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Studies on electron transfer from methanol dehydrogenase to cytochrome c_L, both purified from *Hyphomicrobium* X

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Ferricytochrome c_L isolated from *Hyphomicrobium* X is an electron acceptor in assays for homologous methanol dehydrogenase (MDH), albeit a poor one compared with artificial dyes. The intermediates of MDH seen during the reaction are identical with those observed with Wurster's Blue as electron acceptor, indicating that the reaction cycles are similar. The assay showed a pH optimum of approx. 7.0 and scarcely any stimulation by NH_4Cl, this being in contrast with assays with artificial dyes, where strong activation by NH_4Cl and much higher pH optima have been reported. From the results obtained with stopped-flow as well as steady-state kinetics, combined with the isotope effects found for C^2H_3OH, it appeared that the dissimilarities between the electron acceptors can be explained from different rate-limiting steps in the reaction cycles. Ferricytochrome c_L is an excellent oxidant of the reduced MDH forms at pH 7.0, but the substrate oxidation step is very slow and the activation by NH_4Cl is very poor at this pH. At pH 9.0 the reverse situation exists: ferricytochrome c_L is a poor oxidant of the reduced forms of MDH at this pH. No C^2H_3OH isotope effect was observed under these conditions, indicating that substrate oxidation is not rate-limiting, so that activation by NH_4Cl cannot be found. Since just the opposite holds for assays with artificial dyes, the poor electron-acceptor capability and the different pH optimum of ferricytochrome c_L as well as the insignificant activating effect of NH_4Cl (all compared with artificial assays) can be explained. Although different views have been reported on the rate-limiting steps in the systems from *Methylophilus methylotrophus* and *Methylobacterium* sp. strain AM1, these are most probably incorrect, as rate-limiting electron transfer between ferrocytochrome c_L and horse heart ferricytochrome *c* can occur. Therefore the conclusions derived for the *Hyphomicrobium* X system might also apply to the systems from other methylotrophic bacteria. Comparison of the assays performed *in vitro* (at pH 7.0) having ferricytochrome c_L and Wurster's Blue as electron acceptor with methanol oxidation by whole cells shows that the former has similarity whereas the latter has not, this being although ferricytochrome c_L is a poor electron acceptor in the assay performed *in vitro*. The reason for this is the absence of a (natural) activator able to activate the (rate-limiting) substrate oxidation step at physiological pH values.

INTRODUCTION

The suitability of ferricytochrome *c* to function as electron acceptor for methanol dehydrogenase (MDH) *in vitro* is a controversial point [1]. Methanol-dependent reduction of cytochrome *c* has been observed in anaerobically prepared cell-free extracts from *Hyphomicrobium* X. When the cell-free extracts were brought into contact with O_2, MDH activity became dependent on the presence of NH_4^+ ions in the assay and methanol-dependent reduction of ferricytochrome *c* was no longer observed, either in the absence or in the presence of NH_4^+ [2]. In other reports, however, it was mentioned that methanol-dependent cytochrome *c* reduction occurred under aerobic conditions if the purified components from *Methylomonas* J [3], *Methylophilus methylotrophus* or *Methylobacterium* sp. strain AM1 [4] were assayed with an excess of horse heart ferricytochrome *c* as final electron acceptor. The turnover rates observed were low (e.g. for the components from *M. methylotrophus* a value of 0.025 s^{-1} at pH 7.0 was given) and the authors ascribed this to the slow reaction between reduced MDH and ferricytochrome c_L [4]. It was further concluded that the mechanism operating in this electron-transfer process is similar to that in the autoreduction of ferricytochrome c_L observed at high pH [5].

In previous work [6], with the purified components from *Hyphomicrobium* X, it was found that the reaction between reduced MDH and ferricytochrome c_L at pH 7.0 is instantaneous, that this reaction is much slower at pH 9.0 and that the (slight) autoreduction of ferricytochrome c_L is caused by a mechanism different from that operating in the reaction with reduced MDH. However, this finding makes the unsuitability of ferricytochrome c_L to function as an electron acceptor for MDH in an assay still more intriguing. Recent studies on MDH with Wurster's Blue, as artificial electron acceptor, have extended and confirmed the proposed catalytic cycle [7]. The rate-limiting step in the catalytic cycle in the absence of activator appeared to be the decomposition reaction

Abbreviations used: PQQ, PQQH· and $PQQH_2$, the quinone, the semiquinone and the quinol forms of 2,7,9-tricarboxy-1*H*-pyrrolo-[2,3-*f*]quinoline-4,5-dione respectively; Wurster's Blue, the free radical of *NNN'N'*-tetramethyl-*p*-phenylenediamine; MDH, methanol dehydrogenase; $MDH_{red.}$, reduced MDH; $MDH_{sem.}$, MDH in the semiquinone state; $MDH_{ox.}$, fully oxidized form of MDH; $MDH_{ox.}$·S, the complex of $MDH_{ox.}$ with substrate; Ches, 2-(*N*-cyclohexylamino)ethanesulphonic acid.

