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Kinetic and Spectral Investigations on Ca^{2+}- and Sr^{2+}-containing Methanol Dehydrogenases

Si Wouk Kim*, Chun Sung Kim[1], Jung Sup Lee[1], Moon Joo Koh[2], Song Suk Yang, Johannis A. Duine[3] and Young Min Kim[4]

Department of Environmental Engineering, [1]Department of Genetic Engineering, [2]Department of Chemistry, Chosun University, Kwangju 501-759, Korea; [3]Department of Microbiology and Enzymology, Delft University of Technology, 2628 BC Delft, The Netherlands; [4]Department of Biology, Yonsei University, Seoul 120-749, Korea

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Both Ca^{2+}- and Sr^{2+}-containing methanol dehydrogenases (MDH) were purified to homogeneity with yields of 48% and 42%, respectively, from *Methylobacillus methanolovorus* sp. strain SK5. Most of the biochemical and structural properties were similar to each other. However, some differences were found: (1) although the overall shape of the absorption spectrum of Sr^{2+}-MDH was very similar to that of Ca^{2+}-MDH, the absorption intensity originating from the cofactor in Sr^{2+}-MDH was higher than that in Ca^{2+}-MDH. Small blue shift of the maximum was also observed. These are probably due to a difference in redox state of the cofactors in Ca^{2+}- and Sr^{2+}-MDH; (2) Sr^{2+}-MDH was more heat-stable than Ca^{2+}-MDH above 56°C; (3) the V_{max} values for the methanol-dependent activities of Sr^{2+}- and Ca^{2+}-MDH in the presence of 3 mM KCN were 2,038 and 808 nmol/mg protein/min, respectively. In addition, the K_m values of Sr^{2+}- and Ca^{2+}-MDH for methanol were 12 and 21 μM, respectively; (4) the endogenous activity of Ca^{2+}-MDH was more sensitive than that of Sr^{2+}-MDH in the presence of cyanide; (5) Diethyl pyrocarbonate treatment increased the enzyme activities of Ca^{2+}- and Sr^{2+}-MDH 4.2- and 1.4-folds, respectively. These results indicate that Sr^{2+} stabilizes the structural conformation and enhances the activity of MDH more than Ca^{2+}.

Key words: Methanol dehydrogenase, pyrroloquinoline quinone, *Methylobacillus methanolovorus* sp. strain SK5

Gram-negative methylotrophic bacteria oxidize methanol to formaldehyde via quinoprotein (PQQ-containing) methanol dehydrogenase (MDH, EC 1.1. 99. 8). MDH is a soluble, periplasmic dehydrogenase, and consists of two identical dimers of large and small subunit in an $\alpha_2\beta_2$ conformation (1, 12).

Ca^{2+} is located at the interspace between the two α-subunits and sustains the optimal configuration of PQQ in the active enzyme. Ca^{2+}-lacking mutant forms of the MDH from *Methylobacterium extorquens* show abnormal absorption spectra and are inactive but incubation with Ca^{2+} restores activity and normal spectrum. Ca^{2+} might be important for PQQ-binding to the protein (13).

Ca^{2+} in the active site of MDH can be replaced with Sr^{2+} (8) or Ba^{2+} (6). Sr^{2+}- or Ba^{2+}-MDHs showed several different kinetic and structural properties from those of Ca^{2+}-MDH. Sr^{2+}-MDH has an increased absorption coefficient for the PQQ chromophore, higher V_{max} value for methanol-dependent enzyme activity and was more resistant to cyanide than Ca^{2+}-MDH. The activation energies of Sr^{2+}- or Ba^{2+}-MDHs were shwon to be lower than that of Ca^{2+}-MDH. In addition, Sr^{2+}-MDH exhibited higher thermal stability than Ca^{2+}-MDH (6, 8, 9).

Methylobacillus methanolovorus sp. strain SK5 used in this study was isolated from soil in Kwangju, Korea (10). It is an obligate methylotrophic bacterium, which grows only on methanol, and not on any other single or multicarbon compounds. We describe here the kinetic and spectral differences between Ca^{2+}- and Sr^{2+}-containing MDHs purified from the organism.

