrane (M_r cut-off level 10^4) and stored at −20 °C.

During purification, protein concentrations were determined by the method of Bradford [4], with bovine serum albumin as a standard.

M_r determinations

M_r values of the native proteins were determined with gel filtration, by the procedure of Andrews [5], on a Sephadex G-100 column (58 cm × 1.0 cm) equilibrated with 0.1 M-sodium phosphate buffer, pH 6.5, containing 0.1 M-KCl. The following marker proteins were used for calibration: horse heart cytochrome *c* (M_r 12750), myoglobin (M_r 18800), bovine pancreas chymotrypsinogen A (M_r 25000), ovalbumin (M_r 45000), horse liver alcohol dehydrogenase (M_r 80000) and α-globulin (M_r 150000).

M_r values of the denatured proteins were determined by electrophoresis on gradient (4–30%, w/v) polyacrylamide slab gels (7 cm) (Pharmacia) in SDS-containing buffer, as described below. Electrophoresis was stopped 45 min after Bromophenol Blue had migrated from the gels. Markers were from the low-M_r calibration kit (Pharmacia).

Polyacrylamide-gel electrophoresis

Preparations were checked for homogeneity by electrophoresis on 10% (w/v) polyacrylamide gels (7.6 cm), cross-linked with 0.27% (w/v) bisacrylamide, in a Pharmacia GE-411 electrophoresis apparatus cooled with tap water. Proteins were denatured in the presence of 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol by keeping the solutions at 100 °C for 5 min. Electrophoresis was conducted in 36 mM-Tris/39 mM-glycine buffer, pH 9.0, containing 0.2% (w/v) SDS at 150 V for 80 min. Protein staining was performed with Coomassie Brilliant Blue R-250. Peroxidase activity of the cytochromes was determined by the method of Thomas *et al.* [6].

Isoelectric focusing

Isoelectric focusing was performed according to the instructions of the manufacturer [7] on Phast Gel IEF 3–9 (Pharmacia). Protein staining was performed as recommended [8].

Spectrophotometric determination of cytochrome *c*

Determinations of the amounts of cytochrome c_H and c_L in the cell-free extracts were performed in accordance with Froud & Anthony [9]. The haem content was calculated from the difference spectra of reduced minus oxidized pyridine haemochrome [10] by using a molar absorption coefficient of 19100 $M^{-1} \cdot cm^{-1}$ for the 550 nm–575 nm wavelength pair [11]. The specific absorption coefficients of the α-bands were calculated from the haem contents and also from the specific absorption coefficients at 280 nm, determined in accordance with van Iersel *et al.* [12].

Absorption spectra at 77 K were recorded between 535 and 590 nm, with the absorption at 570 nm as a reference point, with a DW-2a spectrophotometer (American Instrument Co.) [13].

Autoreduction and reduction by MDH

Ferri- and ferro-cytochromes c_H and c_L were prepared by adding a slight excess of $K_3Fe(CN)_6$ or $Na_2S_2O_4$ respectively to the preparations. Low-M_r contaminants were removed by chromatography on a PD-10 gel filtration column (Pharmacia) equilibrated with the appropriate buffer. Cyclopropanol-inactivated MDH was prepared as described by Dijkstra *et al.* [14]. The amounts of enzyme were calculated by using a specific absorption coefficient of 2.02 litre $\cdot g^{-1} \cdot cm^{-1}$ at 280 nm, determined in accordance with van Iersel *et al.* [12]. MDH as isolated by the described procedure is in its fully reduced form ($MDH_{red.}$).