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of the oxidized enzyme–substrate complex ($MDH_{ox.} \cdot S$) into the reduced form of MDH ($MDH_{red.}$) and product [8]. Since this might also apply to the MDH/cytochrome c_L system, it seemed worthwhile to study the 'instantaneous' reaction between $MDH_{red.}$ and the semiquinone form of MDH ($MDH_{sem.}$) and ferricytochrome c_L with stopped-flow kinetic methods. To identify the rate-limiting step in the catalytic cycle and the 'physiological' qualities of the system, steady-state kinetic experiments were performed with MDH and an excess of ferricytochrome c_L as electron acceptor. In addition, the effects of activator, C^2H_3OH and inhibitors on the system were studied with both methods.

MATERIALS AND METHODS

Materials

Wurster's Blue was prepared from *NNN'N'*-tetramethyl-*p*-phenylenediamine as described previously [9]. Deazalumiflavin was kindly given by Dr. J. M. Lhoste. Sephadex gel-filtration PD-10 columns were from Pharmacia. Horse heart cytochrome *c* (type III) and zwitterionic buffers were from Serva (Heidelberg, Germany). All other chemicals were obtained from Janssen Chimica (Beerse, Belgium).

Organism and growth conditions

Hyphomicrobium X was grown in batch culture at 30 °C on a mineral salt medium [9] supplemented with 0.4% (v/v) methanol. Cells were harvested at the end of the exponential growth phase by centrifugation, washed twice with 50 mM-potassium phosphate buffer, pH 7.0, and stored at −20 °C.

MDH and cytochrome c_L

MDH and cytochrome c_L were purified as described previously [6]. MDH was isolated as $MDH_{sem.}$, previously designated as 'MDH_{ox1}', the semiquinone form of the enzyme [10]. $MDH_{red.}$, the fully reduced enzyme [10], was prepared by photoreduction according to the procedure of Massey & Hemmerich for flavoproteins [11]. To 3 ml of $MDH_{sem.}$ in 50 mM-Mops/NaOH buffer, pH 7.0, were added 50 μl of 0.2 M-EDTA and 5 μl of 1 mM-deazalumiflavin in dimethyl sulphoxide. The mixture was made anaerobic by flushing with argon (< 3 p.p.m. of O_2) for 15 min and irradiated for 2–3 min with a 100 W halogen lamp at a distance of 20 cm. Low-M_r contaminants were removed by overnight dialysis at 4 °C.

Determination of MDH, cytochrome c_L and horse heart cytochrome *c*

The amounts of $MDH_{sem.}$ and $MDH_{red.}$ were calculated by using a specific absorption coefficient of 2.02 litre·g^{-1}·cm^{-1} at 280 nm, determined according to the chromatographic procedure of Van Iersel *et al.* [12]. Specific absorption coefficients for cytochrome c_L (2.02 litre·g^{-1}·cm^{-1}) and horse heart cytochrome *c* (1.95 litre·g^{-1}·cm^{-1}) were from the literature [6,13]. Specific absorption coefficients at other wavelengths were determined from the ratio of the absorbance at that wavelength to that at 280 nm. Molar absorption coefficients were calculated from the specific absorption coefficients, by using M_r values of 120000 for MDH, 19600 for cytochrome c_L and 12800 for horse heart cytochrome *c*.

Calculation of absorption spectra

Absorption spectra were measured with a Hewlett–Packard HP 8450A photodiode-array spectrophotometer at 20 °C. The spectra of MDH in a reaction mixture with cytochrome c_L were corrected for the presence of oxidized and reduced cytochrome c_L by calculating their amounts with multicomponent analysis in the wavelength region 492–566 nm, by using the genuine spectra of cytochrome c_L [6] and assuming a negligible contribution of MDH to the absorption in that region. Multicomponent analysis was carried out with the software supplied with the spectrophotometer.

Steady-state kinetics

Specific activities of MDH with Wurster's Blue as electron acceptor were determined as described previously, except that ethanol was replaced by methanol [14]. MDH activity with ferricytochrome c_L as electron acceptor was measured in 1.0 ml assay mixtures containing CH_3OH or C^2H_3OH (1.0 μmol) and ferricytochrome c_L (5.3 nmol) in the appropriate buffer. Reactions were started by adding 0.28 nmol of MDH in the same buffer. Initial reaction rates were measured by monitoring the increase in absorbance at 550 nm. Absorption coefficients at 550 nm of 21.6 mM^{-1}·cm^{-1} for ferrocytochrome c_L and 6.7 mM^{-1}·cm^{-1} for ferricytochrome c_L were used in the calculations. Apparent kinetic parameters for the reduction of ferricytochrome c_L by MDH in the presence of various concentrations of NH_4Cl at pH 7.0 were determined by the direct-linear-plot method [15]. Before use, cytochrome c_L was oxidized with a small excess of $K_3Fe(CN)_6$ or reduced with $Na_2S_2O_4$. After that, contaminants were removed by passing the mixture through a PD-10 gel-filtration column, equilibrated with the appropriate buffer.