Materials and Methods

Bacterial strain and growth conditions

M. methanolovorus sp. strain SK5 was cultured aerobically in a 15 L fermentor (Bioflo 3000, New Brunswick Scientific) at 30°C on a mineral base medium supplemented with 1.0% methanol as a carbon and energy source (10). This medium commonly contained 0.07 mM $CaCl_2$. To obtain Sr^{2+}-

* To whom correspondence should be addressed

MDH, $CaCl_2$ was replaced by 0.27 mM $SrCl_2$. Cells were harvested at the end of the exponential growth phase and stored at -40°C until used.

Purification steps

All purification steps were carried out at 4°C. Frozen cell paste was suspended in an equal volume of 25 mM Tris-HCl buffer, pH 8.0 (standard buffer), with 1 mM methanol as a stabilizer. After cell disruption, the suspension was centrifuged at 15,000× g for 20 min at 4°C. The resulting supernatant (cell-free extract) was ultracentrifuged at 100,000×g for 90 min, and the supernatant was used as soluble fraction. After ammonium sulfate fractionation (40-80%), the fraction was dialysed against the same buffer for 3 hours.

The dialysate was applied to a DEAE-Sephacel column (5.0×20 cm, Sigma) equilibrated with the standard buffer, pH 8.0. The column was washed with the same buffer and the absorbed proteins were eluted with a linear gradient of NaCl from 0 to 1 M at a flow rate of 3.0 ml/min. Active fractions were collected and concentrated with Centriprep-30 (Amicon). The concentrate was loaded on a Sephacryl S-100 HR column (Pharmacia) equilibrated with the standard buffer and the elution was performed at a flow rate of 0.5 ml/min. After the enzyme fraction was pooled and concentrated, aliquots of the concentrate were applied to a Superose 12 HR 10/30 column (Pharmacia) equilibrated with the same buffer. Fractions having high enzyme activity were collected and stored at -70°C. Protein concentrations were determined by the method of Bradford (2).

Enzyme assay

MDH activity was measured spectrophotometrically at 30°C with phenazine ethosulfate as primary electron acceptor and 2,6-dichlorophenol indophenol (DCPIP) as secondary electron acceptor (3). The reduction rate of DCPIP (molar absorption coefficient of 22,000 $M^{-1}cm^{-1}$) was measured by following the decrease in absorbance at 600 nm.

Absorption spectra

The absorption spectra of Ca^{2+}- and Sr^{2+}-bound MDH were determined with a Shimadzu UV-1601PC spectrophotometer at room temperature. Both enzymes (0.36 mg/ml) were incubated in 25 mM Tris-HCl buffer (pH 8.5).

Analysis of prosthetic group

To determine the amounts of PQQ involved in both enzymes, PQQ was extracted by the method of Duine *et al.* (5). One hundred microliter of methanol dehydrogenase in 0.02 M potassium phosphate buffer (pH 7.0) was mixed with 100 µl 1 M NaH_2PO_4, pH 1.0 (with conc HCl), and ther a 100 µl methanol was added. After centrification, the supernatant was analyzed by high performance liquid chromatography using methanol/water/85% H_3PO_4 (45/54.5/0.5, v/v/v) as the solvent and the prosthetic group was detected at 313 nm. The total amount of PQQ was determined after quinol was oxidized to quinone by an excess of DCPIP.

Metal content analysis

Metals (Ca^{2+} and Sr^{2+}) contained in the MDHs were analyzed with an Inductively Coupled Plasma Emission Spectrophotometer (ICP) (Jobin Ybon). Proteins were treated with 6 N HNO_3 and boiled for 2 h. Then the solutions were filtered. The eluted solutions were injected to ICP and then the metal contents were measured.

Results

Purification and biochemical properties

Purification steps and yield of Ca^{2+}-MDH are shown in Table 1. Ca^{2+}-MDH was purified about 4.7-fold in six steps, with a yield of 48%. Sr^{2+}-MDH was also purified through the same procedure with a yield of 42%. The molecular weight of native enzyme was estimated to be 112 KDa, and those of subunits were 61.6 and 11.4 KDa, respectively.