The reduction of ferricytochrome c_L (1.7 μM) and ferricytochrome c_H (4.4 μM) with reduced MDH (equimolar amount) and with cyclopropanol-inactivated MDH (4.8 μM) was measured in 0.05 M-Ches/KOH buffer, pH 9.0, and 0.05 M-Mops/KOH buffer, pH 7.0. The reaction was monitored for 10 min by a Hewlett–Packard 8450 A photodiode-array spectrophotometer, by measuring absorption changes at the 550 nm–556 nm wavelength pair (556 nm is the isosbestic point of the cytochrome c_L spectra) or the 550 nm–558 nm (558 nm is

Table 1. Purification of the soluble cytochromes c_L and c_H from *Hyphomicrobium* X

Both cytochromes were purified from aerobically methanol-grown cells as described in the Materials and methods section. The cytochrome *c* concentrations were calculated from the reduced-minus-oxidized difference spectra by using an absorption coefficient of 19 $mM^{-1} \cdot cm^{-1}$ for the 550 nm–535 nm wavelength pair. Yield and purification factors were based on the amount and specific concentrations found after the initial DEAE-Sepharose step.

Fraction	Cytochrome (nmol)	Protein (mg)	Specific concentration (nmol of cytochrome *c*/mg of protein)	Yield (%)	Purification (fold)
Cytochrome c_H					
DEAE-Sepharose	2097	232	9.0	100	1
Silica gel	2068	70	29.6	98	3.3
CM-Sepharose	1500	18	80.0	71	8.9
Cytochrome c_L					
1st DEAE-Sepharose	754	154	4.9	100	1
Fractogel	544	18	29.9	72	6.1
2nd DEAE-Sepharose	463	8	53.1	61	10.8

the isosbestic point of the cytochrome c_H spectra) every 30 s.

Autoreduction of the ferricytochromes was monitored at the same wavelength pairs in a period of several hours. First-order rate constants were calculated by simulation by using PSI, an interactive simulation program [15].

Determination of the midpoint potentials

Potentiometric titrations of the cytochromes were conducted in 50 mM-Mops/KOH buffer, pH 7.0, at 25 °C. The cytochromes (1.25 μM) were titrated anaerobically in the presence of diaminodurene (400 μM), trimethylhydroquinone (100 μM) and quinhydrone (100 μM) with 1.83 mM-$K_3Fe(CN)_6$ (oxidative way) or with 0.45 mM-$Na_2S_2O_4$ (reductive way). Absorption spectra and redox potentials were measured between +100 and +400 mV for cytochrome c_L and between +100 and +450 mV for cytochrome c_H. Midpoint potentials were calculated in accordance with the method described by van Wielink *et al.* [13].

Materials

Cyclopropanol was prepared by enzymic hydrolysis of cyclopropyl acetate [16]. Silica gel (70–325 mesh) was from Merck (Darmstadt, Germany).

RESULTS

Induction and purification of the soluble cytochromes *c*

By following the indicated procedure (Table 1), two soluble cytochrome *c* fractions were obtained that behaved homogeneously in polyacrylamide-gel electrophoresis. As argued in the Discussion section, the cytochrome eluted first from the column is cytochrome c_H, the second one cytochrome c_L. The conditions of growth had only minor effects on the proportions determined in the cell-free extracts: 73.5% cytochrome c_H/26.5% cytochrome c_L in aerobically grown and 62% cytochrome c_H/38% cytochrome c_L in anaerobically grown cells with nitrate as electron acceptor.

M_r values and subunits

Gel filtration on the calibrated Sephadex G-100 column revealed M_r values of 19500 for cytochrome c_L and 15500 for cytochrome c_H. Gradient polyacrylamide-gel electrophoresis of the denatured samples gave comparable values (19600 and 14000 respectively), indicating that both proteins are monomers.

Isoelectric focusing

The cytochrome c_L preparation showed one main band with a pI of 4.3 and two minor bands with pI values of 4.2 and 4.5. The cytochrome c_H preparation gave two bands of equal intensity of protein staining, having pI values of 7.4 and 7.5.

