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# Properties of Electron Carriers in the Process of Methanol Oxidation in a New Restricted Facultative Marine Methylotrophic Bacterium, *Methylophaga* sp. MP

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**Abstract** Methanol dehydrogenase (MDH) and *c*-type cytochromes from marine methanol-oxidizing bacterium, *Methylophaga* sp. MP, were purified and characterized. The native MDH had a molecular mass of 148 kDa and its isoelectric point was 5.5. Two *c*-type cytochromes, $c_L$ and $c_H$, were found, and their isoelectric points were 3.4 and 8.0, respectively. The purified MDH had higher thermal stability than that of the other soil methylotrophic bacteria. The electron flow rate from MDH to cytochrome $c_L$ was higher than that from MDH to cytochrome $c_H$, indicating that the physiological primary electron acceptor for MDH is cytochrome $c_L$. The electron transfer from MDH to phenazine ethosulfate (PES, artificial electron acceptor) in the two dye (PES/DCPIP)-linked assay system was not inhibited by NaCl, whereas the electron flow from MDH to cytochrome $c_L$ in the cytochrome/DCPIP-linked assay system was suppressed significantly by NaCl. Metal chelating agents such as EDTA showed the same effects on the MDH activity.

**Key words:** Methanol oxidation, methanol dehydrogenase, cytochromes, *Methylophaga* sp. MP

In Gram-negative soil methylotrophic bacteria, methanol is oxidized to formaldehyde by a periplasmic methanol dehydrogenase (MDH, EC 1.1.99.8), which has pyrroloquinoline quinone (PQQ) as a prosthetic group and cytochrome $c_L$ as its primary electron acceptor [2, 3, 4, 20]. MDH consists of two identical dimers of large and small subunits in an $\alpha_2\beta_2$ conformation [21]. It is approximately 140 kDa in size and is a basic protein in many Gram-negative soil methylotrophic bacteria [4]. MDH and cytochrome $c_L$ are present at high concentrations in periplasm and constitute the first part of a methanol oxidation electron transport chain. A typical class I type cytochrome (cytochrome $c_H$) is also involved in methanol oxidation and cytochrome $c_H$ is oxidized by a terminal oxidase:

Methanol→MDH→cytochrome $c_L$
→cytochrome $c_H$→terminal oxidase

Most soil methylotrophic bacteria contain at least two soluble *c*-type cytochromes whose molecular weights, isoelectric points, and absorption spectra are different from each other. The *c*-type cytochromes can be classified based on the isoelectric point. Generally, the isoelectric point of cytochrome $c_H$ is higher than that of cytochrome $c_L$.

A new restricted facultative marine methanol-oxidizing bacterium, *Methylophaga* sp. MP, has been isolated from seawater around Mokpo, Korea, and characterized. The isolate was able to grow on methanol or on fructose as a carbon and energy source in a medium containing 3% NaCl. The evidences from 16S rRNA comparison and other properties suggest that *Methylophaga* sp. MP is closely related to *Methylophaga thalassica* (manuscript in preparation). In this study, a novel MDH and *c*-type cytochromes were purified from this isolate and characterized. The MDH had an unusually low isoelectric point, in contrast to the other MDHs from typical soil methylotrophic bacteria. Such an electron transport from an acidic MDH to the related cytochromes has not been reported previously.

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## MATERIALS AND METHODS

### Organism and Cultivation

*Methylophaga* sp. MP was cultivated on an artificial seawater medium containing 1% (v/v) methanol as a carbon and energy source. Cells were incubated at 30°C and aerated by a continuous air flow (35 l/min) and rotary agitation (200 rpm) in a 100-l fermentor (KF-1001, KoBioTech, Inchon, Korea). Cells from the late exponential growth phase were harvested by continuous centrifugation at 10,000 ×*g* and were kept frozen at −80°C.

### Preparation of Cell-Free Extract

Frozen cells were suspended in 25 mM Tris/HCl buffer (pH 8.0, Standard Buffer) at 4°C and were disrupted by passages through French Pressure Cell (Constant System, Warwickshire, U.K.). Unbroken cells and debris were removed by centrifugation at 15,000 ×*g* for 20 min at 4°C. The resulting supernatant was centrifuged at 100,000 ×*g* for 90 min, and the supernatant was used as a soluble fraction.

