A Novel Quinoprotein Methanol Dehydrogenase Containing an Additional 32-Kilodalton Peptide Purified from *Acetobacter methanolicus*: Identification of the Peptide as a *MoxJ* Product†

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ABSTRACT: *Acetobacter methanolicus* is a unique acetic acid bacterium which has a methanol oxidase respiratory chain in addition to an ethanol oxidase respiratory chain. In this study, two different forms of methanol dehydrogenase (type I and II MDHs) were purified from *A. methanolicus* grown on methanol. Type I MDH was more basic (p*I* of 8.0) and smaller (M_r of 148K) than type II MDH (p*I* of 6.7 and M_r of 177K). Type I MDH consisted of α and β subunits of 62 and 10 kDa, which has the same $\alpha_2\beta_2$ conformation as the enzymes purified so far. The type II MDH contained an additional peptide of 32 kDa, of which a single copy was estimated to bind to the $\alpha_2\beta_2$-MDH judging from the whole molecular weight and the stoichiometry of each subunit determined in sodium dodecyl sulfate–high-performance gel filtration chromatography. Compared with type I MDH, type II MDH exhibited a lower enzyme activity, but the electron-transfer activity to cytochrome c_L was much more resistant to the inhibition with NaCl or EDTA. The possibility could be excluded that type II MDH is an artificial complex of type I MDH with a 32-kDa peptide, since it was inducible with methanol and could be detected in the periplasm, as well as the other subunits. Furthermore, the N-terminal amino acid sequence of the 32-kDa peptide showed a high homology to that of the *moxJ* product deduced from the DNA sequence of *Paracoccus denitrificans* or *Methylobacterium extorquens* AM1. Thus, the results obtained in this study indicate that the newly found type II MDH contains the additional peptide, that can be a *MoxJ* product, besides the $\alpha_2\beta_2$ subunits of MDH. Furthermore, the function of the MoxJ is discussed in relation to the electron-transfer reaction or the assembly of MDH.

Acetobacter methanolicus is an acidophilic methylotroph which has been described as a new species of acetic acid bacteria (Uhlig et al., 1986) and more recently has been reported to belong to the genus *Acidomonas* (Urakami et al., 1989). *A. methanolicus* is able to grow on methanol as well as on glycerol or glucose as carbon and energy sources by using a periplasmic oxidase system and a cytoplasmic ribulose monophosphate pathway (Steudel et al., 1980). The strain has a characteristic methanol oxidase system, besides ethanol oxidase system, functioning at acidic pH (Elliot & Anthony, 1988; Loffhagen & Babel, 1984). When grown on methanol, *A. methanolicus* has been shown to produce large amounts of methanol dehydrogenase (MDH),[1] a quinoprotein containing pyrroloquinoline quinone (PQQ) as the prosthetic group, and cytochromes c_L and c_H in the periplasm (Elliot & Anthony, 1988; Loffhagen & Babel, 1984) and a cytochrome *co*-type cytochrome *c* oxidase in the membrane (Chan & Anthony, 1991a; Matsushita et al., 1992). The methanol oxidase respiratory chain of the organism has been shown to be totally different from the ethanol oxidase respiratory chain in which a membrane-bound alcohol dehydrogenase is linked *via* ubiquinone to cytochrome *bo* ubiquinol oxidase (Matsushita et al., 1992).

MDH of methylotrophs has been purified as a soluble form and shown to exhibit an $\alpha_2\beta_2$ conformation consisting of 62- and 8.5-kDa peptides (Nunn et al., 1989). The quinoprotein dehydrogenase has been shown extensively to be coupled to cytochrome c_L (Anthony, 1988; Chan & Anthony, 1991b), and to further transfer electrons *via* a cytochrome *c* to a cytochrome *c* oxidase embedded in the membrane (Froud & Anthony, 1984; Mukai et al., 1990). However, the *in vitro* electron-transfer rate from MDH to cytochrome c_L was extremely low compared with that of the intact cells (Anthony, 1988). This may be due to one of the following reasons: (i) the purified components do not keep the native function which is retained in the intact cells; or (ii) another unknown factor or component is required for the system. In fact, Dijkstra et al. (1988) have shown that an oxygen-sensitive factor, isolated under anaerobic conditions, is able to activate NH_4-independent reaction of MDH.

Genetics of the methanol oxidation system (*mox*) have been well studied in *Paracoccus denitrificans* and *Methylobacterium extorquens* AM1 where many *mox* genes have been detected to be involved. Recently, the MDH structural gene (*moxF*) has been shown to be a part of the *moxFJGI* cluster, and further the *moxF* and *moxI* genes have been shown to be the structural genes for the α and β subunits, respectively, of MDH and the *moxG* product to be cytochrome c_L (Anderson & Lidstrom, 1988; van Spanning et al., 1991). Several lines of evidence suggest that *moxJ* is indispensable for formation of active MDH (Lee et al., 1991; van Spanning et al., 1991), that *moxJ* seems to be cotranslated together with *moxFGI*, and that the *moxJ* product (30-kDa peptide) can be translocated into periplasm (Anderson & Lidstrom, 1988; van Spanning et al., 1991). However, the product of the *moxJ* gene has never been detected, and thus the function has never been known.

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[1] Abbreviations: DCIP, 2,6-dichlorophenolindophenol; HPLC, high-performance liquid chromatography; MDH, methanol dehydrogenase; native-PAGE, nondenatured polyacrylamide gel electrophoresis; p*I*-PAGE, isoelectric focusing polyacrylamide gel electrophoresis; PMS, phenazine methosulfate; PMSF, phenylmethanesulfonyl fluoride; PQQ, pyrroloquinoline quinone; PVDF, poly(vinylidene difluoride); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone.

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In this study, two different forms of MDH were isolated from *A. methanolicus* grown on methanol. The present paper indicates that the novel type of MDH contains an additional peptide besides α and β subunits and that the peptide might be a *moxJ* product.