Spectral properties

Cytochrome c_H showed an absorption maximum at 408 nm in the oxidized form and maxima at 414, 520 and 551 nm in the reduced form (Fig. 1). A maximum at 547.6 nm was observed for the 77 K spectra. Addition of cyanide at pH 9 did not influence the absorption spectrum of the reduced form, but a shift from 408 to 412 nm was induced in the spectrum of the oxidized form. Subsequent addition of dithionite to the latter preparation induced maxima at 420, 524 and 554 nm. The pyridine haemochrome absorption spectrum showed maxima at 412, 520 and 548 nm, maxima typical for a cytochrome *c* [17]. Since the specific absorption coefficient of cytochrome c_H was determined to be 2.48 litre $\cdot g^{-1} \cdot cm^{-1}$ at 280 nm, on assuming an M_r of 14000 it could be calculated that there are 1.3 haem *c* groups per enzyme molecule, and the molar absorption coefficients of oxidized and reduced cytochrome c_H are 6.56 and 23.7 $mM^{-1} \cdot cm^{-1}$ at 550.6 nm respectively.

Cytochrome c_L has an absorption maximum at 408 nm in the oxidized form and maxima at 414, 520 and 550 nm in the reduced form (Fig. 1). A maximum at 548.5 nm was found in the 77 K spectra. The pyridine ferrohaemochrome spectrum showed maxima at 412, 520 and 550 nm. Addition of cyanide at pH 9.0 did not influence the spectrum of the reduced form, but a shift from 408 to 410 nm was seen on addition of cyanide to the oxidized form. Subsequent addition of dithionite to the latter preparation gave maxima at 418, 522 and 552 nm. Since the specific absorption coefficient at 280 nm is 2.02 litre $\cdot g^{-1} \cdot cm^{-1}$, and assuming an M_r of 19600, it could be calculated that there are 1.2 haem *c*

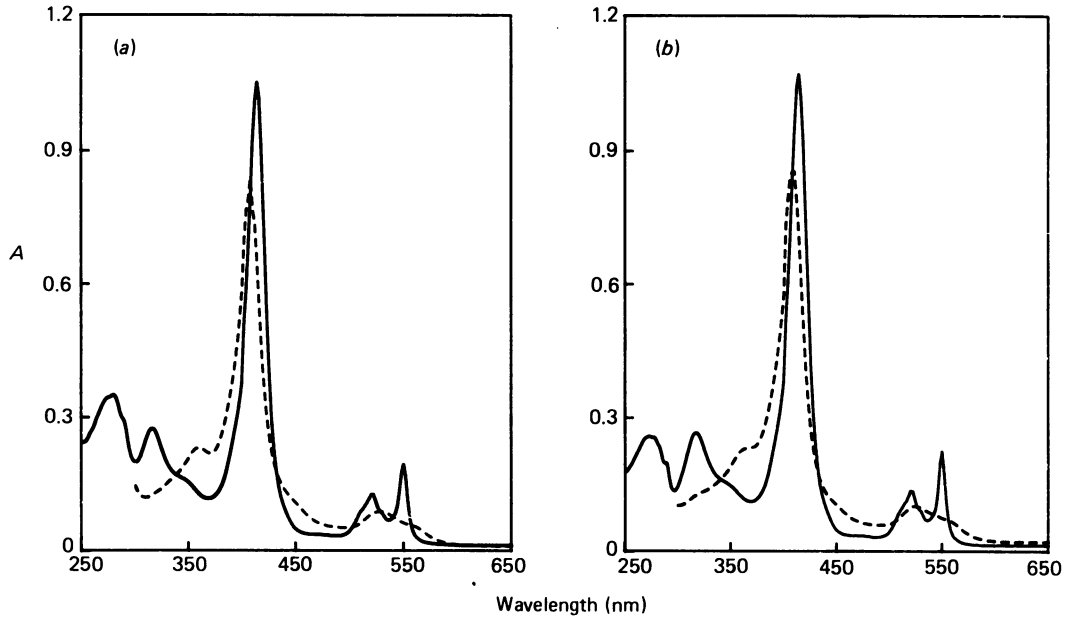

Fig. 1. Absorption spectra of cytochromes c_L and c_H

(*a*) Spectra of oxidized (----) and reduced (——) cytochrome c_L (8.6 μM) at 25 °C in 50 mM-Mops buffer, pH 7.0. (*b*) Spectra of oxidized (----) and reduced (——) cytochrome c_H (8.9 μM) at 25 °C in 50 mM-Mops buffer, pH 7.0. Oxidized and reduced cytochromes were prepared as described in the Materials and methods sections.

groups per enzyme molecule. On the basis of the same data, it could be calculated that the molar absorption coefficients of cytochrome c_L at 550 nm are 6.74 and 21.6 $mM^{-1} \cdot cm^{-1}$ for the oxidized and the reduced form respectively.