### Enzyme Purification

Purification of MDH and cytochromes from *Methylophaga* sp. MP was carried out at 4°C from the soluble fraction. Solid $(NH_4)_2SO_4$ was slowly added to this protein solution to give 60% saturation. After removal of the precipitate by centrifugation at 15,000 ×*g* for 30 min, $(NH_4)_2SO_4$ was added to give 85% final saturation. The precipitated proteins were centrifuged and redissolved in the same buffer. After dialysis for 4 h, the dialysate was concentrated with Centricon (YM-10, Amicon, Bedford, U.S.A.) and the concentrate was applied to a FPLC-Mono Q column (Amersham Bioscience, Uppsala, Sweden) which was equilibrated with standard buffer. MDH and cytochrome *c* proteins were eluted with a linear gradient of 0–100 mM NaCl in standard buffer. Fractions containing respective proteins were pooled, concentrated, and applied to an FPLC Suprose 12 HR 10/30 column (Amersham Bioscience) which was equilibrated with standard buffer containing 0.15 M NaCl.

### MDH Assay

Protein concentration was determined by the Bradford method [6] using bovine serum albumin as a standard. To understand the electron transport mechanism of the three proteins, two methods were used. In the first method, a two dye-linked assay system, PES was used as an artificial primary electron acceptor and 2,6-dichlorophenol indophenol (DCPIP) as a terminal acceptor [10]. In the second method, cytochrome/DCPIP-linked assay system, the primary electron acceptor was replaced with cytochrome $c_L$ and the reduction rate of DCPIP was measured [8]. The reaction mixture (1.0 ml) contained 100 mM Tris/HCl buffer (pH 9.0), 13.4 mM methanol, 1.1 mM PES, 25 μM cytochrome $c_L$, and 30 μM DCPIP. Reactions were started by adding MDH and the reduction of DCPIP was measured by decrease in absorbance at 600 nm.

### Analysis of MDH and Cytochromes

The molecular weights of the native MDH and cytochrome *c*′ were determined by gel filtration on a Superose 12 column using the following standard proteins: cytochrome *c* (12,400 Da), carbonic anhydrase (29,000 Da), bovine serum albumin (66,000 Da), alcohol dehydrogenase (150,000 Da), β-amylase (200,000 Da), and apoferritin (443,000 Da). Molecular weights of subunits of MDH and cytochrome *c* were determined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis as described by Laemmli [18] using 15% polyacrylamide gels. The absorption spectra of purified cytochromes were determined with a Shimadzu UV-2550 spectrophotometer at room temperature.

### Isoelectric Focusing

The isoelectric point of MDH from *Methylophaga* sp. MP was determined by the Multiphor II-system (Amersham Bioscience) using SERVALYT gels in the pH range of 2–9. The proteins were visualized by staining with Coomassie Brilliant Blue R250.

### Determination of *N*-terminal Amino Acid Sequence

Purified MDH was separated into its subunits by SDS gel electrophoresis and blotted to a PVDF-membrane (Bio-Rad, Hercules, U.S.A.). *N*-terminal sequence analysis of polypeptide was performed with an automated sequencer (Model 473A, Applied Biosystems, Forstercity, U.S.A.) equipped with a built-in phenylthiohydantoin-amino acids analyzer.

## RESULTS AND DISCUSSION

### Purification and Properties of MDH

MDH of *Methylophaga* sp. MP was purified about 39-fold through five steps, with a yield of 2.5% (Table 1). After cell disruption, MDH activity was found exclusively in the soluble fraction. The $(NH_4)_2SO_4$ fractionation resulted in a 13-fold enrichment of MDH. The proteins of MDH, cytochrome $c_H$ and cytochrome $c_L$ bound to the MONO Q column were eluted with Tris/HCl buffer (pH 8.0) containing 0.14, 0.16, and 0.2 M NaCl, respectively. Electrophoretically homogeneous preparation was obtained by the final purification step of gel filtration on a Superose 12 HR column.