Stopped-flow kinetic measurements

Stopped-flow experiments were performed at 20.1 °C with an HI-Tech SF 50 stopped-flow spectrophotometer equipped with a beam-splitter and two monochromator photomultiplier assemblies. Data acquisition was performed with a 100 kHz DASH 16F A/D converter controlled by an Olivetti M24 SP computer. Reduction of ferricytochrome c_L by $MDH_{red.}$ and $MDH_{sem.}$ was monitored at 418 and 550 nm (at the latter wavelength the absorption of MDH is negligible). Oxidation of $MDH_{sem.}$ and $MDH_{red.}$ was measured at 337 nm, an isosbestic point for redox conversion of cytochrome c_L. For the determination of the kinetic constants, $MDH_{red.}$ and $MDH_{sem.}$ concentrations (indicated in the Figures as catalytic sites concentrations since MDH is a bifunctional dimer) up to 40-fold compared to ferricytochrome c_L were used.

Pseudo-first-order rate constants were calculated by non-linear regression with the use of a Gauss–Newton algorithm, available with ASYST (Keithley). The data used were the average of at least four experiments (the reproducibility achieved was normally within 6%). Those parts of the curves were used encompassing over at least 4 half-lives. More complex reaction curves were analysed by numerical integration with PSI, an interactive simulation program [16].

Experiments at pH 7.0 and pH 9.0 were carried out by mixing MDH in 10 mM-Mops/NaOH buffer, pH 7.0, and 10 mM-Ches/NaOH buffer, pH 9.0, respectively

(with or without 0.2 M-NaCl), with an equal volume of ferricytochrome c_L in the same buffer. To exclude interference of the substrate-oxidation step in the catalytic cycle (due to contamination with small amounts of alcohols, formaldehyde or endogenous substrate [4,17]) measurements were performed in the presence of 1 mM-C^2H_3OH, ensuring a rate limitation of this step ([18]; the present work). The effects of several compounds were examined: EDTA (0.1 mM and 1.0 mM); potassium phosphate (0.2 M); NaCl (1.0 mM), $MgCl_2$ (0.2 mM and 1.0 mM); NH_4Cl (1.0 mM). Concentrations mentioned in stopped-flow experiments were the final concentrations after mixing.

Electron transfer from cytochrome c_L to horse heart cytochrome *c*

Differences in absorption, measured at 548 and 532 nm, were used to determine the rate of electron transfer between ferrocytochrome c_L (2.75 μM) and horse heart ferricytochrome *c* in 50 mM-Mops/NaOH buffer, pH 7.0. These wavelengths appeared to be most suitable, as determined from difference spectra [(ferricytochrome c_L + ferrocytochrome *c*) − (ferrocytochrome c_L + ferricytochrome *c*)], as described for other cytochromes *c* by König *et al.* [19]. The following concentrations of horse heart cytochrome *c* were used: 20.6 μM, 30.9 μM, 41.8 μM, 51.4 μM and 61.8 μM. The molar absorption coefficients at 532 nm and 548 nm, calculated as described above, were for oxidized and reduced cytochrome c_L 9.6 and 7.8 $mM^{-1} \cdot cm^{-1}$ at 532 nm and 7.0 and 19.2 $mM^{-1} \cdot cm^{-1}$ at 548 nm respectively. For oxidized and reduced horse heart cytochrome *c* the values were 9.9 and 6.2 $mM^{-1} \cdot cm^{-1}$ at 532 nm and 7.4 and 22.8 $mM^{-1} \cdot cm^{-1}$ at 548 nm respectively.

RESULTS

Reduction of horse heart ferricytochrome *c* by ferrocytochrome c_L

In studies on cytochrome c_L as electron acceptor for MDH, assays have been reported that use catalytic amounts of cytochrome c_L and large amounts of horse heart ferricytochrome *c* as final electron acceptor [3,4]. However, since the rate constant for this reaction was not given, uncertainty existed about the reliability of the system. Investigations on the electron-transfer rates between ferrocytochrome c_L from *Hyphomicrobium* X and horse heart ferricytochrome *c* showed that the rates are very low: at all concentrations of ferricytochrome *c* investigated, a pseudo-first-order rate of 0.005 s^{-1} was observed, indicating rapid complex-formation and rate-limiting electron transfer between both cytochromes. Thus, to avoid complications due to the introduction of an external rate-limiting step, horse heart cytochrome *c* was omitted and an excess of ferricytochrome c_L was used in all assays of MDH.

Steady-state kinetics

Experiments at different pH values showed that the pH optimum is 7.0, the rate at pH 9.0 being 4 times lower than that at pH 7.0 (Table 1). Inclusion of NH_4Cl in the assay mixtures increased the rates by 2–3-fold at all pH values. At pH 7.0 the stimulation by NH_4Cl (K_m 0.9 mM) was maximal at about 20 mM (with a rate of 0.5 nmol of cytochrome c_L reduced/s per nmol of MDH), higher concentrations causing a decline in activity. Other salts tested appeared to be inhibitory, some of them even at low concentrations: NaCl (20 mM, 60% inhibition); $MnCl_2$ (0.05 mM, 22% inhibition); $MgCl_2$ (0.5 mM, 22% inhibition).

With C^2H_3OH as substrate, appreciably lower reaction rates were observed compared with those with CH_3OH (Table 2). In the absence of NH_4Cl a 6.8-fold decrease and in its presence (0.8 mM) a 3.9-fold decrease were observed. Such an isotope effect was absent in the assays at pH 9.0.