MATERIALS AND METHODS

Materials. DEAE- and CM-Toyopearls, medium-performance column resins, and TSK G3000SW, a gel filtration column for high-performance liquid chromatography (HPLC), were purchased from Toyo-Soda (Tokyo). Immunoblotting kit and prestained marker proteins were obtained from Bio-Rad. Poly(vinylidene difluoride) (PVDF) microporous membrane was purchased from Millipore Co. Cytochrome *c* (horse heart), phenylmethanesulfonyl fluoride (PMSF) and N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) were obtained from Sigma. Ampholine (pH 3.5–10.0 for IEF) was purchased from Pharmacia LKB. Yeast extract, HPLC marker proteins, and p*I* marker proteins were kindly supplied by Oriental Yeast Co. (Osaka). All other materials were of analytical grade and obtained from commercial sources.

Bacterial Strain, Growth Conditions, and Preparation of Soluble Fraction. A. methanolicus JCM6891 was used in this experiment. The organism was grown at 30 °C with a rotary shaking (200 rpm) up to the late logarithmic phase in a culture medium consisting of 10 mL of methanol, 3.0 g of $(NH_4)_2SO_4$, 1.0 g of KH_2PO_4, 0.16 g of K_2HPO_4, 0.7 g of $MgSO_4$, 0.5 g of NaCl, 0.4 g of $Ca(NO_3)_2{\cdot}4H_2O$, and 1 g of yeast extract per liter (Uhlig et al., 1986). Cells were harvested by centrifugation at 4 °C and then washed with distilled water and with 10 mM potassium phosphate buffer (pH 6.0). The washed cells were resuspended in 5 volumes of ice-cold 10 mM potassium phosphate buffer (pH 6.0) containing 5 mM $MgCl_2$. After the addition of small amounts of DNase, the suspension was passed twice through a French pressure cell press at 16 000 psi. After centrifugation at 10000*g* for 10 min to remove cells and cell debris, the supernatant was centrifuged at 120000*g* and 4 °C for 90 min. The ultracentrifugation was repeated to obtain soluble fraction.

Preparation of Periplasmic and Cytoplasmic Fractions. Freshly prepared cells were washed with 0.2 M Tris-HCl buffer (pH 7.5 at 25 °C), and 2 g of wet cells was then suspended in 10 mL of the same buffer containing 5 mM EDTA and 20% (w/v) sucrose. After addition of 20 mg of lysozyme, the suspension was incubated with gentle shaking at 30 °C for 2 h and then centrifuged for 10 min at 15000*g* and 4 °C. The resultant precipitates were resuspended in 10 mL of ice-cold 5 mM $MgCl_2$ solution and then incubated on ice for 10 min. The suspension was centrifuged for 10 min at 15000*g* and 4 °C to separate the supernatant and the precipitate. The supernatant was used as the periplasmic fraction, and the precipitate was resuspended in 5 mL of ice-cold 5 mM potassium phosphate buffer (pH 6.0). The suspension was treated by a French press as described above and centrifuged at 9000*g* for 10 min to remove the unbroken cell debris. The supernatant was further centrifuged for 90 min at 120000*g* and 4 °C to obtain the supernatant and the precipitate that were used as cytoplasmic and membrane fractions, respectively.

Purification of MDH and Cytochrome c_L. All purification procedures were performed at 0–4 °C unless described otherwise. After being dialyzed against 10 mM potassium phosphate buffer (pH 6.0), the soluble fraction was applied onto a DEAE-cellulose column (1-mL bed volume per 2.3 mg of protein) which had been equilibrated with 10 mM potassium phosphate buffer (pH 6.0). The column was washed with 2 column volumes of the same buffer and then with 30 mM potassium phosphate buffer (pH 6.0). MDH activity was detected in the pass-through fraction, while almost all cytochrome c_L was eluted together with a small amount of MDH with 30 mM potassium phosphate buffer. The fraction that passed through the DEAE-cellulose column was dialyzed against 10 mM Tris-HCl buffer (pH 8.0) and then applied onto a DEAE-Toyopearl column (1-mL bed volume per 2.2 mg of protein) preequilibrated with the same buffer. Elution of the enzyme was performed by 4 column volumes of 10 mM Tris-HCl (pH 8.0 at 25 °C) followed by a linear gradient consisting of 3 column volumes each of the same buffer without and with 100 mM NaCl. In the anion-exchange column chromatography, two fractions exhibiting MDH activity were obtained at the pass-through and adsorbed fractions. Thus, two different forms of MDH were purified by two-step column chromatographies. When there were still some impurities in these enzyme fractions, MDHs were further purified by a CM-Toyopearl column where both enzymes are similarly adsorbed in 10 mM potassium phosphate buffer (pH 6.0) and then eluted by a linear gradient from 10 to 100 mM potassium phosphate buffer (pH 6.0). Fractions having MDH activity were pooled, concentrated up to 2.0 mg of protein/mL by embedding it in sucrose powder or by ultrafiltration with a UK-50 membrane, and then frozen by liquid N_2 and stored at −20 °C before use.

The cytochrome c_L fraction, eluted with 30 mM potassium phosphate from the DEAE-cellulose column, was further purified by Sephadex G-75 gel filtration column chromatography. The partially purified cytochrome c_L (31.7 nmol/mg of protein) was frozen by liquid N_2 and stored at −20 °C before use.