The purified MDH maintained its activity at 4°C for over 24 h. The native MDH had a molecular mass of 148 kDa. The MDH was a PQQ-containing enzyme consisting of two apparently identical subunits in an $\alpha_2\beta_2$ conformation. Each of the α and β subunits had molecular masses of 64 and 10 kDa, respectively. The molecular mass and subunit

**Table 1.** Purification of MDH from *Methylophaga* sp. MP.

| Purification step | Total protein (mg) | Total activity (mmol/min) | Specific activity (μmol/mg protein/min) | Purification fold | Yield (%) |
|---|---|---|---|---|---|
| Cell-free extract | 7,390 | 591 | 80 | 1.0 | 100 |
| Soluble fraction | 5,145 | 484 | 94 | 1.2 | 82 |
| $(NH_4)_2SO_4$ (60–80% saturation) | 192 | 207 | 1,077 | 13.5 | 35 |
| Mono Q | 10.7 | 19 | 1,774 | 22.2 | 3.2 |
| Superose 12 HR | 4.8 | 15 | 3,117 | 39.0 | 2.5 |

structure of MDH of *Methylophaga* sp. MP are similar to those of MDHs isolated from typical Gram-negative soil methylotrophic bacteria. Only two marine methylotrophic bacteria, *Methylophaga marina* and *Methylophaga thalassica*, have been isolated and studied so far, and the properties of MDH have been investigated only in *M. marina* [16]. The MDH of *M. marina* is made of two apparently identical subunits giving a total molecular mass of 145,000 Da. The MDH of *Methylophaga* sp. MP was an acidic protein whose isoelectric point was 5.5. Most MDHs have high isoelectric points (7–10.5) except for the enzymes from *Paracoccus denitrificans* [5] and *M. marina* [16] which are characterized by their low isoelectric points (3.7–6.4).

The MDH from *Methylophaga* sp. MP had a $K_m$ value of 0.11 mM for methanol. The $K_m$ values of MDHs from *M. marina* and *Methylobacillus* sp. SK1 were 20 μM and 0.66 mM [16, 17], and those of MDHs from *Methylocystis* sp. GB 25 and *Methylosinus* sp. WI 14 were 0.34 mM and 0.45 mM, respectively [13, 14]. The MDH investigated in this study had a lower $K_m$ value than those of MDHs from most of methylotrophs except *M. marina*.

The purified MDH from *Methylophaga* sp. MP was more stable than those of the typical soil methylotrophic bacteria. The MDH was stable at 60°C for 40 min (Fig. 1), but most MDHs from soil methylotrophic bacteria were unstable at the same temperature. The present MDH lost most of its activity within 10 min at 70°C. A similar pattern was observed with MDH from *Methylobacillus methanolovous* sp. SK5, which lost its activity at 64°C within 10 min [17].

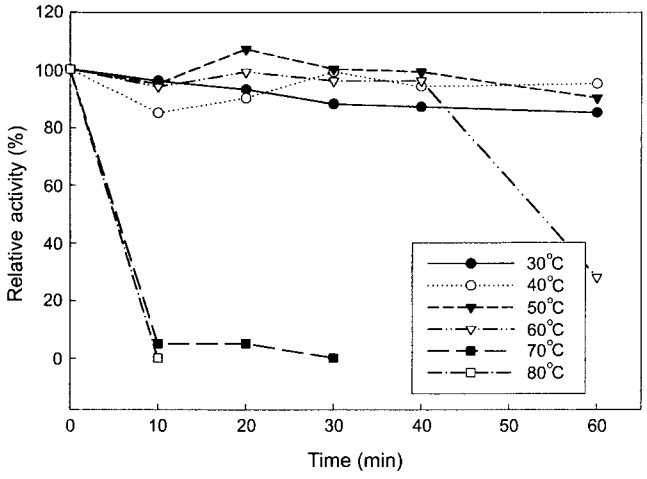


**Fig. 1.** Effect of temperature on the stability of MDH purified from *Methylophaga* sp. MP.
MDH activity was assayed by the method described in Materials and Methods.

## Properties of Cytochromes

Two *c*-type cytochromes, $c_L$ and $c_H$, were found in *Methylophaga* sp. MP. The cytochromes $c_H$ and $c_L$ had isoelectric points of 8.0 and 3.4, respectively. The reduced cytochrome $c_H$ had three absorption peaks at 551.0, 522.8, and 417.6 nm, while the oxidized form showed a single peak at 410.8 nm. Similarly, the reduced cytochrome $c_L$ had three peaks at 551.0, 521.4, and 415.0, while the oxidized protein had a single peak at 408.2 nm. The molecular masses of the cytochrome $c_H$ and cytochrome $c_L$ were estimated to be 15.5 and 18.4 kDa, respectively. General properties of the two cytochromes are summarized in Table 2.

