Preparation and Characterization of Ca^{2+}-free Methanol Dehydrogenase from *Hyphomicrobium denitrificans* A3151

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Ca^{2+}-free Methanol dehydrogenase (MDH) has been prepared from *Hyphomicrobium denitrificans* A3151 and spectroscopically characterized. Ca^{2+}-free MDH had full-oxidized pyrroloquinoline quinone (PQQ). Incubation of Ca^{2+}-free MDH with Ca^{2+} ion led to reconstituted MDH containing full-reduced PQQ, which was tightly bound with Ca^{2+} ion and had the enzyme activity.

Methanol dehydrogenase (MDH; EC 1.1.99.8), which is a pyrroloquinoline quinone (PQQ)-dependent enzyme, catalyzes the oxidation of methanol to formaldehyde in the carbon metabolism of various methylotrophic bacteria.[1] It has been known that a single Ca^{2+} ion is tightly bound to MDH in the active site.[2] It is very difficult to remove the Ca^{2+} ion from MDH, although Ca^{2+}-free enzymes have been reported in other quinoproteins such as soluble glucose dehydrogenase.[3] Only one example of Ca^{2+}-free MDH has been reported by Anthony et al.[2a] They prepared Ca^{2+}-free MDH from the mutant strains of bacteria lacking of genes for proteins related the incorporation of Ca^{2+} ion into MDH (MoxA, K, and L). However, Ca^{2+}-free MDH from native bacteria and its characterization have never been reported so far. Therefore, we report here that the Ca^{2+}-free MDH were prepared from *Hyphomicrobium denitrificans* A3151 and characterized by electronic absorption and electronic paramagnetic resonance spectroscopies.

The *H. denitrificans* strain was cultured in a mineral salt medium containing 1% methanol and 0.5% potassium nitrate at 30 °C for 3 days under static conditions.[4] The cell suspended 40 mM of Tris-HCl buffer (pH 7.5) containing phenylmethylsulfornyl fluoride (protease inhibitor) were sonicated at 180 W for 30 min, followed by centrifugation (15,000 rpm for 1 h). Ammonium sulfate was added to the supernatant solution to give a concentration of 20%, and precipitate was collected by centrifugation. The concentration of ammonium sulfate in the supernatant was raised to 80% of the saturation, and the solution was stirred and centrifuged as before. A resulting precipitate containing MDH was dialyzed against 40 mM of Tris-HCl buffer (pH 7.5). Further purification was performed with SuperQ-Toyopearl, Q-Sepharose, and Phenyl-Sepharose column chromatographies.[5] SDS-PAGE of the purified MDH showed two bands (65 and 9 kDa), which correspond to α and β subunits of MDH from other methylotrophs, respectively.[1] The 65- and 9-kDa subunits of the MDH had the amino-acid sequences of NDKLIELSNSNE and YDGTHCKAPGN at the N-terminus, respectively. The sequences of both subunits are almost identical to those of MDH from *H. methylovorum* GM2 (α: NDKLIELSKSNE and β: YDGTKCKAPGN).[6] The amount of Ca^{2+} ion in the MDH was estimated to be less than 0.03 atoms by atomic absorption spectrophotometery, and hence the MDH lacks Ca^{2+} ion. Other metal ions (Fe, Cu, V, Cr, Mn, Co, Ni, Zn, Mo, Ru, Cd, and W) were also not detected in the Ca^{2+}-free MDH by ICP measurement.

In the same purification process except ammonium sulfate precipitation steps before SuperQ-Toyopeal column chromatography, Ca^{2+}-containing MDH, in which amount of Ca^{2+} ion is 1.43 atoms per $\alpha_2\beta_2$, was obtained. After that the Ca^{2+}-containing MDH was kept in 80% ammonium sulfate solution with 50 mM EDTA for 1 h at pH 7.5 and 4 °C, the amount of Ca^{2+} ion in the enzyme was not changed, therefore, the binding of Ca^{2+} ion to MDH from *H. denitrificans* is tightly as the same as MDH from other bacteria. We found that Ca^{2+}-free MDH was prepared by the ammonium sulfate precipitation procedures of the cell extract, although further studies would be need for the elucidation of the Ca^{2+} ion elimination mechanism from MDH.

The UV–visible absorption spectrum of Ca^{2+}-free MDH exhibits a peak at 386 nm and a shoulder band around 412 nm (**a** in Figure 1). The A_{386}/A_{412}, A_{280}/A_{386}, and A_{280}/A_{412} ratios were 1.34, 16.30, and 21.84, respectively. The spectrum is obviously different from those of resting states of MDH (semiquinone forms) from other bacteria, and similar to that of full-oxidized MDH (quinone form).[7] In MDH model complex studies,[8] the absorption band due to the quinone is observed at 354 nm in anhydrous acetonitrile. The spectral difference between Ca^{2+}-free MDH and quinone compound would be due to the environment of PQQ. In the active site of MDH, the PQQ forms hydrogen bonds in its equatorial plane with surrounding protein residues and is sandwiched between a tryptophan side chain and a characteristic disulfide bridge formed by adjacent cysteine residues.[9,10] In the EPR spectrum of Ca^{2+}-free MDH, no signal was detected (**a** in Figure 2), while Ca^{2+}-containing MDH