By recording spectra during the reaction, and correcting them for the presence of oxidized and reduced cytochrome c_L (Fig. 1), intermediates of MDH were detected in the catalytic cycle, since the spectra were different from those of $MDH_{red.}$ and $MDH_{sem.}$ [8,10] and could not be fitted by mixtures of the two. The spectra depicted in Fig. 1 strongly resemble that of an intermediate found in studies on the reaction of MDH with

Table 1. Cytochrome c_L-linked MDH activity at various pH values

MDH activity in an assay with ferricytochrome c_L as electron acceptor was measured as described in the Materials and methods section. The following concentrations were used: MDH, 0.28 μM (specific activity 10.9 μmol of Wurster's Blue/min per mg of protein); ferricytochrome c_L, 5.3 μM; methanol, 1.0 mM; NH_4Cl, 20 mM.

Buffer	pH	Rate (nmol of cytochrome c_L reduced/s per nmol of MDH)	
		$-NH_4Cl$	$+NH_4Cl$
50 mM-Mops	6.0	0.045	0.226
50 mM-Mops	7.0	0.079	0.237
50 mM-Hepes	8.0	0.057	0.151
50 mM-Tricine	8.0	0.049	0.112
50 mM-Ches	9.0	0.015	0.046
100 mM-Sodium borate	9.0	0.010	0.022

Table 2. Isotope effects on the steady-state kinetics at pH 7.0 and pH 9.0

MDH activity in assays with ferricytochrome c_L as electron acceptor was measured as described in the Materials and methods section. The following components and concentrations were used: MDH, 0.28 μM (specific activity 13.8 μmol of Wurster's Blue/min per mg of protein); ferricytochrome c_L, 5.3 μM; CH_3OH or C^2H_3OH, 1.0 mM; NH_4Cl, 0.8 mM. Buffers used were 50 mM-Mops buffer, pH 7.0, and 50 mM-Ches buffer, pH 9.0. Values in parentheses are those obtained at pH 9.0.

Substrate	$10^2 \times$ Rate (nmol of cytochrome c_L reduced/s per nmol of MDH)	
	$-NH_4Cl$	$+NH_4Cl$
CH_3OH	10.0 (1.9)	14.2 (3.0)
C^2H_3OH	1.5 (2.0)	3.6 (2.8)

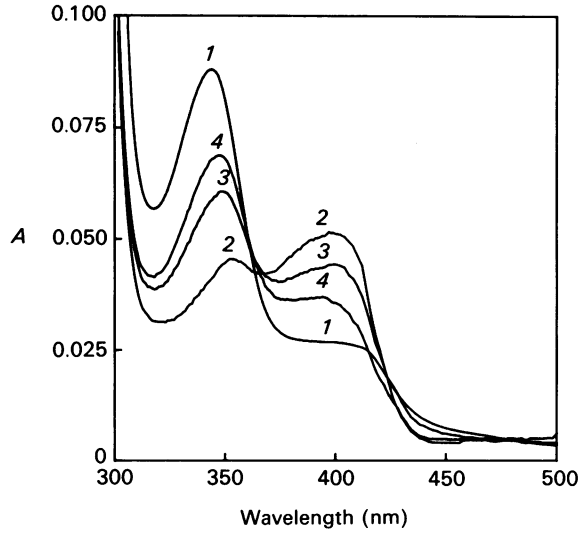


Fig. 1. Spectra of a mixture of MDH species observed during the reaction of $MDH_{red.}$ with ferricytochrome c_L and CH_3OH at pH 7.0

The mixture consisted of Mops buffer, pH 7.0 (50 mM), $MDH_{red.}$ (2.3 μM), ferricytochrome c_L (5.1 μM) and CH_3OH (1 mM). The reaction was started by adding $MDH_{red.}$. The sum of the spectra of MDH species was calculated from the recorded spectra by correcting them for the presence of oxidized and reduced cytochrome c_L. Spectrum *1* represents the spectrum of $MDH_{red.}$ in the assay mixture in the absence of cytochrome c_L. The time elapsed after mixing was 36 s (spectrum *2*), 72 s (spectrum *3*) and 96 s (spectrum *4*).

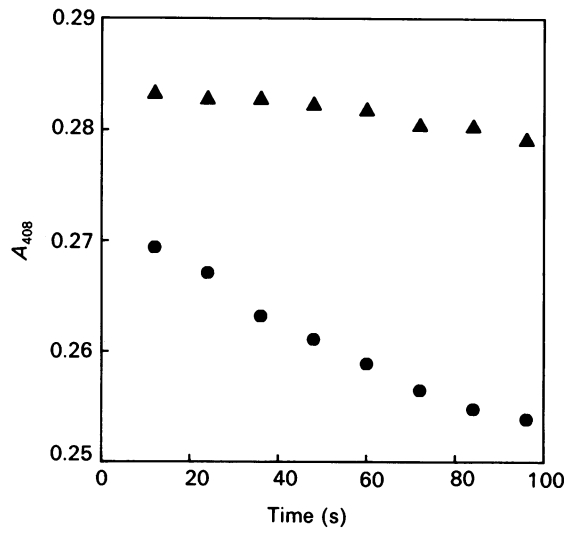


Fig. 2. Reaction progress curves for the reaction of $MDH_{red.}$ with ferricytochrome c_L and CH_3OH or C^2H_3OH

Reaction progress curves at 408 nm were recorded after the addition of $MDH_{red.}$ (0.28 μM) to the reaction mixture described in the legend to Fig. 1, for CH_3OH (●) and for C^2H_3OH (▲). The absorbance traces were constructed from a series of spectra taken at 10 s intervals with a measuring time of 2 s.