Methylotrophic bacteria contain at least two soluble *c*-type cytochromes. *Methylobacterium extroquens* AM1 contains three *c*-type cytochromes, *c*-553, $c_L$, and $c_H$ [22], and *Methylophilus methylotrophus* also has three cytochromes, $c_L$, $c_H$, and *c*" [9]. In contrast, *Methylomonas J* contains two cytochromes, *c*-551 (I) and *c*-551 (II) [23], and *Hyphomicrobium* X also has cytochromes $c_L$ and $c_H$ [11]. The present study revealed that *Methylophaga* sp. MP had two *c*-type soluble cytochromes, $c_L$ and $c_H$. However, how many *c*-type cytochromes exist in other marine methylotrophs remains unknown.

## *N*-Terminal Sequence of the α-Subunit of MDH

The sequence of the first 20 amino acid residues of the *N*-terminal region of the large subunit of MDH provided a measure of the similarity in analogous proteins between *Methylophaga* sp. MP and other methylotrophs (Fig. 2). It showed a very high homology (80% identity in amino acid sequence) to the α-subunit of MDH from *Hyphomicrobium methylovorum* [26] and high similarities (a match of 75% of identical amino acids) to the MDHs from *Methylobacterium extorquens* AM1 [20], *Methylobacterium organophilum* [19], and *Methylobacterium nodulans* [25]. The MDH of *Methylophaga* sp. MP showed relatively low similarities to the MDHs from *A. methanolicus* [7] and *P. denitrificans* [15] (a match of about 60% identity) and showed lower similarity to the MDH of *Methylocystis* sp. GB25 [13].

**Table 2.** Comparison of the soluble *c*-type cytochromes from methylotrophic bacteria.

| | *Methylophaga* sp. MP[a] | | *Methylobacterium extorquens* AM1[b] | | | *Methylophilus methylotrophus*[c] | | | *Methylomonas* J[d] | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $c_H$ | $c_L$ | $c_H$ | $c_L$ | *c*-553 | $c_H$ | $c_L$ | $c''$ | *c*-551(I) | *c*-551(II) |
| Molecular weight (kDa) | 15.5 | 18.4 | 11 | 20.9 | 23 | 8.5 | 17–21 | 15 | 16 | 12.5 |
| Isoelectric point | 8.0 | 3.4 | 8.8 | 4.2 | Low | 8.55 | 4.0–4.3 | 8.7 | 5.3 | 4.3 |
| Absorption maxima Ferrocytochrome (α) | 551.0 | 551.0 | 550.5 | 549.0 | 553.0 | 551.25 | 549.75 | 550.0 | 551 | 551 |
| Ferrocytochrome (γ) | 417.6 | 415.0 | 416.5 | 416.0 | 419.0 | 416.25 | 416.0 | 520.0 | 417 | 416 |
| Ferricytochrome (γ) | 410.8 | 408.2 | 410.0 | 410.0 | 414.0 | 408.0 | 410.0 | 413.0 | 409 | 409 |
| Ratio of γ-/α-absorption | 6.25 | 2.55 | 5.23 | 6.25 | 6.05 | 2.72 | 5.16 | n.d | n.d | n.d |

[a]This study.
[b]O'Keeffe and Anthony [22].
[c]Cross and Anthony [9].
[d]Ohta and Tobari [23]. n.d, not determined.

| | 1 | 5 | 10 | 15 | 20 |
|---|---|---|---|---|---|
| *Methylophaga* sp. MP | N | D | K | L | V E L S K S N E N C V M Q G K D |
| *H. methylovorum* | N | D | K | L | I E L S K S N E N W V M P G K N |
| *M. organophilum* | N | D | K | L | V E L S K S D D N W V M P G K N |
| *M. extorquens* | N | D | K | L | V E L S K S D D N W V M P G K N |
| *M. nodulans* | N | D | K | L | V E L S K S D G N W V M P G K N |
| *A. methanolicus* | N | E | K | L | V E L H K T N G N W V M N G R T |
| *P. denitrificans* | N | D | E | L | V E L A K D P A N W V M T G R D |
| *Methylocystis* sp. GB25 | N | D | R | L | E V L T K S E N N X A M Q G K N |
| *Methylosinus* sp. WI14 | E | D | R | L | E A L A K S E D N W A M Q G K N |

**Fig. 2.** *N*-terminal sequences of α-subunits of MDH purified from *Methylophaga* sp. MP and related proteins.
The α-subunits are from MDHs of *Hyphomicrobium methylovorum* GM2 [26], *Methylobacterium organophilum* XX [19], *Methylobacterium extorquens* AM1 [20], *Methylobacterium nodulans* [25], *Acetobacter methanolicus* [7], *Paracoccus denitrificans* [15], *Methylocystis* sp. GB25 [13], and *Methylosinus* sp. WI14 [14].

