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Purification and Characterization of an Activator Protein for Methanol Dehydrogenase from Thermotolerant *Bacillus* spp*

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Nico Arfman, Jozef Van Beeumen‡, Gert E. De Vries, Wim Harder, and Lubbert Dijkhuizen

From the Department of Microbiology, University of Groningen, NL-9751 NN Haren, The Netherlands and the ‡Laboratory of Microbiology and Microbial Genetics, University of Gent, B-9000 Gent, Belgium

All thermotolerant methanol-utilizing *Bacillus* spp. investigated by us possess a NAD-dependent methanol dehydrogenase (MDH) activity which is stimulated by a protein present in the soluble fraction of *Bacillus* sp. C1 cells. This activator protein was purified to homogeneity from *Bacillus* sp. C1 cells grown at a low dilution rate in a methanol-limited chemostat culture. The native activator protein (M_r = 50,000) is a dimer of M_r = 27,000 subunits. The N-terminal amino acid sequence revealed no significant similarity with any published sequences. Stimulation of MDH activity by the activator protein required the presence of Mg^{2+} ions. Plots of specific MDH activity *versus* activator protein concentration revealed Michaelis-Menten type kinetics. In the presence of activator protein, MDH displayed biphasic kinetics (*v versus* substrate concentration) toward C_1-C_4 primary alcohols and NAD. The data suggest that in the presence of activator protein plus Mg^{2+} ions, MDH possesses a high affinity active site for alcohols and NAD, in addition to an activator- and Mg^{2+}-independent low affinity active site. The activation mechanism remains to be elucidated.

In Gram-negative methylotrophic bacteria, the conversion of methanol to formaldehyde is catalyzed by a methanol dehydrogenase (EC 1.1.99.8), containing pyrroloquinoline quinone (PQQ)[1] as prosthetic group, which is located in the periplasmic space. This NAD(P)-independent classical methanol dehydrogenase oxidizes various primary alcohols with phenazine methosulfate (PMS) as an *in vitro* electron acceptor (1–4). Studies with Gram-positive methylotrophs, such as *Nocardia* sp. 239 (5), *Mycobacterium gastri* (6), and thermotolerant *Bacillus* spp. (7, 8) have shown that these organisms, which lack a periplasmic space, do not possess this classical methanol dehydrogenase. As an exception, PMS-linked methanol dehydrogenase activity could be demonstrated in *Corynebacterium* sp. XG (9), although the data do not exclude a possible role of NAD(P). Duine *et al.* (10) reported the presence of a NAD-dependent, PQQ-containing methanol dehydrogenase (nMDH) in *Nocardia* sp. 239. In this organism, nMDH activity was thought to reside in a multienzyme complex together with NAD-dependent formaldehyde dehydrogenase and NADH dehydrogenase. In contrast, in thermotolerant methylotrophic *Bacillus* strains, the initial oxidation of methanol is catalyzed by a novel NAD-dependent (and PQQ-independent) methanol dehydrogenase (MDH) (7, 8). We previously reported the purification and characterization of MDH from *Bacillus* sp. C1 (8). Several peculiar observations, such as disproportionality of MDH activity with respect to protein concentration and considerable loss of MDH activity during purification (but not of the reverse formaldehyde reductase activity), encouraged us to analyze the MDH system in more detail. Here we report the purification and characterization of a MDH activator protein and discuss its effect on the kinetics of alcohol oxidation by MDH from *Bacillus* sp. C1. Further biochemical properties and structural features of MDH are presented in the accompanying paper (11).