Wurster's Blue as electron acceptor [8] and which was identical with a complex of $MDH_{ox.}$ and substrate ($MDH_{ox.}$·S). With C^2H_3OH as substrate the spectra were identical but the lifetime of the intermediate was appreciably longer, as is also obvious from the absorbance traces at 408 nm (Fig. 2), a wavelength that is an isosbestic point for the cytochrome c_L redox conversion.

Stopped-flow kinetics

On analysing the reaction curves measured at 337 nm (specific for $MDH_{red.}$ and $MDH_{sem.}$ oxidation) and 550 nm (specific for ferricytochrome c_L reduction) in 10 mM-Mops buffer, pH 7.0, single exponentials with the same pseudo-first-order rate constants were found. Therefore, for practical reasons, reactions of MDH with ferricytochrome c_L could be and were performed at 418 nm, the wavelength with the largest absorbance amplitude.

At pH 7.0 under the conditions used (excess $MDH_{red.}$ or $MDH_{sem.}$, 10 mM-Mops buffer), the reduction of ferricytochrome c_L was monophasic and obeyed pseudo-first-order kinetics. Within the concentration range of $MDH_{red.}$ or $MDH_{sem.}$ applied, no limiting value of $k_{obs.}$ was reached (Fig. 3). From the slope of the plot of the pseudo-first-order rate constants ($k_{obs.}$) versus the concentration of the MDH catalytic sites (Fig. 3) the bimolecular rate constants were determined: $(1.9\pm0.4)\times10^5$ $M^{-1}\cdot s^{-1}$ for $MDH_{red.}$ (k_{+1}) and $(2.1\pm0.4)\times10^5$ $M^{-1}\cdot s^{-1}$ for $MDH_{sem.}$ (k_{+2}). In the presence of 0.2 M-NaCl the rate constant decreased to 6000 ± 1200 $M^{-1}\cdot s^{-1}$ for $MDH_{red.}$ and to 3200 ± 670 $M^{-1}\cdot s^{-1}$ for $MDH_{sem.}$. Not only NaCl but also high concentrations of potassium phosphate (0.2 M) were inhibitory (approx. 99 % inhibition).

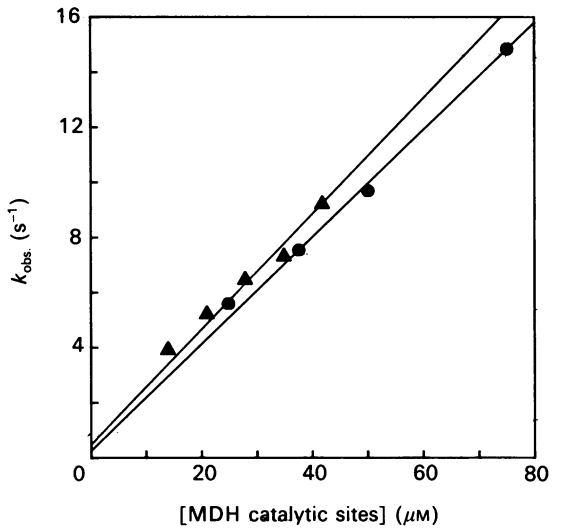


Fig. 3. Kinetics of the oxidation of $MDH_{red.}$ and $MDH_{sem.}$ by ferricytochrome c_L at pH 7.0

The pseudo-first-order rate constants ($k_{obs.}$) were determined with the stopped-flow kinetic method and the values were plotted as a function of the concentration of the catalytic sites of $MDH_{red.}$ (●) and $MDH_{sem.}$ (▲). The reactions were performed in 10 mM-Mops buffer, pH 7.0, containing 1 mM-C^2H_3OH, as described in the Materials and methods section. The ferricytochrome c_L concentration was 2.0 μM.

At pH 9.0 (10 mM-Ches buffer) a quite different picture emerged. The reaction-rate progress curves appeared to be biphasic but they could be fitted with two exponentials.

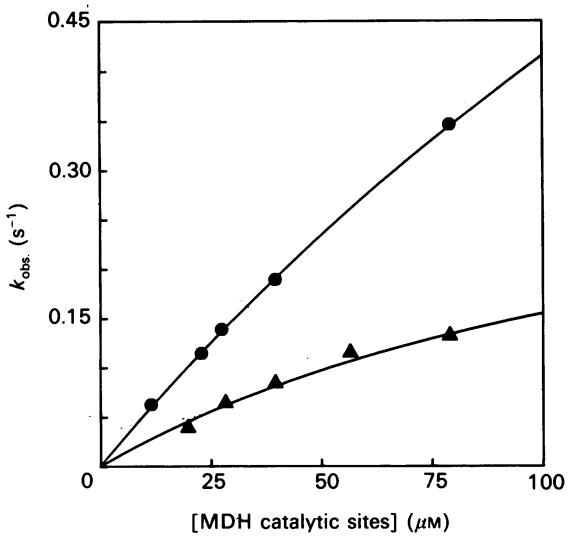


Fig. 4. Kinetics of the oxidation of $MDH_{red.}$ and $MDH_{sem.}$ by ferricytochrome c_L at pH 9.0 in the presence of NaCl