## Substrate Specificity and Effect of Divalent Cations

MDH from *Methylophaga* sp. MP showed broad substrate specificity (Table 3). The enzyme had the ability to oxidize primary alcohols up to heptanol, aldehydes and methylamines. The MDH showed the highest affinity to methanol, and the affinity decreased as the chain length increased. It showed a $K_m$ value of 0.11 mM for methanol. Similar to other MDHs described, the MDH in *Methylophaga* sp. MP could oxidize only primary but not secondary alcohols [12, 13, 24].

The effects of divalent cations (1 mM in final concentration) on MDH activity were shown in Table 4. MDH was totally inhibited by $Co^{2+}$, $Mn^{2+}$, and $Cu^{2+}$ and strongly inhibited by $Fe^{+2}$ and $Sr^{+2}$. The metal ions showed similar effects on MDHs from *Methylobacillus glycogenes*, *Methylobacillus* sp. SK1, and *Methylosinus* sp. WI 14 [1, 14, 17].

## Electron Flow from MDH to Cytochrome *c*

The electron flow between electron carriers in methanol oxidation was determined by using the artificial electron transport system in which potassium ferricyanide was provided as a final electron acceptor. The decrease of absorbance at 420 nm by the reduction of potassium ferricyanide was monitored. Potassium ferricyanide was reduced by MDH in the presence of cytochrome $c_L$, but not in the presence of cytochrome $c_H$ (Fig. 3A).

Figure 3B shows the rates of electron transfer from MDH to the two types of cytochromes. Cytochrome $c_L$ was reduced completely within 90 s, whereas cytochrome $c_H$ was reduced completely after 12 min. This indicated that the reduction rate of cytochrome $c_L$ was higher than that of

**Table 3.** Substrate specificity and corresponding $K_m$-value of methanol dehydrogenase from *Methylophaga* sp. MP.

| Substrates | Relative activity (%) | $K_m$-value (μM) | Substrates | Relative activity (%) | $K_m$-value (μM) |
|---|---|---|---|---|---|
| Methanol | 100 | 0.111 | 1-Heptanol | 20 | 0.802 |
| Ethanol | 101.2 | 0.401 | Iso-propanol | 19 | n.d* |
| 1-Propanol | 88.6 | 0.560 | Iso-butanol | 13 | n.d |
| 1-Butanol | 81.8 | 0.463 | Formaldehyde | 86 | n.d |
| 1-Pentanol | 80.1 | 0.389 | Acetaldehyde | 18 | n.d |
| 1-Hexanol | 84.2 | 0.602 | Monomethylamine | 16 | n.d |
| | | | Dimethylamine | 12 | n.d |

*n.d. not determined.

**Table 4.** Effect of metal ions on the MDH activity.

| Ions | Final conc. (mM) | Relative activity (%) |
|---|---|---|
| None | - | 100 |
| $Mn^{2+}$ | 1 | 0 |
| $Ba^{2+}$ | 1 | 87 |
| $Cu^{2+}$ | 1 | 2.5 |
| $Co^{2+}$ | 1 | 0 |
| $Hg^{2+}$ | 1 | 93 |
| $Zn^{2+}$ | 1 | 58 |
| $Sr^{2+}$ | 1 | 28 |
| $Mg^{2+}$ | 1 | 94 |
| $Ca^{2+}$ | 1 | 97 |
| $Fe^{2+}$ | 1 | 21 |

cytochrome $c_H$, and that cytochrome $c_L$ was the primary electron acceptor for the MDH.

In *M. extorquens* AM1 and *M. methanolovorus* sp. strain SK5, cytochrome $c_L$ acts as a primary electron acceptor for MDH [10, 17]. These organisms carry acidic cytochrome $c_L$, as *Methylophaga* sp. MP does. It appears that the cytochrome $c_L$ with a low isoelectric point serves as a primary electron accepter in the methanol oxidation process.