Enzyme Assays. MDH activity was measured spectrophotometrically at 25 °C with phenazine methosulfate (PMS) and 2,6-dichlorophenolindophenol (DCIP) as electron acceptors. The reaction mixture (total 3 mL) for MDH contained 67 mM glycine–NaOH buffer (pH 9.5), 0.20 mM PMS, 0.11 mM DCIP, 30 mM NH_4Cl, 33 mM methanol, and enzyme solution. The reduction of DCIP was measured by following the decrease of the absorbance at 600 nm. Cytochrome c_L reduction activity of MDH was measured spectrophotometrically by the reduction of cytochrome *c* (horse heart) *via* cytochrome c_L. The reaction mixture (total 1 mL) contained 10 mM acetate buffer (pH 5.5), 50 μM cytochrome *c*, 0.5 μM cytochrome c_L, and purified MDH. The reduction rate of cytochrome *c* was determined at 25 °C by measuring the increase of the absorbance at 549–541 nm. Millimolar extinction coefficients of 15.9 and 19.0 cm^{-1} were used for DCIP at pH 9.5 and cytochrome *c*, respectively. Glucose dehydrogenase activity was also measured spectrophotometrically with PMS and DCIP (Matsushita et al., 1992). Glucose-6-phosphate dehydrogenase was measured at 50 mM Tris-HCl (pH 7.5) by the standard method (Langdon, 1966).

Polyacrylamide Gel Electrophoresis. Three different gel electrophoreses were performed in this study. Nondenatured polyacrylamide gel electrophoresis (native-PAGE) was performed using a slab gel consisting of a 7% polyacrylamide separating gel part and a 3% stacking gel part without any treatment of the sample. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250 or with an activity staining which was done in the same solution as the reaction mixture for PMS–DCIP assay except for including a few grains of nitroblue tetrazolium instead of DCIP. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using a slab gel with a 13% polyacrylamide

separating gel and a 5% stacking gel after the sample was treated with 2% SDS at 60 °C for 30 min. The gel was stained with Coomassie brilliant blue R-250. The molecular weight of the sample peptides was estimated from comparison with the marker proteins consisting of phosphorylase *b* (92 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (31 kDa), and lysozyme (14 kDa). Isoelectric focusing polyacrylamide gel electrophoresis (p*I*-PAGE) was performed with a disc gel containing 7.5% polyacrylamide and 5.0% ampholine (pH 3.5–10). Electrophoresis was carried out at 200 V for 4 h with 0.02 M phosphoric acid as the anodic solution and 1 M NaOH as the cathodic solution. Proteins on the gel were stacked with 3.0% perchloric acid and then stained with Coomassie brilliant blue G-250 in 3.0% perchloric acid. Activity staining was done as with native-PAGE. The p*I* value of MDH was determined by comparing with p*I* marker proteins consisting of horse heart cytochrome *c* (p*I* of 10.6) and several acetylated horse heart cytochromes *c* having p*I*'s of 4.1, 4.9, 6.4, 8.3, and 9.7.

HPLC Gel Filtration Chromatography. High-performance gel filtration was carried out with a TSK G3000SW gel column (21.5 mm × 60 cm) in denatured and nondenatured conditions. In the nondenatured condition, elution was done in 0.1 M Mops buffer (pH 7.0) containing 0.4 M NaCl with a flow rate of 1.0 mL/min. The molecular weight of MDH was calibrated using marker proteins consisting of glutamate dehydrogenase (290 kDa), lactate dehydrogenase (140 kDa), enolase (67 kDa), adenylate kinase (32 kDa), and cytochrome *c* (12.4 kDa). In the denatured condition, MDH was treated with 2% SDS and eluted with a flow rate of 0.5 mL/min in 0.1 M Tris-HCl (pH 7.5) containing 0.4 M NaCl and 2% SDS. The same marker proteins as for SDS–PAGE were used for molecular weight calibration.

Molecular Weight Determination of Whole MDH. The molecular weight of whole MDH was determined by monitoring the elution from HPLC by low-angle laser light scattering photometry and precision differential refractometry (Takagi & Hizukuri, 1984). MDH (2 mg/mL, 100 μL) was applied to HPLC gel filtration with a TSK gel G3000SW column (21.5 mm × 30 cm) after filtration through a Millipore filter with a pore size of 1.0 μm. Elution was performed with a flow rate of 0.5 mL/min, and monitored with a low-angle laser light photometer and then with a precision differential refractometer equipped with a double pen recorder. The molecular weight of MDH was calculated from the equation

$$MW = k(\text{area})_{LS}/(\text{area})_{RI}$$

where $(\text{area})_{LS}$ and $(\text{area})_{RI}$ are the total peak area under the elution curve of the scattering photometer and that of the refractometer, respectively. k is a constant depending on the instrumental and experimental conditions, and determined to be 90 476 using ovalbumin (M_r 45 000) as the standard.

Immunoblotting Analysis. Anti-MDH was obtained from rabbits immunized with the type II MDH. The enzyme (1.0 mg of protein) mixed with an equal volume of Freund's complete adjuvant was injected subcutaneously into a New Zealand white rabbit. Booster injection was done with 0.5 mg of the same enzyme mixed with the incomplete adjuvant 1 month later. The rabbit was bled 2 weeks later, and the blood was stored at 25 °C for 4 h and centrifuged to remove red cells. The antisera thus obtained were used without further purification. For immunoblotting, samples were treated with 2% SDS, SDS–PAGE was performed with a 13% polyacrylamide gel, and proteins on the gel were transferred onto a PVDF membrane electrophoretically (Matsudaira, 1987).

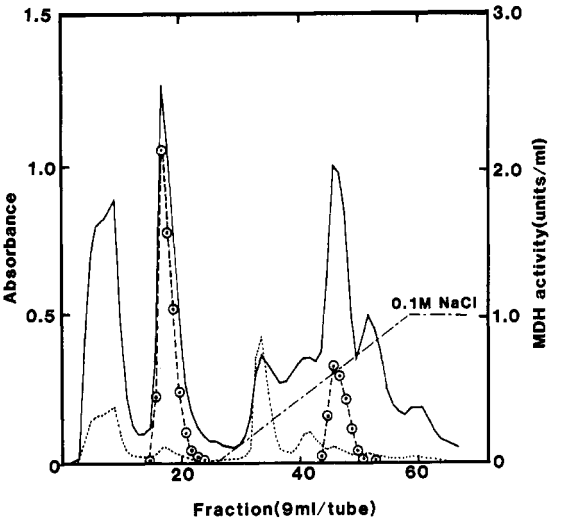

FIGURE 1: Elution profile of MDH in DEAE-Toyopearl column chromatography. Soluble fraction passed through a DEAE-cellulose column (114 mg of protein) was applied onto a DEAE-Toyopearl column (a column volume of 50 mL) preequilibrated with 10 mM Tris-HCl buffer (pH 8.0). Elution was made by using a linear gradient of NaCl from 0 to 100 mM (– • –) as described under Materials and Methods. (—) Absorbance at 280 nm; (···) absorbance at 415 nm. (⊙– –⊙) MDH activity (units/mL).