In the dye-linked assay system, no activity was found in either Ca^{2+}- or Sr^{2+}-MDHs in the absence of ammonium ion. The apparent K_m values of Ca^{2+}- and Sr^{2+}-MDHs for ammonium chloride were estimated to be 4.5 and 25.7 mM, respectively.

The V_{max} for the methanol-dependent activity of Sr^{2+}-MDH was 2.5-fold higher than that for Ca^{2+}-MDH. On the other hand, V_{max} values for the en-

Table 1. Purification scheme of Ca^{2+}-MDH from *M. methanolovoru* sp. SK5

Purification step	Total protein (mg)[a]	Total activity (µmol/min)	Sp. act. (nmol/mg protein/min)	Yield (%)	Purifi. fold
Cell-free extract	672	151	225	100	1.00
Soluble fraction	640	134	210	88	0.93
Ammnium sulfate (40~80%)	400	135	338	89	1.50
DEAE Sephacel	112	101	907	67	4.03
Sephacryl S-100	77	77	997	51	4.43
Superose 12 (HR 10/30)	70	72	1048	48	4.66

[a] Determined by the method of Bradford (1976).

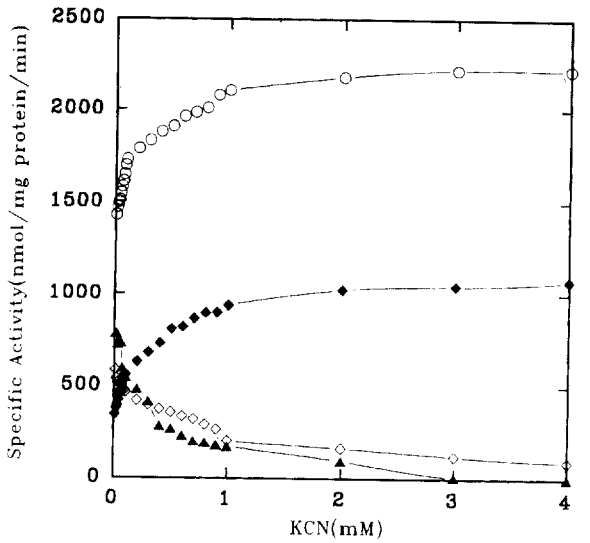

Fig. 1. Effect of KCN on the methanol-dependent activities of Sr^{2+}-(▼) or Ca^{2+}-MDH (◇), and endogenous activities of Sr^{2+}-(◆) or Ca^{2+}-MDH (○).

dogenous activities of Sr^{2+}- and Ca^{2+}-MDHs were influenced by the KCN concentration. At low KCN concentration (below 0.3 mM), the endogenous activity of Ca^{2+}-MDH was higher than that of Sr^{2+}-MDH. However, at high concentration, V_{max} value of Ca^{2+}-MDH was lower than that of Sr^{2+}-MDH (Fig. 1). As shown in Fig. 2, the endogenous activity of Ca^{2+}-MDH was more sensitive to cyananide than that of Sr^{2+}-MDH. At high KCN concentration (above 3 mM), the endogenous activity of Ca^{2+}-MDH

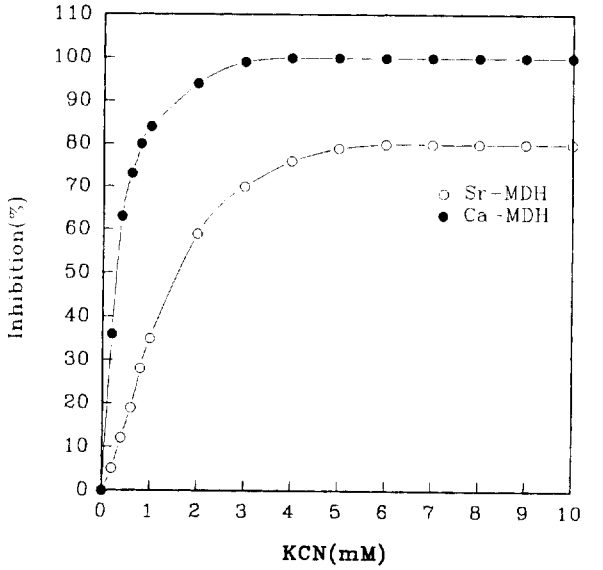

Fig. 2. Inhibition by KCN of the endogenous activities of Ca^{2+}- and Sr^{2+}-MDH. MDH activity was measured spectrophotometrically at 30°C with phenazine ethosulfate as primary electron acceptor and 2,6-dichlorophenol indophenol as secondary acceptor.