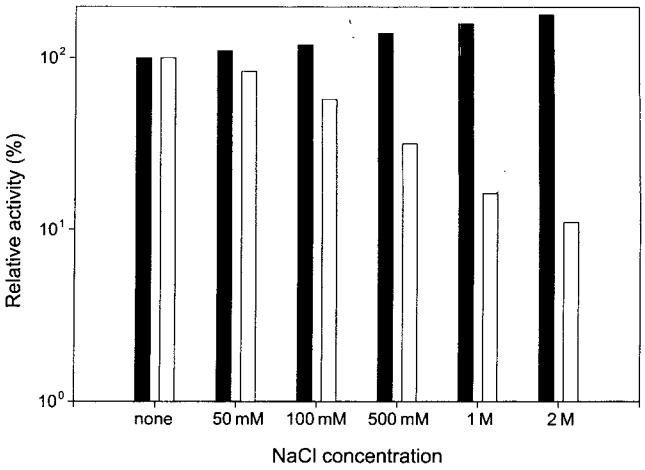


**Fig. 4.** Effect of sodium chloride on the MDH activity.
MDH activity was determined by two different assay systems as described in Materials and Methods. All reaction mixtures contained different concentration of NaCl, and 20 μM MDH. Two different assay systems were as follows: (■), MDH activity was measured by the two dye-linked assay system using PES as a primary electron acceptor and DCPIP as a terminal acceptor; (□), MDH activity was measured by the method using cytochrome $c_L$ as a primary electron acceptor and DCPIP as a terminal acceptor.

## Effects of Salts on Electron Transport System

The interactions of MDH and cytochromes involve electrostatic reactions between lysyl residues on the MDH and carboxyl groups of the cytochrome $c_L$ [7, 8]. If similar types of electrostatic interactions occur between MDH and the cytochrome $c_L$ in *Methylophaga* sp. MP, the electron transfer should be affected by salt concentrations. The effect of NaCl concentrations on the activity of MDH was determined by the two different assay systems (Fig. 4). The two dye-linked system used PES, and the cytocrome/DCPIP linked system used cytochrome $c_L$ as respective