EXPERIMENTAL PROCEDURES AND RESULTS[2]

DISCUSSION

NAD-dependent alcohol dehydrogenases (ADH, EC 1.1.1.1) can be found in a wide variety of organisms, but only horse liver ADH and ADH from *Bacillus stearothermophilus* strain 2334 thus far were reported to possess significant reactivity toward methanol (19–21). Both these enzymes, however, are specialized in ethanol oxidation (Table IV), and their activity with methanol has no immediate physiological relevance. All 14 recently isolated methanol-utilizing thermotolerant *Bacillus* spp. investigated were found to possess an immunologically related NAD-dependent (and PQQ-independent) alcohol dehydrogenase. The enzyme oxidizes C_1-C_4 primary alcohols and 1,3-propanediol, but none of the resulting aldehydes (7, 8, 11, 12). The alcohol dehydrogenase (MDH) activity in these organisms is strongly stimulated in the presence of a M_r = 50,000 protein plus Mg^{2+} ions (Table III). Studies with the purified proteins of *Bacillus* sp. C1 revealed that the activator protein has a profound effect on the kinetic properties of MDH. At physiological methanol concentrations (0.1 to 1 mM), the methanol turnover rate of *Bacillus* sp. C1 MDH is increased up to 40-fold by the activator protein, as based on the kinetic constants shown in Table III. A comparison of kinetic data of the *B. stearothermophilus* and horse liver ADH enzymes (19) with those of MDH from *Bacillus* sp. C1 shows that the latter enzyme, in the absence of activator protein,

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[1] The abbreviations used are: PQQ, pyrroloquinoline quinone; MDH, NAD-dependent methanol dehydrogenase; ADH, alcohol dehydrogenase; nMDH, NAD-dependent PQQ-containing methanol dehydrogenase; SDS, sodium dodecyl sulfate; PMS, phenazine methosulfate.

[2] Portions of this paper (including "Experimental Procedures," "Results," Figs. 1–7, and Tables I–IV) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

has a similar k_{cat}/K_m value for methanol but a 500-fold lower k_{cat}/K_m value for ethanol. In contrast, fully activated MDH from *Bacillus* sp. C1 shows a 35- to 65-fold higher catalytic efficiency with methanol while the k_{cat}/K_m value for ethanol remains 30-fold lower (Table IV). Activation of NAD-dependent alcohol dehydrogenases by a specific activator protein has not been described before. The present data suggest that MDH from *Bacillus* sp. C1 is a representative of a novel class of alcohol dehydrogenases, displaying strongly increased methanol conversion rates when stimulated by the activator protein.

Studies with different activator/MDH ratios (Fig. 7) indicated that the activator protein primarily increases the V_{max} of MDH with a concomitant slight decrease in K_m for methanol. In addition, at higher activator concentrations, a second catalytic mechanism becomes apparent which possesses a significantly higher affinity for methanol. The observed kinetics could not be explained in terms of cooperativity as Hill plots of the primary data yielded Hill coefficients of about unity. Rather, the biphasic kinetics could be resolved in two independent catalytic mechanisms, each obeying Michaelis-Menten type kinetics but with different K_m and V_{max} values. The data therefore suggest that a high affinity site appears next to an activator-independent (and Mg^{2+}-independent) low affinity site.

Regulatory mechanisms controlling enzyme activity and involving interactions between separate proteins may either be based on product channelling (22), covalent enzyme modification, or the formation of a protein complex (23). The presence of activator protein did not affect the stoichiometry of the MDH reaction, which rules out the possibility that MDH activation is due to a sequential enzyme activity (22), using NADH or aldehyde as a substrate. The involvement of covalent enzyme modification in MDH activation is also unlikely, as stimulation of MDH activity is a reversible process (Fig. 6) and does not require additional substrates. Various techniques, including gel filtration chromatography (Fig. 1), electron microscopy (11), and activation studies with immobilized MDH (Fig. 6), were used to demonstrate the formation of a protein complex between MDH and activator protein, but none of them was successful. This indicates that MDH activation does not require the formation of a stable MDH-activator protein complex, but more likely involves a loose interaction between the two proteins.