Table I: Summary of a Typical Purification of Two Forms of MDH from *A. methanolicus*

step	protein (mg)	MDH activity		recovery (%)
		units	units/mg	
soluble fraction	580	119	0.205	100
DEAE-cellulose	114	107	0.935	89.8
DEAE-Toyopearl				
pass-through fraction	19.8	52.7	2.66	44.1
adsorbed fraction	15.6	20.4	1.30	17.1

After being blocked with 3% gelatin and washed, the membrane was incubated for 2 h with anti-MDH. After being washed, the membrane was incubated with protein A–peroxidase conjugate solution for 2 h and then visualized by the addition of color reagent and H_2O_2.

Amino-Terminal Sequence Analysis. SDS–PAGE and electrophoretic transfer of peptides onto a PVDF membrane were done as described above. The peptide on the membrane was stained with 0.1% Coomassie brilliant blue for 1 min and then washed. After the membrane containing the peptide was visualized, it was cut out, and the N-terminal sequence was directly analyzed by a gas-phase protein sequenator.

Other Analytical Procedures. Absorption spectrophotometry was performed with a Hitachi 557 dual-wavelength spectrophotometer. Cytochrome *c* content was estimated from the reduced minus oxidized difference spectrum using the millimolar extinction coefficient of 19.1 at 550–540 nm. Protein content was determined by the modified Lowry method using bovine serum albumin as a standard (Dulley & Grieve, 1975).

RESULTS

Purification of MDH from the Soluble Fraction of A. methanolicus. In this study, as summarized in Table I, two different forms of MDH were purified by two anion-exchange column chromatographies from the soluble fraction of *A. methanolicus*. MDH activity was separated into pass-through (type I MDH) and adsorbed (type II MDH) fractions on the DEAE-Toyopearl column chromatography, of which a typical elution profile is shown in Figure 1. The percentage of type II MDH in MDHs purified from the soluble fraction varies

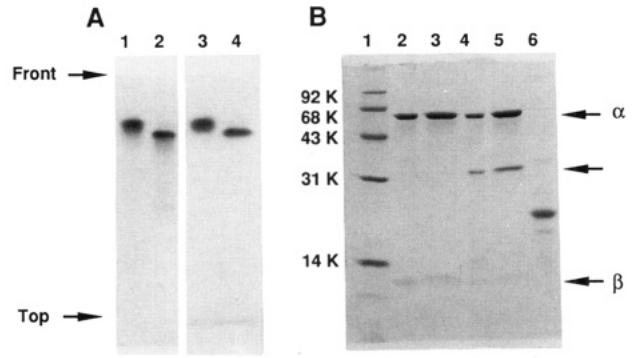

FIGURE 2: Electrophoretic analysis of two forms of purified MDH. (A) Native-PAGE; 10 μg of protein each of type I MDH (lanes 1, 3) or type II MDH (lanes 2, 4) was applied to each lane. Gels were stained with Coomassie blue R-250 (lanes 1, 2) and with activity staining using nitroblue tetrazolium (lanes 3, 4). (B) SDS–PAGE; type I MDH (lanes 2, 3) and type II MDH (lanes 4, 5) were applied to each lane; lanes 2 and 4, 4 μg of proteins; lanes 3 and 5, 6 μg of protein. Lane 1 shows molecular marker proteins, and lane 6 shows purified cytochrome c_L (5 μg of protein).

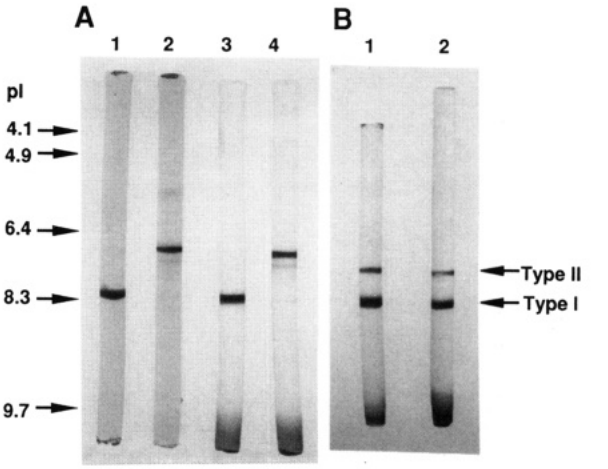

FIGURE 3: p*I*-PAGE of two forms of purified MDH and of MDH in the periplasmic and soluble fractions. (A) Type I MDH (24 μg of protein) was applied to gels 1 and 3, and type II MDH (20 μg of protein) was applied to gels 2 and 4. Gels 1 and 2 were stained for protein and gels 3 and 4 for enzyme activity. (B) Periplasmic fraction (18 μg of protein) and soluble fraction (86 μg of protein) prepared as described under Materials and Methods were applied to gels 1 and 2, respectively. The gels were stained for the enzyme activity.