Both cytochromes (in their oxidized state) displayed rather broad absorption bands at 695 nm, measured at pH 7.

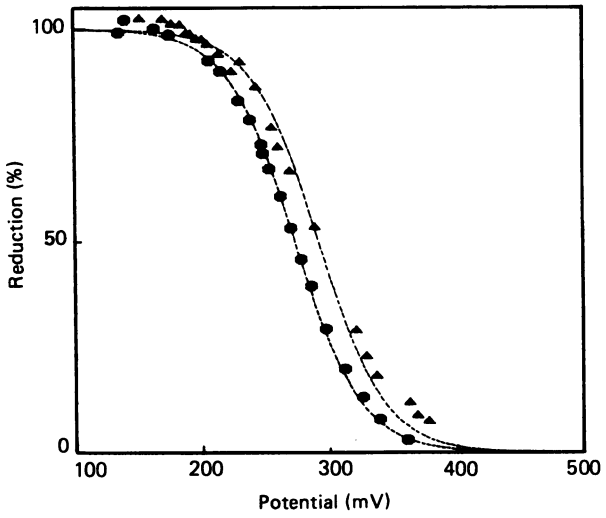

Fig. 2. Potentiometric titrations of cytochromes c_L and c_H

Oxidative titrations of ferrocytochrome c_L (1.25 μM, ●) and ferrocytochrome c_H (1.25 μM, ▲) were performed in 50 mM-Mops buffer, pH 7.0, at 25 °C with $K_3Fe(CN)_6$. Peak areas of the α-bands in the 535–590 nm range, expressed as percentage reduction, are plotted versus the measured redox potentials. The lines represent best fits for one component, simulated according to the procedure described elsewhere [13]. Reductive titrations (not shown) gave identical results.

Midpoint redox potentials

A series of 19 spectra in the 530–590 nm region were taken during redox titrations. The percentage reduction, calculated from the area of the α-band, was plotted versus the measured redox potential (Fig. 2). The calculated midpoint potentials at pH 7.0 and 25 °C were +270 and +292 mV for cytochrome c_L and c_H respectively.

Reduction and autoreduction of the ferricytochromes

Autoreduction of the cytochromes *c* was negligible at pH 7.0 but occurred with substantial rates at pH 9.0 ($k = 1.2 \times 10^{-5}$ s^{-1} for cytochrome c_L and $k = 3.5 \times 10^{-5}$ s^{-1} for cytochrome c_H). Inhibition of autoreduction was observed in the presence of KCN (10 mM), but not in the presence of cyclopropanol.

Very rapid reduction of ferricytochrome c_L, but not of ferricytochrome c_H ($k = 2.8 \times 10^{-3}$ s^{-1}), occurred on addition of an equimolar concentration of reduced MDH (Fig. 3). The rate of reduction at pH 7 was so high that it could not be measured. In contrast, at pH 9.0 the rate was lower so that reduction could be measured and the rate calculated (Table 2). On addition of an equimolar concentration of cyclopropanol-inactivated MDH, the rate of reduction was much lower than that in the presence of active enzyme ($k = 2.7 \times 10^{-4}$ s^{-1}), but higher than the autoreduction rate of ferricytochrome c_L (Fig. 3). Since the cyclopropanol-inactivated enzyme still showed some residual activity (0.7%) in the usual assay of the enzyme [18], the low but measurable activity with ferricytochrome c_L may be related to this. Cyanide (10 mM) was an inhibitor for the reaction between MDH and cytochrome c_L, but only when ferricytochrome *c* was preincubated with cyanide. Methanol (10 mM) was unable to abolish the inhibition. When cyanide was added to ferrocytochrome c_L in the presence of an equimolar concentration of MDH, on subsequent addition of an