The pseudo-first-order rate constants ($k_{obs.}$) were determined with the stopped-flow kinetic method and the values were plotted as a function of the concentration of the catalytic sites of $MDH_{red.}$ (●) and $MDH_{sem.}$ (▲). The reactions were performed in 10 mM-Ches buffer, pH 9.0, containing 0.2 M-NaCl and 1 mM-C^2H_3OH, as described in the Materials and methods section. The ferricytochrome c_L concentration was 2.0 μM. The curves depicted were simulated by using the values for K_a, k_{+1b} and k_{+2b} from Table 3.

Similar biphasic kinetics at low ionic strength has also been observed for the reduction of *c*-type cytochromes with flavodoxin [20,21] and for the ascorbate- and dithionite-mediated reduction of horse heart cytochrome *c* [22,23] at pH 7.0. It was described to be due to the existence of two conformers of cytochrome *c*. The rate constants for the fast phase increased proportionally with the concentrations of MDH until a maximum value was reached. In the presence of 0.2 M-NaCl the reaction-rate progress curves become monophasic. This may be due to a higher interconversion rate between the two conformers, to a shift in their equilibrium or to a comparable reduction rate of both conformers with MDH at high ionic strength. The plot of $k_{obs.}$ versus the concentration of the catalytic sites showed saturation (Fig. 4), indicating that electron transfer is rate-limiting rather than complex-formation.

Table 3. Rates and association constants for the oxidation of $MDH_{red.}$ and $MDH_{sem.}$ with ferricytochrome c_L at pH 9.0

The $k_{obs.}$ values for the oxidation of $MDH_{red.}$ and $MDH_{sem.}$ with ferricytochrome c_L at pH 9.0 were obtained as described in the legend to Fig. 4. By using the equations given in the text and applying non-linear regression (Marquardt's algorithm), association constants (K_a values) and electron-transfer rate constants (k_{+1b} and k_{+2b}) could be calculated. Values in parentheses are those obtained in the presence of NaCl (0.2 M).

MDH form	k_{+1b} or k_{+2b} (s^{-1})	$10^{-3} \times K_a$ (M^{-1})
$MDH_{red.}$	0.33±0.07 (1.8±0.15)	8.2±2.1 (3.0±0.3)
$MDH_{sem.}$	0.23±0.04 (0.38±0.07)	8.0±2.4 (6.9±2.1)

The reaction between ferricytochrome c_L and $MDH_{red.}$ or $MDH_{sem.}$ is visualized in the following reaction sequences {as the redox potential of cytochrome c_L (+270 mV, [6]) is probably much higher than that of $MDH_{red.}$ and $MDH_{sem.}$ (the redox potential of the PQQ/$PQQH_2$ couple being +90 mV), k_{-1b} and k_{-2b} will be very small so that they can be neglected}:

$$\text{cyt. } c_L^{ox.} + MDH_{red.} \underset{k_{-1a}}{\overset{k_{+1a}}{\rightleftharpoons}} \text{cyt. } c_L^{ox.} \cdot MDH_{red.} \xrightarrow{k_{+1b}} \text{cyt. } c_L^{red.} \cdot MDH_{sem.}$$

and

$$\text{cyt. } c_L^{ox.} + MDH_{sem.} \underset{k_{-2a}}{\overset{k_{+2a}}{\rightleftharpoons}} \text{cyt. } c_L^{ox.} \cdot MDH_{sem.} \xrightarrow{k_{+2b}} \text{cyt. } c_L^{red.} \cdot MDH_{ox.}$$

Applying the steady-state approximation in accordance with Hiromi [24] and assuming that ferricytochrome c_L, reduced MDH and the complex are in rapid binding equilibrium (i.e. k_{-1a}, $k_{-2a} \gg k_{+1b}$, k_{+2b}), the following hyperbolic functions can be derived:

$$k_{obs.} = \frac{k_{+1a} \cdot k_{+1b} \cdot [MDH_{red.}]}{k_{+1a} \cdot [MDH_{red.}] + k_{-1a}}$$