was inhibited completely; however, 25 % of Sr^{2+}-MDH activity remained. The K_m values of both Ca^{2+}- and Sr^{2+}-MDH for methanol were found to be 21 and 12 μM, respectively.

Absorption spectra

The absorption spectrum of the native Ca^{2+}-MDH showed a peak at 343 nm with a wide shoulder around 400 nm. This result indicates that the enzyme is a quinoprotein containing PQQ as a prosthetic group. The absorption spectrum of

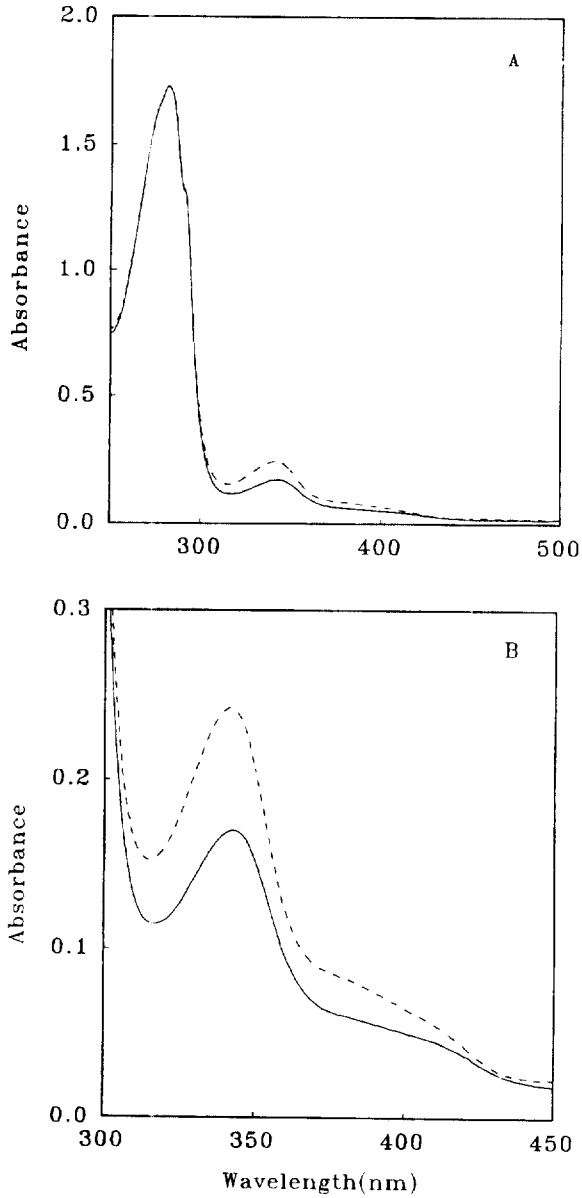

Fig. 3. (A) Absorption spectra of Ca^{2+}-MDH (—) and Sr^{2+}-MDH (···). (B) Enlargement of the absorption spectra of Ca^{2+}- and Sr^{2+}-MDH for the PQQ chromophore. Same amounts of Ca^{2+}- and Sr^{2+}-MDH (0.36 mg/ml) were diluted in 25 mM Tris-HCl buffer (pH 8.5).