Table 2. Reduction rates of ferricytochrome c_L with reduced MDH

Cytochrome c_L (1.7 μM) of *Hyphomicrobium* X was reduced in the absence and in the presence of an equimolar concentration of reduced MDH. Reduction rates are compared with the values given in the literature for the systems of *Methylobacterium* sp. strain AM1 [2] and *Methylophilus methylotrophus* [2] in which similar ratios of concentrations were used. All the cytochromes showed very low autoreduction rates at pH 7.0, too low to be detectable by the method employed.

Source	Reduction rate in the presence of MDH at pH 7: $10^3 \times k$ (s^{-1})	Autoreduction rate at pH 9: $10^5 \times k$ (s^{-1})	Reduction rate in the presence of MDH at pH 9: $10^3 \times k$ (s^{-1})
Hyphomicrobium X	–*	1.2	20
Methylophilus methylotrophus	1.0	100	6
Methylobacterium sp. strain AM1	4.0	200	14
Horse heart cytochrome *c*	n.d.†	3.8	n.d.†

* Too fast to measure.
† Not determined.

equimolar concentration of ferricyanide (to ferrocytochrome c_L), partial reduction (30%) took place. The absorption maxima of this preparation were similar to those of ferrocytochrome c_L and different from those obtained after addition of cyanide to ferricytochrome *c* and subsequent reduction with dithionite.

DISCUSSION

Hyphomicrobium X, grown aerobically as well as anaerobically with nitrate as electron acceptor, always contained two soluble cytochromes *c*. Comparison of their properties with those of cytochromes *c* from other methylotrophic bacteria (Table 3) revealed that they are similar to the so-called cytochromes c_L and c_H. Not only the physicochemical properties but also the physiological behaviour (see below) is in accordance with this view.

Although a clear distinction exists between cytochromes c_H and c_L (Table 3), it should be noted that a significant variation in properties exists among the members of each group. This might be connected with species differences, but another reason could be the different isolation procedures used to prepare the cytochromes, resulting in different conformations responsible for variation in redox potentials, autoreduction rates etc. (Tables 2 and 3) among the members of the groups of cytochromes c_H and c_L.

Cytochromes c_H and c_L from *Hyphomicrobium* X are comparable with horse heart cytochrome *c* in the following respects: autoreduction rates are more or less similar and cyanide induces changes in the absorption spectrum of the ferricytochromes *c* (a shift in the maximum at 408 nm to higher wavelengths and a decrease of the absorption band at 695 nm), but not in that of ferrocytochromes *c* [26,27]. In case of the horse heart cytochrome *c*, these effects have been attributed to the displacement of methionine as sixth ligand to the iron atom in the haem by cyanide [26]. Displacement of this ligand is not possible in the ferrocytochrome *c*, since it has a more compact conformation. The complex of ferricytochrome *c* and cyanide can be reduced, however, by dithionite, giving a complex with absorption maxima different from that of ferrocytochrome *c*. This reduction does not occur when reduced MDH is added to the ferricytochrome c_L–cyanide complex, although the inhibition of electron transfer must also be due to binding of cyanide to the cytochrome. The latter conclusion follows from the fact that addition of excess methanol had no effect on the cyanide inhibition of the ferricytochrome c_L reduction (cyanide is a competitive inhibitor for methanol in the dye-linked assay [18]). Although autoreduction, ligand-binding of methionine to iron and reduction of ferricytochrome c_L by MDH are all effected by cyanide, this does not mean that the phenomena are due to the same cause. For instance, it has been demonstrated by spectroscopic techniques that distortion of the ligand-binding of methionine and autoreduction occur independently [28].