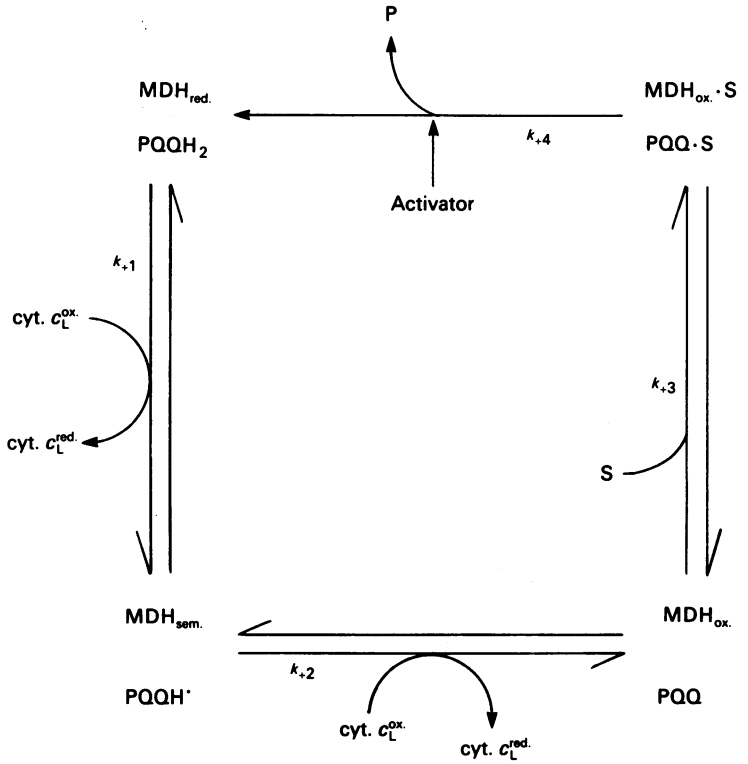
and

$$k_{obs.} = \frac{k_{+2a} \cdot k_{+2b} \cdot [MDH_{sem.}]}{k_{+2a} \cdot [MDH_{sem.}] + k_{-2a}}$$

With non-linear-regression analysis of the measured points (Marquardt's algorithm), the association constants K_a (k_{+1a}/k_{-1a} and k_{+2a}/k_{-2a}) and the electron-transfer first-order rate constants (k_{+1b} and k_{+2b}) could be determined (Table 3). As is apparent from Table 3, the association constants of $MDH_{red.}$ and $MDH_{sem.}$ are more-or-less similar. Whereas high concentrations of NaCl were inhibitory at pH 7.0, an increase of the electron-transfer rates (k_{+1b} and k_{+2b}) was observed at pH 9.0 (Table 3). Addition of low concentrations of NH_4Cl (1.0 mM) as well as NaCl led to an increase of the pseudo-first-order rate constants by 1.4-fold at pH 7.0 and by 1.8-fold at pH 9.0 ($MDH_{red.}$ and $MDH_{sem.}$ concentrations used were 37.5 μM). Addition of C^2H_3OH to the reaction mixtures had no effect, either at pH 7.0 or at pH 9.0. EDTA (0.1 mM) inhibited the oxidation of $MDH_{red.}$ and $MDH_{sem.}$ almost completely (95–99 %) at pH 7.0. Subsequent addition of up to 1.0 mM-$MgCl_2$ had only a slight effect, decreasing the inhibition by EDTA to 84 %. $MgCl_2$ (0.2 mM) as such enhanced the pseudo-first-order rate constants by 1.4-fold as was also observed for NH_4Cl and NaCl. Adding $MgCl_2$ before the addition of EDTA did not change the extent of the inhibition.

DISCUSSION

The present work shows that ferricytochrome c_L is an electron acceptor in the assay for MDH, albeit poor. The spectra of the MDH intermediates occurring during the oxidation of MDH with ferricytochrome c_L are the same as observed with the artificial electron acceptor Wurster's Blue, so that a similar scheme is proposed (Scheme 1) as



Scheme 1. Reaction cycle of MDH with ferricytochrome c_L as electron acceptor

The scheme refers to a single catalytic site of dimeric MDH. S represents substrate and P represents product.

has been deduced for the latter [7,8]. However, significant differences exist with respect to the rate-limiting step in the cycles, as is discussed in the following paragraphs.

The catalytic cycle of MDH at pH 7.0 and pH 9.0 with different electron acceptors

Two conditions are required for efficient turnover: (i) rapid oxidation of MDH$_{red.}$ and MDH$_{sem.}$ by ferricytochrome c_L or Wurster's Blue and (ii) rapid intramolecular oxidation of substrate in the enzyme–substrate complex (MDH$_{ox.}$·S). Depending on the oxidant, pH, ionic strength and activator, the oxidation of MDH$_{red.}$ and MDH$_{sem.}$ or the substrate oxidation becomes rate-limiting.

(i) From the stopped-flow experiments it appeared that ferricytochrome c_L is an excellent oxidant of MDH$_{red.}$ and MDH$_{sem.}$ at pH 7.0 (Fig. 3), but a rather poor oxidant at pH 9.0 (Fig. 4 and Table 3). The slower oxidation of MDH$_{red.}$ and MDH$_{sem.}$ at the latter pH could result from the stronger negative charge of cytochrome c_L (pI 4.3; [6]) and MDH (pI approx. 6.5; M. Dijkstra, J. Frank, Jzn. & J. A. Duine, unpublished work) at this pH, which could prevent an adequate interaction between the two proteins. At pH 9.0 $k_{obs.}$ reaches a limiting value, which can be explained by assuming that in the MDH·cytochrome c_L complex geometrical reorientation or electron transfer becomes rate-limiting.