the native Sr^{2+}-MDH was almost similar to that of Ca^{2+}-MDH; however, the maximum peak of Sr^{2+}-MDH was observed at 342 nm whereas that of Ca^{2+}-MDH was detected at 343 nm. And the absorption coefficient of Sr^{2+}-MDH for the PQQ chromophore had increased (Fig. 3). The ratios of A_{343}/A_{400}, A_{280}/A_{343} and A_{280}/A_{400} for Ca^{2+}-MDH were 3.4, 10.1, and 34.6, respectively, whereas the ratios of A_{342}/A_{400}, A_{280}/A_{342} and A_{280}/A_{400} for Sr^{2+}-MDH were 3.7, 7.1, and 26.3, respectively. It indicates that not only the absorption coefficient but also the shape of the spectrum are different between Ca^{2+}- and Sr^{2+}-MDH. However, the molar ratio of the PQQ involved in the Ca^{2+}-MDH was 1.9 molecules of PQQ per molecule of enzyme, and 2.1 molecules of PQQ per molecule of enzyme in the Sr^{2+}-MDH.

Substrate specificities

To test the substrate specificities of Ca^{2+}-MDH, various substrates were used in a final concentration of 6.7 mM. Aliphatic primary alcohols were relatively good substrates; however, their oxidation rates decreased with increasing carbon chain length. Interestingly, allylalcohol and formaldehyde were oxidized well. However, the enzyme did not oxidize secondary alcohols, (di)methylamines, formate, cyclohexanol, and catechol. Substrate specificity of Sr^{2+}-MDH was almost similar to that of Ca^{2+}-MDH (Table 2).

Table 2. Substrate specificities of Ca^{2+}- and Sr^{2+}-MDH

Substrate[a]	Relative activity (%)[b]	
	Ca^{2+}-MDH	Sr^{2+}-MDH
Methanol	100	100
Ethanol	90	81
n-Propanol	75	56
Isopropanol	5	56
1-Phenyl-1-propanol	0	0
n-Butanol	5	43
Isobutanol	0	0
t-Butanol	0	0
n-Octanol	4	2
n-Nonanol	35	14
n-Dodecanol	16	10
Benzylalcohol	0	0
Allylalcohol	88	96
Cyclohexanol	7	8
Catechol	0	0
Formaldehyde	89	98
Acetaldehyde	19	32
Formate	0	0
Monomethylamine	0	0
Dimethylamine	0	0

[a] Activity was measured in the presence of 6.7 mM of each substrate.
[b] Activity in the presence of methanol was taken as 100%.

Table 3. Effect of inhibitors on enzyme activity of Ca^{2+}- and Sr^{2+}-MDH

Inhibitors	Conc. (mM)	Relative activity (%)	
		Ca^{2+}-MDH	Sr^{2+}-MDH
Dicalent Cations[a]			
None		100	100
Ba^{2+}	1	96	85
Ca^{2+}	1	111	98
Cd^{2+}	1	52	62
Fe^{2+}	1	37	75
Mg^{2+}	1	108	98
Ni^{2+}	1	48	58
Hg^{2+}	1	42	52
Zn^{2+}	1	50	49
Co^{2+}	1	0	1
Cu^{2+}	1	0	3
Cr^{2+}	1	0	0
Mn^{2+}	1	0	0
Protein Modification Reagents[b]			
Diethylpyrocarbonate	1	422	140
N-acetylimidazole	2	95	90
Pyridoxal 5-phosphate	2	94	99
Phenyl glyoxal	2	91	103
DTNB[c]	2	92	100

[a] Inchubation with divalent cation was carried out for 1 min before the reaction was started by adding 6.7 mM methanol.
[b] Incubation with protein modification reagent was carried out for 20 min before the reaction was started by adding 6.7 mM methanol.
[c] 5,5-Dithio-bis-(2- nitrobenzioic acid).

Effects of divalent cations and protein modification reagents

Divalent cations (1 mM) such as Co^{2+}, Cu^{2+}, Cr^{2+}, and Mn^{2+} inhibited the enzyme activity completely. Most of protein modification reagents did not influence the activity. However, the activities of Ca^{2+}- and Sr^{2+}-MDHs increased to 4.2- and 1.4-folds, respectively, by incubation in the presence of 1 mM of diethyl pyrocarbonate (DEPC) (Table 3). To eliminate the possibiliity that DEPC acts as a substrate (it decomposes to ethanol and CO_2), DEPC-treated enzyme solution was passed through the Sephadex G-25 column (1×10 cm, Sigma). Although DEPC was removed completely, they still maintained 90% of increased activities. DEPC itself was not used as a substrate.