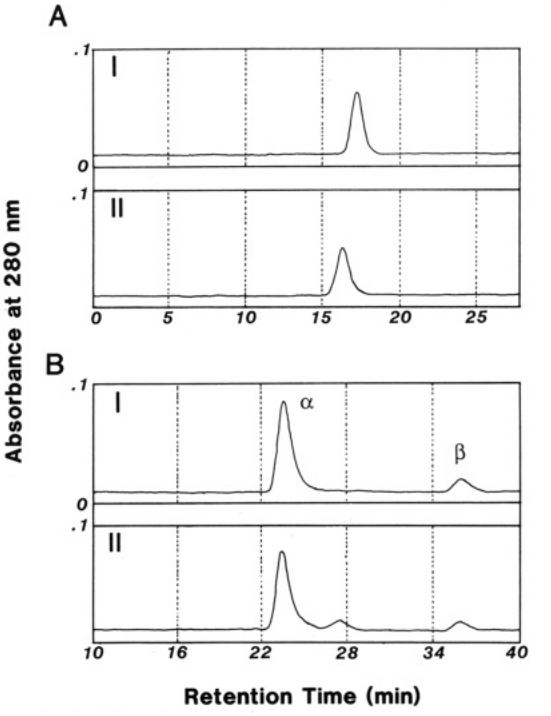

FIGURE 4: Gel filtration on HPLC of two forms of purified MDH. Purified type I MDH and type II MDH were analyzed with a TSK-Gel G-3000SW column in the presence or absence of SDS. Elution conditions are described under Materials and Methods. (A) In the absence of SDS, type I MDH (I; 51 μg) and type II MDH (II; 50 μg) were eluted with a flow rate of 1.0 mL/min. (B) In the presence of SDS, type I MDH (I; 49 μg) and type II MDH (II; 51 μg) were eluted with a flow rate of 0.5 mL/min.

from 10 to 44%, in terms of protein content, from experiment to experiment. This is in clear contrast with the previous observations that only a single species of MDH was purified from several methylotrophs and even from *A. methanolicus* (Elliot & Anthony, 1988). As described under Materials and Methods, another minor but significant MDH activity, corresponding to 3–4% of the total activity, was also detected in the adsorbed fraction of the DEAE-cellulose column, which was eluted together with cytochrome c_L. The MDH fraction, although not purified, seemed to contain type II MDH, judging from a 34-kDa band seen in SDS–PAGE (see below).

The presence of two forms of MDH was examined in the soluble fraction prepared by several different conditions. Since both MDHs could be distinguished as different bands in p*I*-PAGE as will be described later, the ratio of both MDHs in the crude soluble fraction could be seen by staining gels of p*I*-PAGE for activity (see Figure 3). Two forms of MDH could be seen with almost the same ratio, regardless of (i) addition of proteinase inhibitors (PMSF and TLCK), (ii) addition of chelator (EDTA or EGTA), (iii) pH or ionic strength of the buffer used, or (iv) growth phase of cells used, during the preparation of the soluble fraction (data not shown). In any case, in the p*I*-PAGE of the soluble fraction, 20–30% of MDH activity was always detected in the band corresponding to type II MDH.

Structural Properties of Type I MDH and Type II MDH. Homogeneity of both purified MDHs was confirmed by four different means: native-PAGE, p*I*-PAGE, SDS–PAGE, and HPLC gel filtration as shown below. Both MDHs showed a single band exhibiting the enzyme activity in native-PAGE; type II MDH migrated slightly faster than type I MDH (Figure 2). The MDHs also gave a single band with MDH activity in p*I*-PAGE where type I and type II MDHs exhibited p*I* values of 8.0 and 6.7, respectively (Figure 3). As seen in SDS–PAGE (Figure 2), type I MDH consists of two subunits of 62 and 10 kDa that correspond to α and β subunits, respectively, of MDH purified from several methylotrophs (Chan & Anthony, 1991b; Nunn et al., 1989), while type II MDH consisted of three subunits whose molecular weights were estimated to be 62K, 34K, and 10K. The purity of both MDHs was also confirmed by HPLC gel filtration where a single and symmetric peak was observed with both enzymes (Figure 4); type II MDH (retention time of 16.3 min) was eluted faster than type I MDH (17.1 min). Molecular weights estimated from comparison with marker proteins by this method were 96K and 126K for type I and type II MDHs, respectively. HPLC gel filtration of MDHs was also performed in the presence of SDS (Figure 4). As in the case of SDS–PAGE, type I MDH was separated into two subunits (retention times of 23.6 and 35.8 min) and type II MDH into three subunits (23.5, 27.4, and 35.8 min). From the calibration with marker proteins, the molecular weights of these three subunits were estimated to be 58K, 32K, and 8.7K, respectively. The molar ratio of each subunit could be estimated to be 1:0.44:0.87 by integrating the A_{280} elution profile with an

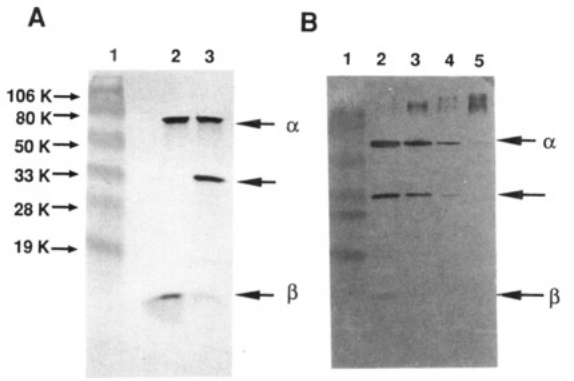

FIGURE 5: Immunoblotting analysis with an antibody prepared against type II MDH of purified type I and type II MDHs, and of soluble and membrane fractions of the cells grown on methanol or glycerol. (A) One microgram of protein each of type I (lane 2) and type II (lane 3) MDHs was subjected to SDS–PAGE and immunoblotted. (B) Twenty micrograms of protein each of the soluble (lanes 2 and 4) and membrane (lanes 3 and 5) fractions prepared, as described under Materials and Methods, from the cells grown on methanol (lanes 2 and 3) or on glycerol (lanes 4 and 5) was subjected to SDS–PAGE and immunoblotted. Lane 1 of both (A) and (B) shows prestained marker proteins.

assumption that each peptide has the same extinction.