High salt concentrations were inhibitory at pH 7.0, but slightly stimulatory at pH 9.0 (Table 3). Since low concentrations of NH_4Cl as well as NaCl were stimulatory at both pH values, a specific role of NH_4Cl as activator for these steps can be excluded. At pH 7.0 low concentrations of EDTA inhibited the oxidation of MDH$_{red.}$ and MDH$_{sem.}$ nearly completely. This inhibition was not reversed by the subsequent addition of Mg^{2+} ions, indicating that one of the components has a site with a high affinity for EDTA. This might be related to the dramatic effect of EDTA and high salt concentrations on the turnover of methanol *in vitro* [4] as well as *in vivo* [25,26], although it should be admitted that the inhibition in the latter case could be largely suppressed by the addition of Mg^{2+} ions [26].

The reverse holds for Wurster's Blue as an oxidant. This compound is an excellent oxidant of reduced MDH at pH 9.0, but a very poor one at pH 7.0 (a 100-fold lower rate compared with ferricytochrome c_L [8]).

(ii) Measurements under steady-state conditions showed that the turnover at pH 9.0 was slower than that at pH 7.0 (Table 1), which can be ascribed to the fact that oxidation of MDH$_{red.}$ and MDH$_{sem.}$ becomes rate-limiting. The absence of a deuterium isotope effect at pH 9.0 (Table 2), indicating that substrate conversion (k_{+4}; Scheme 1) is not rate-limiting, supports this view.

Although NH_4Cl enhanced the overall rate of reduction of ferricytochrome c_L at pH 7.0 and pH 9.0 (Table 1), only at pH 7.0 did it act as a real activator. This increase in the overall rate was related to the substrate oxidation, as was shown by a decrease of the deuterium isotope effect from 6.8 to 3.9 (Table 2). All these observations are in agreement with the view that substrate conversion (k_{+4}; Scheme 1) is the rate-limiting step at pH 7.0 in a system with ferricytochrome c_L as electron acceptor.

Here too the reverse holds with Wurster's Blue as electron acceptor. Since substrate oxidation is the rate-

limiting step at pH 9.0 and NH_4Cl is an activator at this pH, a substantial increase in the rate is observed on addition of NH_4Cl, whereas only a slight non-specific salt effect is observed at pH 7.0 (the oxidation of reduced MDH probably becomes rate-limiting [8]).

Comparison with the components from other organisms

Quite different results were reported for assays with the purified components from *Methylobacterium* sp. strain AM1 and *Methylophilus methylotrophus* [4]. It should be noted, however, that Beardmore-Gray *et al.* [4] used equimolar concentrations of reduced MDH and cytochrome c_L and that this reaction was monitored with an excess of horse heart ferricytochrome *c*. However, the reaction between horse heart ferricytochrome *c* and ferrocytochrome c_L was not investigated. Our experiments indicate a rather slow electron transfer between horse heart ferricytochrome *c* and ferrocytochrome c_L from *Hyphomicrobium* X. The pseudo-first-order rate constant ($k_{obs.} = 0.005\ s^{-1}$), was independent of the concentration of horse heart cytochrome *c* used in this study, indicating rapid complex-formation and rate-limiting electron transfer between both cyctochromes. Although it cannot be excluded that the rate constant for the reaction between *Methylobacterium* sp. strain AM1 ferrocytochrome c_L and horse heart ferricytochrome *c* is higher than that of the *Hyphomicrobium* X system, it is not unlikely that it is also the rate-limiting step in that system, so that the real reduction rate of ferricytochrome c_L was not observed.

The rate-limiting electron transfer between horse heart ferricytochrome *c* and ferrocytochrome c_L could also explain the observation made by Beardmore-Gray *et al.* [4] that the K_m and $V_{max.}$ values were similar for methanol and the endogenous substrate in the assays with components from *Methylophilus methylotrophus*, rendering incorrect their conclusion that electron transfer from reduced MDH to ferricytochrome c_L is the rate-limiting step.

Ferricytochrome c_L as electron acceptor *in vivo*

In *Hyphomicrobium* X both MDH and cytochrome c_L constitute about 10% of the soluble protein [6,9]. Assuming that both proteins are located in the periplasmic space [27–29], which is about 20% of the total cell volume [4], then the concentrations of MDH (catalytic sites) and cytochrome c_L are 0.5 mM and 1.6 mM respectively. From the bimolecular rate constant of $1.9 \times 10^5\ M^{-1} \cdot s^{-1}$ derived in the present study, the cytochrome c_L reduction rate will be 9.6 μmol of cytochrome c_L reduced/min per mg of cells or 4.8 μmol of O_2 reduced/min per mg of cells. Since an average consumption of 0.4–0.6 μmol of O_2/min per mg of cells has been found for methanol oxidation by whole bacteria [30], it is clear that the bimolecular rate constants of the oxidation of MDH (k_{+1} and k_{+2}; Scheme 1) with ferricytochrome c_L observed *in vitro* can explain the physiological turnover rates. As discussed above, at pH 7.0 the overall rate of the assay *in vitro* is very low, since the rate-limiting step in the cycle is the slow decomposition of the $MDH_{ox.} \cdot S$ complex. Recently we reported the existence of a low-M_r component capable of enhancing the rate-limiting step with ferricytochrome c_L as electron acceptor at pH 7.0 [31,32]. This factor might be the 'natural' activator for MDH, activating the substrate oxidation step *in vitro* at pH 7.0 (k_{+4}; Scheme 1) to a rate comparable with that observed *in vivo*.

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