Effects of pH and temperature

The optimal pH and the pI value for both MDHs were 9.5 and 9.3, respectively. These results indicate that the enzymes are basic proteins. The highest enzyme activity of Ca^{2+}-MDH was found at 60°C whereas that of Sr^{2+}-MDH appeared at 70°C. When thermal stability was analyzed at the various temperatures using the standard assay systems, the enzyme activity of Ca^{2+}-MDH signi-

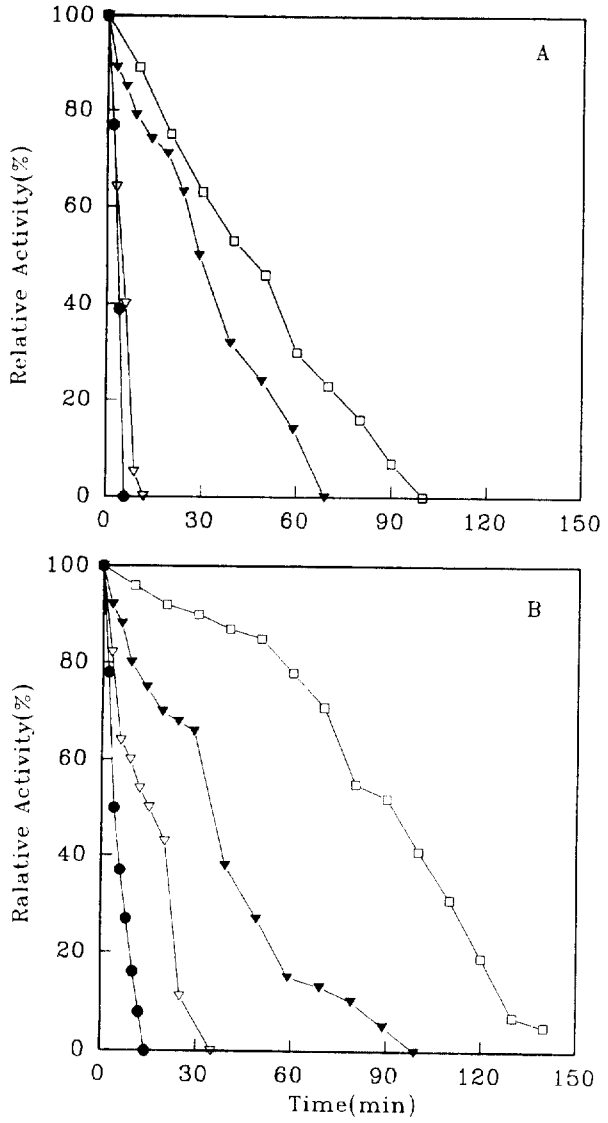

Fig. 4. Time course for thermal inactivation of (A) Ca^{2+}-(0.88 mg/ml) and (B) Sr^{2+}-MDH (0.6 mg/ml) at 56 (□), 50 (▼), 64 (▽), and 70 (●)°C.

ficantly decreased after a 10 min incubation at 56°C. However, even at 64°C, 90% of enzyme activity of Sr^{2+}-MDH was retained for 10 min (Fig. 4).

Metal contents

Sr^{2+} contained in Sr^{2+}-MDH was estimated to be 1.7 atoms per molecule of native MDH; however, a small amount of Ca^{2+} was detected. Ca^{2+} in Ca^{2+}-MDH was found to be 1.6 atoms per molecule of MDH.