Although the whole molecular weight of both MDHs was estimated by nondenatured HPLC gel filtration as described above, the values obtained were extremely lower than estimated. Since the TSK gel column used for HPLC is known to have an electrostatic interaction with some proteins, especially with highly basic proteins (Inouye, 1991), such slow elution of MDH from the TSK gel column seems to be due to the interaction of basic MDH having a p*I* of 8.0 with the column matrix. In fact, the deletion of high salt (0.4 M NaCl) from the elution buffer further retarded the elution of MDH. In this study, therefore, the molecular weight of MDH was determined by a more reliable light-scattering method that determines the molecular size on the basis of laser-dependent light scattering of a protein eluted from HPLC as described under Materials and Methods. This method gave much larger values; type I and II MDHs are estimated to be 148K and 177K, respectively.

As shown in Figure 5, antibody raised against type II MDH cross-reacted with both subunits of type I MDH much the same as the subunits of type II enzyme. Together with the largest and smallest subunits between both MDHs having identical molecular weights, it is suggested that type II MDH has the same peptides as the α and β subunits of type I MDH.

Enzyme Activities of Type I MDH and Type II MDH. When MDH activity was measured by using a dye-linked assay with PMS/DCIP, both MDHs showed the maximum activity at an unnatural alkaline pH of 9.5, which is the same as MDHs of other methylotrophs (Anthony, 1988). As summarized in Table II, type I MDH exhibited a specific activity of 2.65–4.49 units/mg of protein from preparation to preparation, under the assay condition described under Materials and Methods, which is about 2-fold higher than that of type II MDH (1.21–1.91 units/mg of protein), while an affinity for PMS was lower in type I MDH than in type II enzyme. Both MDHs required NH_4Cl as activator and exhibited the same substrate specificity to oxidize primary alcohols, formaldehyde, and acetaldehyde besides methanol. Both MDHs could also reduce cytochrome c_L, which can be monitored by the reduction of horse heart cytochrome *c*. The reduction of cytochrome c_L with both MDHs was independent of the presence of methanol, though the reason is unknown.

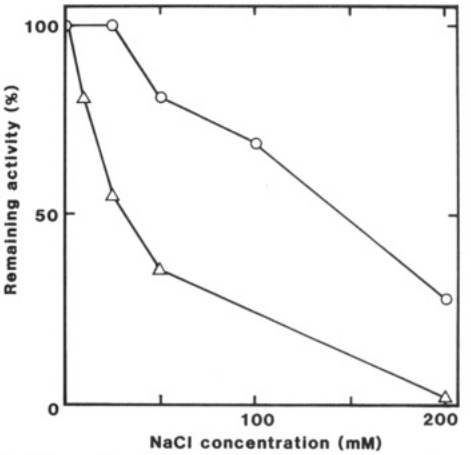

FIGURE 6: Effect of ionic strength on the interaction of MDHs with cytochrome c_L. In the presence of NaCl (0–200 mM), cytochrome c_L-dependent cytochrome *c* reductase activity was measured in 10 mM acetate buffer (pH 5.5) as described under Materials and Methods. The assay was performed by using 0.22 μM type I MDH (△) and 0.54 μM type II MDH (○).

Table II: Enzyme Activities of Two Forms of MDH Purified from *A. methanolicus*

enzyme activities	type I MDH	type II MDH
PMS/DCIP reductase[a]		
activity (μmol min^{-1} mg^{-1})	3.54 ± 0.75 (6)[b]	1.62 ± 0.30 (6)
turnover number (e s^{-1} mol^{-1})	8.7	4.8
K_m for PMS (mM)	5.0	2.2
cytochrome c_L-dependent cytochrome *c* reductase[c]		
activity (nmol min^{-1} mg^{-1})	147	61.7
turnover number (e s^{-1} mol^{-1})	0.36	0.18
K_m for cytochrome c_L (μM)	1.6	4.8

[a] The activities were measured at pH 9.5 as described under Materials and Methods. [b] The values are an average and standard deviations with six different samples. [c] The activity was measured with 0.5 μM cytochrome c_L at pH 5.5 as described under Materials and Methods.

The maximum cytochrome *c* reductase activity was observed at pH 5.5 for both MDHs. With 0.5 μM cytochrome c_L, the reduction rate of cytochrome *c via* cytochrome c_L with type I MDH was 2–3-fold higher than that of type II enzyme. The affinity for cytochrome c_L was also 2–3-fold higher in type I MDH. However, the turnover number of the cytochrome c_L-dependent reaction is much lower than that of the dye–reductase activity. As shown in Figure 6, although the interaction with cytochrome c_L of both MDHs was inhibited with high concentrations of NaCl, type II MDH was much more resistant against such an ionic strength than type I MDH was. Similarly, the cytochrome c_L-dependent activity of type II MDH was more resistant against EDTA; type I and II MDHs were inhibited 95% and 35%, respectively, with 5 mM EDTA.

Localization of Type II MDH or the 32-kDa Peptide in A. methanolicus. To learn the localization of type II MDH, periplasmic and cytoplasmic fractions were prepared from *A. methanolicus* cells. As shown in Table III, separation of the compartments can be confirmed by comparison of MDH activity with glucose-6-phosphate dehydrogenase, a cytoplasmic marker enzyme, and membrane-bound glucose dehydrogenase activities. Thus, almost all of the MDH activity was detected together with cytochrome *c* in the periplasmic fraction. As can be seen in Figure 3, the periplasmic fraction contained two forms of MDH as in the case of the soluble fraction in which periplasm is not separated from cytoplasm.

