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

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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 $\text{NH}_4^+$ ions in the assay and methanol-dependent reduction of ferricytochrome $c$ was no longer observed, either in the absence or in the presence of $\text{NH}_4^+$ [2]. In other reports, however, it was mentioned that methanol-dependent cytochrome $c$ reduction occurred under aerobic conditions if the purified components from *Methylophilus methylotrophus* and *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...
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\textsubscript{6}H\textsubscript{12}OH 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]. Deazaflavin 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_{ox}’ or the semiquinone form of the enzyme [10]. MDH_{red}, the fully reduced enzyme [10], was prepared by photo-reduction 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-deazaflavin 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_{s} 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 \cdot g^{-1} \cdot 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 \cdot g^{-1} \cdot cm^{-1}) and horse heart cytochrome c (1.95 litre \cdot g^{-1} \cdot 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_{s} 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_{3}OH or C\textsubscript{6}H\textsubscript{12}OH (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} \cdot cm^{-1} for ferrocyanochrome 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_{4}Cl 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_{2}Fe(CN)_{6} or reduced with Na_{2}S_{2}O_{3}. 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 26F 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.
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(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$_2$H$_5$OH, 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 mM); NaCl (1.0 mM), MgCl$_2$ (0.2 mM and 1.0 mM); NH$_4$Cl (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 $\mu$M) 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 ([(ferrocytochrome $c_L$ + 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 $\mu$M, 30.9 $\mu$M, 41.8 $\mu$M, 51.4 $\mu$M and 61.8 $\mu$M. 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$^{-1}$ cm$^{-1}$ at 532 nm and 7.0 and 19.2 mm$^{-1}$ cm$^{-1}$ at 548 nm respectively. For oxidized and reduced horse heart cytochrome $c$ the values were 9.9 and 6.2 mm$^{-1}$ cm$^{-1}$ at 532 nm and 7.4 and 22.8 mm$^{-1}$ 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$_4$Cl in the assay mixtures increased the rates by 2–3-fold at all pH values. At pH 7.0 the stimulation by NH$_4$Cl ($K_m$ 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$_2$H$_5$OH as substrate, appreciably lower reaction rates were observed compared with those with CH$_3$OH (Table 2). In the absence of NH$_4$Cl a 6.8-fold decrease in and 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 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

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>$-\text{NH}_4\text{Cl}$</th>
<th>$+\text{NH}_4\text{Cl}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM-Mops</td>
<td>6.0</td>
<td>0.045</td>
<td>0.226</td>
</tr>
<tr>
<td>50 mM-Mops</td>
<td>7.0</td>
<td>0.079</td>
<td>0.237</td>
</tr>
<tr>
<td>50 mM-Hepes</td>
<td>8.0</td>
<td>0.057</td>
<td>0.151</td>
</tr>
<tr>
<td>50 mM-Tricine</td>
<td>8.0</td>
<td>0.049</td>
<td>0.112</td>
</tr>
<tr>
<td>50 mM-Ches</td>
<td>9.0</td>
<td>0.015</td>
<td>0.046</td>
</tr>
<tr>
<td>100 mM-Sodium borate</td>
<td>9.0</td>
<td>0.010</td>
<td>0.022</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$10^3$ Rate (nmol of cytochrome $c_L$ reduced/s per nmol of MDH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{CH}_3\text{OH}$</td>
<td>$-\text{NH}_4\text{Cl}$</td>
</tr>
<tr>
<td>C$_2$H$_5$OH</td>
<td>10.0 (1.9)</td>
</tr>
<tr>
<td>C$_2$H$_5$OH</td>
<td>1.5 (2.0)</td>
</tr>
</tbody>
</table>

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Fig. 1. Spectra of a mixture of MDH species observed during the reaction of MDHred. with ferricytochrome cL and CH₃OH at pH 7.0

The mixture consisted of Mops buffer, pH 7.0 (50 mM), MDHred. (2.3 μM), ferricytochrome cL (5.1 μM) and CH₃OH (1 mM). The reaction was started by adding MDHred.. 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 cL. Spectrum / represents the spectrum of MDHred. in the absence of cytochrome cL. The time elapsed after mixing was 36 s (spectrum 2), 72 s (spectrum 3) and 96 s (spectrum 4).

Wurster’s Blue as electron acceptor [8] and which was identical with a complex of MDHox. and substrate (MDHox..S). With C₂H₃OH 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 cL. redox conversion.

Stopped-flow kinetics

On analysing the reaction curves measured at 337 nm (specific for MDHred. and MDHsem. oxidation) and 550 nm (specific for ferricytochrome cL 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 cL could be and were performed at 418 nm, the wavelength with the largest absorbance amplitude.

At pH 7.0 under the conditions used (excess MDHred. or MDHsem., 10 mM-Mops buffer), the reduction of ferricytochrome cL was monophasic and obeyed pseudo-first-order kinetics. Within the concentration range of MDHred. or MDHsem. applied, no limiting value of kobs. was reached (Fig. 3). From the slope of the plot of the pseudo-first-order rate constants (kobs.) versus the concentration of the MDH catalytic sites (Fig. 3) the bimolecular rate constants were determined: (1.9 ± 0.4) x 10⁵ M⁻¹·s⁻¹ for MDHred. (k₁₁) and (2.1 ± 0.4) x 10⁴ M⁻¹·s⁻¹ for MDHsem. (k₁₂). In the presence of 0.2 mM-NaCl the rate constant decreased to 6000 ± 1200 M⁻¹·s⁻¹ for MDHred. and to 3200 ± 670 M⁻¹·s⁻¹ for MDHsem.. Not only NaCl but also high concentrations of potassium phosphate (0.2 M) were inhibitory (approx. 99% inhibition).