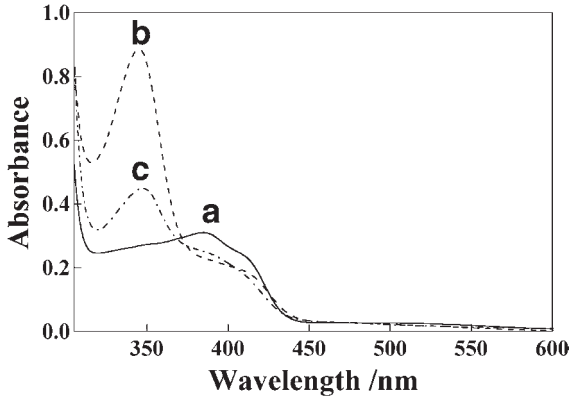

Figure 1. Electronic absorption spectra of Ca^{2+}-free MDH (**a**, solid line), Ca^{2+}-treated MDH before and after addition of Wurster's Blue (**b**, broken and **c**, dot and dash lines, respectively) in 40 mM of Tris-HCl buffer (pH 7.5).

showed a sharp intensity organic radical signal at $g = 2.005$, which is characteristic of semiquinone free radical in quinone-containing proteins. Consequently, the Ca^{2+}-free MDH has quinone-type PQQ in the active site. Generally, oxidized MDH containing quinone-type PQQ is unstable because of the endogenous activity.[11] The results suggest that Ca^{2+} ion in MDH active site would be concerned with the endogenous reaction.

The Ca^{2+}-free MDH was incubated at 25 °C for 1 h in 40 mM of Tris-HCl buffer (pH 8.5) containing 100 mM of $CaCl_2$, and dialyzed twice against the buffer without Ca^{2+} ion. The amount of Ca^{2+} ion in the Ca^{2+}-treated MDH was estimated to be 1.06 atoms per $\alpha_2\beta_2$. The MDH activity of Ca^{2+}-treated MDH was 13.0 unit/mg protein, which is similar to the enzymatic activity of Ca^{2+}-containing MDH (18.0 unit/mg protein), although Ca^{2+}-free MDH had no activities.[12] The UV–vis spectrum of Ca^{2+}-treated MDH exhibits a peak at 345 nm and a shoulder band around 410 nm ($A_{345}/A_{410} = 4.65$, **b** in Figure 1), which is similar to those of full-reduced Ca^{2+}PQQ complex[8] and MDH (quinol form).[13] The Ca^{2+}-treated MDH had no EPR signal, as shown in Figure 2. When one equivalent of oxidant was added to the Ca^{2+}-treated MDH, the absorption spectrum changed into almost the same as that of Ca^{2+}-containing MDH, which has semiquinone state in the active site (**c** in Figure 1), and EPR signal at $g = 2.005$ characteristic of semiquinone free radical was detected (**c** in Figure 2).

We also determined the amino-acid sequences of MDH and the concerning proteins by cloning of their genes from *H. denitrificans*.[14] The gene cluster for methanol oxidation, *moxFJGIRSAC*, was found to exist in *H. denitrificans*. The α and β subunits (MoxF and MoxI) exhibited 64–95 and 57–92% identities, respectively with those of other bacteria reported previously. The amino-acid residues related to the active site of MDH (E55, C103, C104, R109, T159, S174, E177, T243, W243, N261, D303, R331, N394, and W476 in *H. denitrificans*) are completely conserved. One of the proteins (MoxA) for insertion of Ca^{2+} ion into MDH also exhibited identities with those of other bacteria (28–34%).

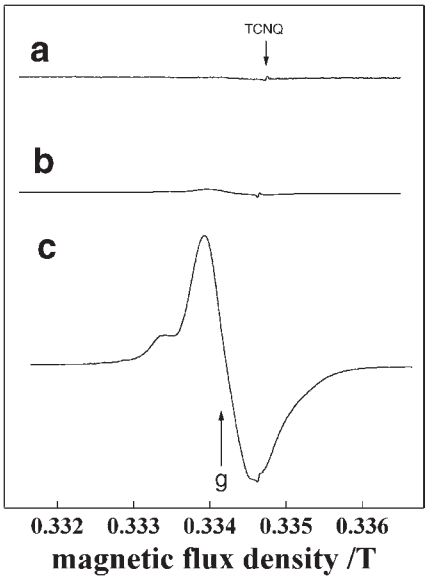

Figure 2. 77 K X-band EPR spectra of (**a**) Ca^{2+}-free MDH, (**b**) Ca^{2+}-treated MDH before and (**c**) after addition of Wurster's Blue in 40 mM of Tris-HCl buffer (pH 7.5). TCNQ: external standard radical marker. Sample concentrations: 400 μM.

The Ca^{2+}-free MDH isolated from native bacteria will give an insight into the elucidation of role of Ca^{2+} ion in quinoenzymes. The more detailed analysis of the elimination mechanism of Ca^{2+} ion from MDH and the roles of Ca^{2+} ion in MDH are in progress.

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