The MDH detected in the periplasmic fraction was purified by the same procedure as in the case of the purification of

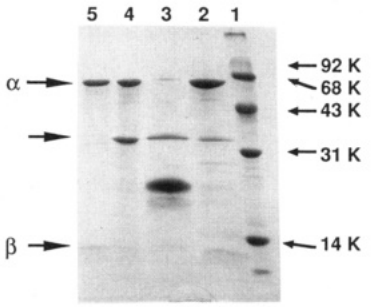

FIGURE 7: SDS–PAGE of several fractions obtained during purification of MDH from periplasm. Lane 1, molecular weight marker proteins; lane 2, pass-through fraction having the MDH activity of DEAE-cellulose (15 μg of protein); lane 3, cytochrome *c* fraction of DEAE-cellulose (10 μg of protein); lane 4, MDH fraction in DEAE-Toyopearl exhibiting lower enzyme activity (8.9 μg of protein); lane 5, MDH fraction in DEAE-Toyopearl exhibiting higher enzyme activity (5.0 μg of protein).

Table III: Separation of Periplasm and Cytoplasm from *A. methanolicus* Cells by the Osmotic Shock Method[a]

components	periplasm	cytoplasm	membrane
protein (mg)	31.8 (38)	29.2 (35)	22.7 (27)
MDH (units)	48.3 (80)	9.28 (16)	2.00 (4)
GDH (units)	0.00 (0)	0.36 (28)	0.91 (72)
G-6-PDH (units)	1.79 (6)	22.9 (85)	2.39 (9)
cytochrome *c* (nmol)	43.0 (77)	9.68 (17)	3.42 (6)

[a] Periplasm, cytoplasm, and membrane were prepared from 2 g of wet cells as described under Materials and Methods. Data express the total activity of MDH, glucose dehydrogenase (GDH), and glucose-6-phosphate dehydrogenase (G-6-PDH) or the total contents of protein and cytochrome *c*. Parentheses show the percentage of each fraction per whole cell.

MDH from the soluble fraction. As in the previous purification, MDH activity (a specific activity of 2.40 units/mg of protein) was eluted in the pass-through fraction of DEAE-cellulose. In this case, however, cytochrome *c* (22.4 nmol of cytochrome/mg of protein) was eluted with a delay from MDH activity in the same pass-through fraction of DEAE-cellulose, which is not observed in the purification of MDH from the soluble fraction. Then, the MDH detected in the pass-through fraction was separated into two fractions, with specific activities of 3.70 and 0.47 units/mg, in DEAE-Toyopearl column chromatography as in the purification from the soluble fraction. As shown in Figure 7, the two forms of MDH with high and low enzyme activities correspond to type I and type II MDHs, respectively. It should be noted that the cytochrome *c* fraction eluted with a delay contained a 34-kDa band besides a 20-kDa band corresponding to cytochrome c_L. The peptide band was shown to be the 32-kDa peptide involved in type II MDH by immunoblotting analysis (data not shown).

Induction of the 32-kDa Peptide of Type II MDH with Methanol. As shown in a previous paper (Matsushita et al., 1992), *A. methanolicus* contains about 50-fold higher MDH activity when the cells are grown on methanol than grown on glycerol. Therefore, it was examined by immunoblotting with an antibody against type II MDH whether the 32-kDa peptide is induced with methanol as well as other larger and smaller subunits of MDH. As shown in Figure 5, the glycerol-grown cells contained only little of the peptides when compared with the methanol-grown cells, suggesting that the 32-kDa peptide is also inducible with methanol as well as α and β subunits of MDH.

N-Terminal Amino Acid Sequence of the 32-kDa Peptide of Type II MDH. Twenty-nine amino acids in the N-terminal of the 32-kDa peptide were determined and compared with the amino acid sequences of the N-terminal portion in the

Type II MDH 32 kDa Peptide	1	Ser	Asp	Gly	Asp	Lys	Val	Leu	Arg	Ile	?	Ala	Ser	12																	
P. denitrificans Mox J		1	Asp	-	Thr	Thr	Asn	Leu	Arg	Val	Cys	Ala	Ser	10																	
M. extorquens Mox J		23	Pro	Asp	Ala	Gly	Thr	-	Leu	Arg	Val	Cys	Ala	Ala	33																
	13	Thr	Gln	Asn	Ala	Pro	Phe	Ser	Arg	Ser	Asp	Ala	Ser	Gly	Phe	Glu	Asp	Lys	29												
	11	Thr	Lys	Asp	Ala	Pro	Phe	Ser	Asp	Ala	Gln	Gly	Ala	Gly	Phe	Glu	Asn	Lys	27												
	34	Glu	Gln	-	Pro	Pro	Leu	Ser	Met	Lys	Asp	Gly	Ser	Gly	Leu	Glu	Asn	Arg	49												

FIGURE 8: N-Terminal amino acid sequence of the 32-kDa peptide of type II MDH compared with those of the expected mature MoxJ of *P. denitrificans* and *M. extorquens* AM1. The sequence of the 32-kDa peptide of type II MDH was determined in this experiment, and those of of *P. denitrificans* and *M. extorquens* AM1 were deduced from the DNA sequences of the respective *moxJ* (Anderson & Lidstrom, 1988; van Spanning et al., 1991). Boxed regions indicate identical amino acids among these sequences. The tenth amino acid residue of the 32-kDa peptide could not be determined in this experiment.

expected mature protein of the *moxJ* products of *P. denitrificans* and *M. extorquens* AM1 which can be deduced from their DNA sequences of *moxJ* (Anderson et al., 1990; van Spanning et al., 1991). As shown in Figure 8, of the N-terminal 29 amino acids in the 32-kDa peptide of type II MDH, 14 and 11 amino acid residues in the sequence were identical with those of *P. denitrificans* and of *M. extorquens* AM1, respectively. Incidentally, 13 identical amino acid residues were observed in the N-terminal sequences of these expected *moxJ* products between *P. denitrificans* and *M. extorquens* AM1. Since the expected whole mature portion of MoxJ has 41% homology between *P. denitrificans* and *M. extorquens* AM1 (Anderson et al., 1990), the N-terminal sequence homology (48% or 41%) between the 32-kDa peptide of type II MDH and MoxJ of *P. denitrificans* or *M. extorquens* AM1, respectively, can be considered to be reasonably high.