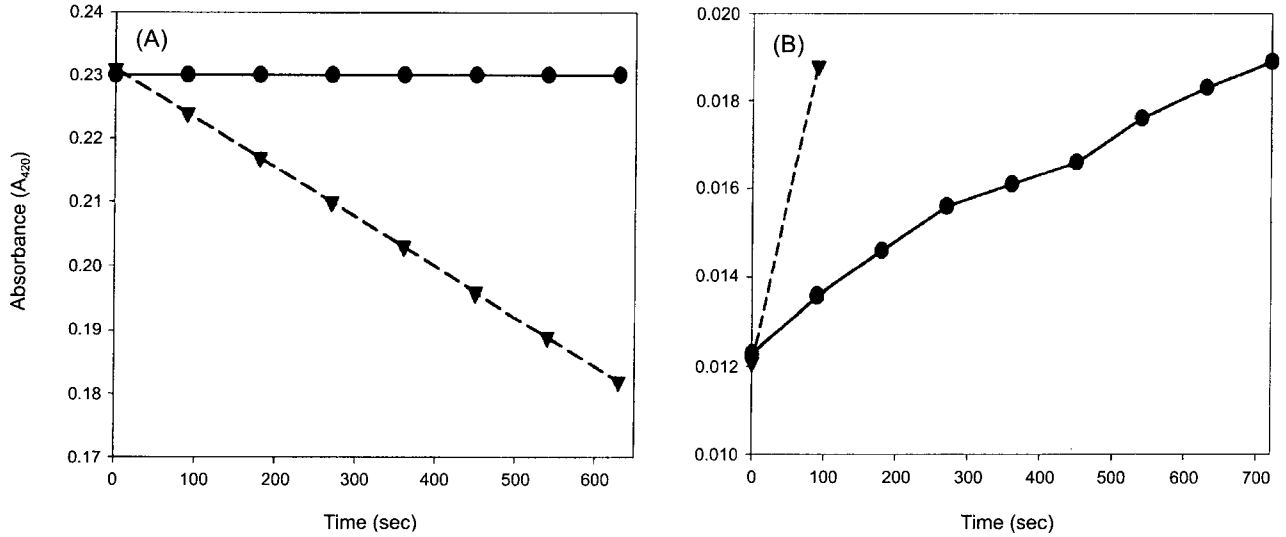


**Fig. 3.** (A) Reduction of potassium ferricyanide with MDH, and cytochrome $c_H$ and $c_L$.
Reduction rates were measured by the decrease of absorbance at 420 nm at room temperature. The assay systems differed from each other as follows: (●), 3 μM $K_3Fe(CN)_6$, 13.4 mM MeOH, and 30 μM cytochrome $c_H$; (▼), 3 μM $K_3Fe(CN)_6$, 13.4 mM MeOH, and 25 μM cytochrome $c_L$. Reactions were started by the addition of MDH (20 μM).
(B) Comparison of reduction rates of cytochromes $c_H$ and $c_L$.
Cytochrome $c_H$ (112 μM) and cytochrome $c_L$ (25 μM) were oxidized with 3 μM potassium ferricyanide and the oxidant was removed by passage through a PD-10 column. Reaction rates were measured by the increase of absorbance at 550 nm. Each reaction mixture contained oxidized cytochrome $c_H$ (●) or cytochrome $c_L$ (▼), and reactions were started by the addition of MDH (20 μM).

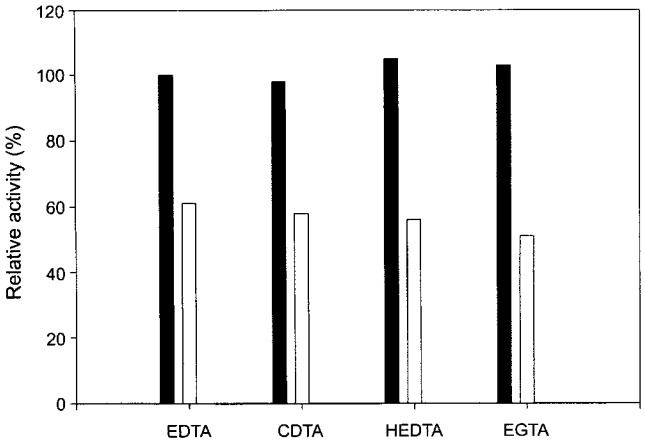


**Fig. 5.** Effect of metal chelating agents on the MDH activity.
MDH was purified and assayed as described in Materials and Methods. All reaction mixtures contained 10 μM metal chelating agents and 20 μM MDH: (■), MDH activity was measured with the two dye-linked assay system, using PES as a primary electron acceptor and DCPIP as a terminal acceptor; (□), MDH activity was measured by the method using cytochrome $c_L$ as a primary electron acceptor and DCPIP as a terminal acceptor.

primary electron acceptors. NaCl was added to the reaction mixture at the concentration from 50 mM to 2.0 M, and MDH activity was then measured in each assay. In the two dye-linked system, the activity of MDH was not significantly affected by NaCl concentrations. The MDH activity increased to the factor of 1.8, when the concentration of NaCl reached 2.0 M. In the cytochrome/DCPIP-linked assay system, however, the MDH activity decreased significantly as NaCl concentration increased. The relative activities of MDH dropped to 83%, 33%, and 12% at NaCl concentrations of 50 mM, 500 mM, and 2.0 M, respectively.

In addition, oxidized cytochrome $c_L$ was reduced by MDH after 360 s of incubation in the presence of 500 mM NaCl, whereas it was reduced completely within 90 s without NaCl (data not shown). These results indicate that the electron transfer from MDH to cytochrome $c_L$ was inhibited by high concentration of NaCl, suggesting that the interactions between MDH and cytochrome in *Methylophaga* sp. MP might also involve electrostatic interactions.

The effect of different chelating agents on the MDH activity was measured using the same assay systems (Fig. 5). In the two dye-linked assay system, chelating agents such as HEDTA, EDTA, CDTA, and EGTA did not inhibit MDH activity at all. However, when cytochrome $c_L$ was used as a primary electron acceptor, the addition of EDTA, CDTA, HEDTA, or EGTA in the final concentration of 10 μM inhibited the MDH activity up to 50%. The chelating agents did not inhibit the electron transfer from MDH to PES, but inhibited the electron transfer from MDH to cytocrome $c_L$. As suggested by Anthony *et al.* [4], chelating agents might have inhibited the activity of MDH by binding to lysyl or arginyl residues of MDH, thus preventing its docking with cytochrome $c_L$ [4].

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