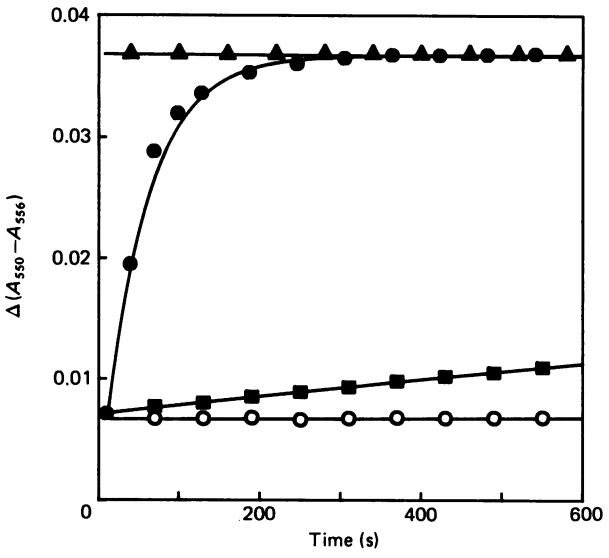

Fig. 3. Reduction and autoreduction of ferricytochrome c_L at pH 7.0 and pH 9.0

Reduction rates of ferricytochrome c_L (1.7 μM) were measured either in 50 mM-Mops buffer, pH 7.0, or in 50 mM-Ches buffer, pH 9.0. ○, Autoreduction at pH 9.0; ●, reduction at pH 9.0 in the presence of reduced MDH (1.7 μM); ▲, reduction at pH 7.0 in the presence of reduced MDH (1.7 μM); ■, reduction at pH 9.0 in the presence of cyclopropanol-inactivated MDH (4.8 μM).

Table 3. Properties of the soluble cytochromes *c* of methylotrophic bacteria

The cytochromes *c* were incorporated in this Table on the base of their pI and redox potential, high values giving rise to classification as cytochrome c_H, low values to classification as cytochrome c_L. Abbreviations: cyt, cytochrome; i, inducible; n.d., not determined.

	Designation of the cytochrome	Proportion in cell-free extract (%)	M_r (native)	M_r (denatured)	pI	E_0 (mV)	Maxima of the γ-band (nm)		Maxima of the α-band (nm)		References
							Ferricyt *c* at 25 °C	Ferrocyt *c* at 25 °C	Ferrocyt *c* at 25 °C	Ferrocyt *c* at 77 K	
Methylobacterium sp. strain AM1	cyt c_H	72	11000	11000	8.8	+294	410	416.5	550.5	548	[19]
Methylophilus methylotrophus	cyt c_H	50	n.d.	8500	8.85	+373	408	416.25	551.25	546,550 (split)	[20]
Methylomonas J	cyt *c*-551 (I)	73	16000	15800	5.3	+290	409	417	551	550	[21]
Methylomonas sp. YK 56	cyt *c*-II	71.5	16000	16000	4.1	n.d.	410	416.5	551.5	551	[22]
Paracoccus denitrificans	cyt *c*-550	n.d.	n.d.	15000	4.5	+253	410	415	550	n.d.	[23,24]
Hyphomicrobium ZV 580	cyt *c*-I	10	n.d.	12600	8.7	+333	410.4	416	550	n.d.	[25]
	cyt *c*-II	15	n.d.	12600	5–6	+245	n.d.	n.d.	n.d.	n.d.	
Hyphomicrobium X	cyt c_H	73.5	15500	14000	7.3–7.5	+292	408	414	550.6	547.6	Present paper
Methylobacterium sp. strain AM1	cyt c_L	28	20900	20900	4.2	+256	410	416	549	548	[19]
M. methylotrophus	cyt c_{LM}	8	n.d.	16800	4.55	+336	408	416.5	550.75	548	[20]
	cyt c_L	42	n.d.	17000–20000	4.0–4.35	+310	410	416	549.75	545, 547 (split)	
Methylomonas J	cyt *c*-551 (II)	27	12500	12700	4.3	+240	409	416	551	547, 550 (split)	[21]
Methylomonas sp. YK 56	cyt *c*-III	26	20000	16800	3.5	n.d.	410.5	416	551.5	546, 550.5 (split)	[22]
Paracoccus denitrificans	cyt *c*-551 (i)	n.d.	n.d.	32000	3.5	+190	409	416	551	n.d.	[23,24]
	cyt *c*-553 (i)	n.d.	n.d.	30000	3.8	+148	410	418	553	n.d.	
Hyphomicrobium ZV 580	cyt *c*-II	50	n.d.	12600	4.3	+218	409	416	550	n.d.	[25]
	cyt *c*-IV	25	n.d.	21600	3.3	+235	410	417	552.6	n.d.	
Hyphomicrobium X	cyt c_L	26.5	19500	19600	4.3	+270	408	414	550	548.5	Present paper