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.
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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$_2$H$_5$OH, 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.

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 PQ/ PQQ$_4$ couple being +90 mV), $k_{+1b}$ and $k_{+2b}$ will be very small so that they can be neglected):

$$
\text{cyt. } c_L^{ox} + \text{MDH}_{red} \overset{k_{+1a}}{\longrightarrow} \text{cyt. } c_L^{ox} \cdot \text{MDH}_{red}.
$$

and

$$
\text{cyt. } c_L^{ox} + \text{MDH}_{sem} \overset{k_{+1b}}{\longrightarrow} \text{cyt. } c_L^{ox} \cdot \text{MDH}_{sem}.
$$

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. $k_{-1a}$, $k_{-2a}$ > $k_{+1b}$, $k_{+2b}$), the following hyperbolic functions can be derived:

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

and

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

With non-linear-regression analysis of the measured points (Marquardt's algorithm), the association constants ($K_a$ values) and electron-transfer 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$_4$Cl (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$_2$H$_5$OH 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$_4$Cl 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

<table>
<thead>
<tr>
<th>MDH form</th>
<th>$k_{+1b}$ or $k_{+2b}$ (s$^{-1}$)</th>
<th>$10^{-3} \times K_a$ (M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDH$_{red}$</td>
<td>0.33 ± 0.07 (1.8 ± 0.15)</td>
<td>8.2 ± 2.1 (3.0 ± 0.3)</td>
</tr>
<tr>
<td>MDH$_{sem}$</td>
<td>0.23 ± 0.04 (0.38 ± 0.07)</td>
<td>8.0 ± 2.4 (6.9 ± 2.1)</td>
</tr>
</tbody>
</table>

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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]; 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_{4}Cl as well as NaCl were stimulatory at both pH values, a specific role of NH_{4}Cl 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_{4}Cl 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-
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limiting step at pH 9.0 and NH$_4$Cl is an activator at this pH, a substantial increase in the rate is observed on addition of NH$_4$Cl, 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 *Methylphilus methyloptrophus* [4]. It should be noted, however, that Beardmore-Gray et al. [4] used equimolar concentrations of reduced MDH and cytochrome $c_5$ and that this reaction was monitored with an excess of horse heart ferricytochrome $c$. However, the reaction between horse heart ferricytochrome $c$ and ferrocytochrome $c_5$ was not investigated. Our experiments indicate a rather slow electron transfer between horse heart ferricytochrome $c$ and ferrocytochrome $c_5$ from *Hyphomicrobium* X. The pseudo-first-order rate constant ($k_{\text{obs}} = 0.055 \text{s}^{-1}$), 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 cytochromes. Although it cannot be excluded that the rate constant for the reaction between *Methylphilus methyloptrophus* sp. strain AM1 ferrocytochrome $c_5$ 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 $c_5$ was not observed.

The rate-limiting electron transfer between horse heart ferricytochrome $c$ and ferrocytochrome $c_5$ could also explain the observation made by Beardmore-Gray et al. [4] that the $K_m$ and $V_{\text{max}}$ values were similar for methanol and the endogenous substrate in the assays with components from *Methylphilus methyloptrophus*, rendering incorrect their conclusion that electron transfer from reduced MDH to ferricytochrome $c_5$ is the rate-limiting step.

Ferricytochrome $c_5$ as electron acceptor in *vivo*

In *Hyphomicrobium* X both MDH and cytochrome $c_5$ 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 $c_5$ are 0.5 mM and 1.6 mM respectively. From the bimolecular rate constant of $1.9 \times 10^4 \text{M}^{-1}\text{s}^{-1}$ derived in the present study, the cytochrome $c_5$ reduction rate will be 9.6 $\mu$mol of cytochrome $c_5$ reduced/min per mg of cells or 4.8 $\mu$mol of O$_2$ reduced/min per mg of cells. Since an average consumption of 0.4–0.6 $\mu$mol 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 ferrocytochrome $c_5$ observed in *vivo* can explain the physiological turnover rates. As discussed above, at pH 7.0 the overall rate of the assay in *vivo* is very low, since the rate-limiting step in the cycle is the slow decomposition of the MDH$_{ox}$.S complex. Recently we reported the existence of a low-M$_r$ component capable of enhancing the rate-limiting step with ferrocytochrome $c_5$ as electron acceptor at pH 7.0 [31,32]. This factor might be the ‘natural’ activator for MDH, activating the substrate oxidation step

in *vivo* at pH 7.0 ($k_{+4}$; Scheme 1) to a rate comparable with that observed in *vivo*.

We are grateful to Arie Braat, who interfaced the stopped-flow apparatus to the Olivetti M24 SP computer and who developed the software used.

REFERENCES


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