Furthermore, the N-terminal sequence of the largest and smallest subunits of type II MDH was also determined to confirm the identity with α and β subunits of type I MDH. Only the first 2 amino acids, Asn and Glu, were obtained in the largest subunit, and the first 12 amino acids, Ala, Tyr, Asp, Gly, Thr, His, X, Lys, Lys, Pro, Gly, and Val, were determined in the smallest subunit. These are consistent with the reported sequences of α and β subunits of MDH from *A. methanolicus* (Chan & Anthony, 1991b).

DISCUSSION

The results presented here clearly indicate that two different forms of MDH are presented in *A. methanolicus* grown on methanol, one of which (type I MDH) consists of two subunits but the other (type II MDH) consists of three subunits. This finding is in clear contrast with previous studies where only a single species of MDH (type I) has been purified from a wide variety of methylotrophs (Anthony, 1982) including *A. methanolicus* (Elliot & Anthony, 1988). In these previous studies, type II MDH may have been overlooked since it exhibits relatively low enzyme activity (see Figure 1). That the presence of the 32-kDa peptide in type II MDH is not artificial but physiological can be reasonably concluded on the basis of the following observations. (i) Type II MDH behaves as a single protein, like type I MDH, in several different analytical procedures such as gel filtration on HPLC, native-PAGE, and p*I*-PAGE. (ii) Type II MDH is detected at an almost constant level in any conditions for cell disruption or in cells of any growth phases. (iii) Type II MDH is present in the periplasmic fraction and can also be purified from the periplasm. (iv) The 32-kDa peptide is induced with methanol as well as the other subunits of MDH.

Type I MDH consists of large and small subunits whose molar ratio was estimated to be approximately 1:1 by HPLC gel filtration in the presence of SDS. The molecular size of the large subunit was estimated to be 62 or 58 kDa in SDS–PAGE or in SDS–HPLC gel filtration, respectively, and that of the small subunit to be 10 or 8.7 kDa, respectively, whereas the whole molecular weight of MDH was determined to be 148K by a light-scattering method. Thus, type I MDH can be concluded to have an $\alpha_2\beta_2$ conformation. Since MDH has been shown to have an $\alpha_2\beta_2$ structure consisting of large (α; 62 kDa) and small subunits (β; 8.5 kDa) in *M. extorquens* AM1 (Nunn et al., 1989) and more recently in MDH purified from *A. methanolicus* (Chan & Anthony, 1991b), type I MDH purified in this study seems to be the same as the one purified so far from several methylotrophs. On the other hand, the novel enzyme found in this study, type II MDH, consists of three subunits, of which the largest and the smallest subunits were the same as the α and β subunits, respectively, of type I MDH: their molecular sizes are identical, both subunits of type I MDH cross-react with the antibody prepared for type II MDH, and their N-terminal sequences are identical, whereas the middle-sized subunit of type II MDH was estimated to be 34 or 32 kDa by SDS–PAGE or SDS–HPLC gel filtration, respectively. Judging from the whole molecular weight of MDH, 177K, and the molar ratio of the three subunits (2:1:2), type II MDH can be considered to have a single copy of the 32-kDa peptide interacting with $\alpha_2\beta_2$-MDH.

The data obtained in this study also indicate that the N-terminal amino acid sequence of the 32-kDa peptide is highly homologous to that of MoxJ of *P. denitrificans* or *M. extorquens* AM1. Although a protein corresponding to MoxJ has never been detected in MDHs purified from these methylotrophs, genetic data have suggested that MoxJ can be translated and translocated into the periplasm, together with the α and β subunits of MDH and cytochrome c_L, and is also involved in the formation of active MDH (Anderson & Lidstrom, 1988; Anderson et al., 1990; Lee et al., 1991; van Spanning et al., 1991). In fact, the 32-kDa peptide was shown in this study to be present partly as a complex with $\alpha_2\beta_2$-MDH and partly as a free form or a complex with cytochrome c_L in the periplasm. Thus, the 32-kDa peptide of type II MDH can be concluded to be the *moxJ* product in *A. methanolicus*, and to function by interacting with $\alpha_2\beta_2$ subunits of MDH. Since some of the 32-kDa peptide is detected as the free form apart from $\alpha_2\beta_2$-MDH, it seems to be easily dissociated from the complex and thus is possible to interact with other $\alpha_2\beta_2$-MDHs to form $\alpha_2\beta_2\gamma$ or with $\alpha_2\beta_2\gamma$ to form $\alpha_2\beta_2\gamma_2$. Anyway, more search would be required to get some conclusion on the argument.

Since type II MDH exhibits a lower enzyme activity and is more acidic than type I MDH, the 32-kDa peptide may interact with the positively-charged portion of type I MDH that is involved in the interaction with cytochrome c_L (Chan & Anthony, 1991b). Therefore, the 32-kDa peptide may not be a catalytically essential component of MDH for the electron transfer to cytochrome c_L. However, the *in vivo* electron-transfer rate from MDH to cytochrome c_L cannot be reproduced with the $\alpha_2\beta_2$-type MDH *in vitro* (Anthony, 1988). Also, the electron transfer of type II MDH to cytochrome c_L was shown in this study to exhibit some tolerance to high ionic strength or chelating agents that are harmful for methanol oxidation even in intact cells (Matsushita et al., 1992). Thus, the possibility still remains that the MoxJ may be a component enabling MDH to work properly *in vivo*. Since the 32-kDa peptide is present in only part of MDH (type II MDH) with a half-stoichiometry or present as a free form, instead, it may be a protein enabling MDH to make the right conformation or to assemble the essential cofactor, PQQ or calcium ion, in the right way. This notion is the same idea as suggested already by van Spanning et al. (1991). Thus, it still remains ambiguous whether type II MDH is a final active complex made from type I MDH or an intermediate to make an active type I MDH. Further study is required to elucidate the function of the 32-kDa peptide or MoxJ in methanol oxidation.

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