Addition of reduced MDH resulted in instantaneous reduction of ferricytochrome c_L at pH 7, but in a reduction with a measurable rate at pH 9.0 (Fig. 3 and Table 2). The reduction rates of ferricytochrome c_H at pH 9.0 and pH 7.0 were similar, whereas the rate at pH 9.0 was 7-fold lower than for ferricytochrome c_L at this pH. Although the higher reactivity of ferricytochrome c_L is in accordance with the view that it is the natural electron acceptor of MDH, the higher activity at pH 7 than at pH 9 is quite remarkable. For systems from other bacteria, much lower activities have been reported and they show the opposite behaviour with respect to pH influence (Table 2), the latter observation leading Anthony and co-workers to the idea that MDH stimulates autoreduction [2,29]. Obviously this is not the case for the system described here, autoreduction occurring at high pH but reaction rates with MDH being higher at pH 7.0 than at pH 9.0. The view that different mechanisms are at hand is substantiated by the fact that cyclopropanol-inactivated enzyme was unable to reduce ferricytochrome c_L (the rate observed resulting from uninhibited enzyme). Binding of enzyme to ferricytochrome c_L has been assumed to be necessary for stimulation of autoreduction [29,30]. Although a change in conformation of inhibited enzyme cannot be excluded completely, so far only modification of PQQ to an adduct has been found as the reason for inhibition [14]. It therefore seems likely that the electrons from the reduced cofactor ($PQQH_2$) in the enzyme are necessary to convert ferricytochrome c_L into ferrocytochrome c_L.

The contradictory results described here compared with those of other systems are hard to explain at the moment. However, it should be noted that the system described here shows very high reaction rates at a physiological pH and is inhibited by EDTA, a compound that is not active in the dye-linked assay but is an inhibitor for the more natural system [31]. Moreover, the cytochromes *c* from *Hyphomicrobium* X showed very low autoreduction rates, suggesting that they were isolated in a native conformation. Therefore it is tempting to speculate that the different results in other systems [2] could be due either to inherent instability of certain cytochromes *c* and/or to the adverse effect of certain isolation procedures or assay conditions on their conformation. The modified conformation could result in high autoreduction rates and in low activity with reduced MDH. With respect to assay conditions, it should be noted that Beardmore-Gray *et al.* [2] used glycerol in their experiments. However, alcohols have been reported to affect both the structure and reactivity of cytochrome *c* [26], possibly as a result of opening of the haem crevice, disruption of the co-ordination of iron with methionine and alteration of the water–protein structure [32]. This could result in higher autoreduction rates and lower reduction rates of ferricytochrome c_L with MDH, as found by Beardmore-Gray *et al.* [2]. Addition of glycerol decreased indeed the reduction rate of ferricytochrome c_L by MDH from *Hyphomicrobium* X (results not shown), so that this compound is not included in the assay mixtures.

Given the high reaction rate at pH 7.0 in the system consisting of equimolar concentrations of MDH and cytochrome c_L from *Hyphomicrobium* X, the intriguing question is still unanswered why the overall reduction rate is so low in an assay for MDH with excess ferricytochrome c_L as electron acceptor. It has been shown that for this system at pH 7.0 methanol oxidation by oxidized MDH is the rate-limiting step in the catalytic cycle. On the other hand, the rate-limiting step of this system at pH 9.0 is the reoxidation of MDH with ferricytochrome c_L ([33]; M. Dijkstra, J. Frank, Jzn. & J. A. Duine, unpublished work).

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