Discussion

In gram-negative methylotrophic bacteria, most of MDHs studied so far contain PQQ as a prosthetic group (4), and are composed of $\alpha_2\beta_2$ tetramers (1, 12). However, *Acetobacter methanolicus* contains two types of MDHs; one of which is a classical type I MDH and another is a novel type II MDH. A novel MDH involves an additional 32 KDa peptide which binds to the normal $\alpha_2\beta_2$-MDH and seems to be a *mxaJ* gene product (11). The purified Ca^{2+}- and Sr^{2+}-MDHs from *M. methanolovorus* sp. strain SK5 consisted of two large subunits and two small subunits in an $\alpha_2\beta_2$ configuration, indicating that they are classical type MDH. The molecular weight of native purified MDH obtained from non-denatured HPLC gel filtration was smaller than expected. This can be explained by the fact that the extremely basic proteins like MDH interact with column matrix so that they are eluted slowly (11).

Although Ca^{2+} is required for the correct PQQ binding to the active site of MDH, the binding can be changed by the replacement with Sr^{2+}. A slight conformational change may result in the affinity of Sr^{2+}-MDH for substrates to increase (8). Sr^{2+}-MDH purified in this study had higher V_{max} value and lower K_m value than Ca^{2+}-MDH, and are resistant to KCN. Two types of MDH activities, methanol-dependent and endogenous activity, were found in the dye-linked assay system *in vitro* (4). It has been known that the endogenous activity is inhibited by cyanide but methanol-dependent activity is maintained in the presence of cyanide. Methanol-dependent activity of Sr^{2+}-MDH from *M. methanolovorus* sp. strain SK5 is higher than that of Ca^{2+}-MDH; whereas, endogenous activity of Sr^{2+}-MDH is more resistant than Ca^{2+}-MDH to cyanide. This can be explained by the fact that MDH contains two cyanide-binding sites; one of which is related to the activation of methanol-dependent activity and is also correlated to the inhibition of endogenous activity by cyanide. Interestingly the properties of cyanide-binding site in Sr^{2+}-MDH are affected significantly by cyanide so as to facilitate the binding of substrate to MDH (7, 8). In addition, Sr^{2+}-MDH activity is more stable than Ca^{2+}-MDH at high temperatures and to air exposure. Thus we can imagine that Sr^{2+}-MDH is a more efficient enzyme for catalyzing methanol.

As shown in Fig. 3, the absorption spectra of Ca^{2+}- and Sr^{2+}-MDHs from this study are different. Harris and Davidson (8) showed that Sr^{2+}-MDH had an increased absorption coefficient for PQQ chromophore but they did not determine the PQQ content. However, in this study, PQQ contents of both enzymes were shown to be the same with a little blue shift of maximum observed at around 340 nm. Thus there is a possibility exists in that the different spectra may be due to the different redox

states of cofactors involved in each MDH.

We also found that DEPC stimulates Ca^{2+}-MDH activities 4.2-fold. DEPC could be decomposed to ethanol which is a substrate for MDH. However, even after DEPC and ethanol were removed by gel filtration, most of the increased activities did not changed. When DEPC was added to assay mixtures as a substrate for MDH, the enzyme was not able to oxidize DEPC. This suggests that DEPC may increase MDH activity through a chemical modification and not by supplying additional substrate, ethanol. However, the increased methanol-dependent MDH activities were rapidly turned to endogenous activities. Now this phenomenon is more difficult to explain. Thus further investigations for the effect of DEPC are required.

When *M. methanolovorus* sp. strain SK5 was cultured in the Ca^{2+}- or Sr^{2+}-containing medium, cells in the Sr^{2+}-medium grew faster than those in the Ca^{2+}-medium although they aggregated to small masses in high Sr^{2+} concentrations (more than 0.81 mM) during the early exponential phase (data not shown). However, cells in the Ba^{2+} (0.27 mM)-containing medium grew until the absorbance affer that reached to 0.8, with no further growth after that. And no methanol-dependent MDH activity from the cell-free extract was detected. Although Goodwin and Anthony (6) investigated some biochemical properties of Ba^{2+}-MDH using Ca^{2+}-free enzyme (from a processing mutant), it may be difficult to characterize enzymological properties of Ba^{2+}-MDH from the cells cultured in the Ba^{2+}-containing medium.

In conclusion, most of the enzymological properties were found to be similar between Ca^{2+}- and Sr^{2+}-MDH. However, Sr^{2+}-MDH seems to be the more efficient enzyme for catalyzing methanol.

Acknowledgment

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