The molar ratio of dimeric activator protein (M_r = 50,000) over decameric MDH (M_r = 430,000) (11) required for maximal stimulation of MDH activity *in vitro*, was determined as 3:1 (Fig. 5). The activator function is highly sensitive to dilution inactivation, however, as indicated by the disproportional MDH activities observed *in vitro* with crude extracts (Fig. 2) and the reconstituted system (purified MDH plus activator protein). This suggests that MDH stimulation *in vivo, i.e.* at high protein concentrations, occurs already at a considerably lower activator/MDH ratio. The relative concentrations of activator protein and MDH in the cell can be approximated from their overall purification factors. When assuming a 70% recovery of activator protein activity in the phenyl-Sepharose step (5-fold purification; Table I), the overall purification factor becomes approximately 425 (0.24% of total soluble protein). Under the applied growth conditions, MDH constitutes 38% of total soluble protein, based on the specific formaldehyde reductase activities of purified MDH (19.6 units/mg of protein) (8) and crude extract (7.4 units/mg of protein; result not shown). The intracellular molar ratio of dimeric activator protein over decameric MDH thus is estimated as 1:17.5. Clearly, when assuming that a substantial part of the MDH molecules in the cell are activated, a single activator molecule must be capable of stimulating a multiple number of MDH molecules. It is therefore not surprising that a protein complex between MDH and activator protein could not be demonstrated, as the two proteins probably interact only temporarily.

It becomes increasingly clear that methanol dehydrogenases in general require additional components to ensure full activity. The PQQ-dependent methanol dehydrogenases from Gram-negative methylotrophs require a high NH_4^+ concentration *in vitro* to facilitate the (rate-limiting) release of formaldehyde from the enzyme (4). An oxygen-labile, low molecular weight factor, has been described which may function *in vivo* (24). An analogous activation mechanism could play a role in the methylotrophic *Bacillus* spp, where the activator protein may act as an aldehyde or NADH releasing factor. The precise mechanism of activation, however, remains to be elucidated. Uncertainty therefore also remains with respect to the physiological role of the activator. These questions and the regulation of MDH and activator protein synthesis will be dealt with in future studies.

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Supplementary Material to

Purification and Characterization of an Activator Protein for Methanol Dehydrogenase from Thermotolerant Bacillus Spp.

by Nico Arfman, Jozef Van Beeumen, Gert E. De Vries, Wim Harder and Lubbert Dijkhuizen

EXPERIMENTAL PROCEDURES

Growth Conditions-The organisms used, Bacillus sp. C1, PB1, AR2, TS1, TS2 and TS4 and their maintenance have been described before (7). Bacillus sp. TF, WMS.1, S2, 4(55) and KA are related strains (12). Batch cultivation was performed at 50°C in a 50 mM methanol-mineral salts medium of pH 7.5 supplemented with 1 ml/l of a vitamin solution as described by Dijkhuizen et al. (7). Cells from early to mid-exponential phase were harvested by centrifugation at 3,800 x g for 10 min at 4°C, washed twice with 50 mM potassium phosphate buffer, pH 7.5, containing 5 mM $MgSO_4$, and stored at -80°C. For continuous cultivation of Bacillus sp. C1, the organism was grown at 50°C in a methanol-limited (100 mM methanol) chemostat using the mineral medium described by Levering et al. (13). The pH was maintained at 7.3 by automatic adjustment with 1 M NaOH.

Preparation of Extracts-Cells were disrupted in the presence of 5 mM dithiothreitol (DTT) by passage through a French pressure cell operating at 1.4×10^5 kN/m². Low speed extracts of Bacillus sp. C1 were obtained by centrifugation at 3,800 x g for 10 min at 4°C. Cell wall and membrane fragments were removed by centrifugation at 25,000 x g for 30 min at 4°C. The resulting high speed supernatants were used as crude extracts for enzyme assays.

Enzyme Assays-Spectrophotometric assays were performed with a Hitachi model 100-60 spectrophotometer. Unless stated otherwise, enzyme assays were performed at 50°C, using prewarmed buffer solutions. NAD-dependent alcohol dehydrogenase activity was assayed by following the alcohol-dependent production of NADH ($\varepsilon_{340} = 6.22$ mM^{-1} cm^{-1}), using the procedure described previously (8). This assay also served as the basic procedure for measuring the MDH-stimulating activity of the activator protein. The assay mixture (1 ml) contained: glycine/KOH buffer, pH 9.5, 100 µmol; $MgSO_4$, 5 µmol; NAD, 2 µmol; and purified MDH, 2.68 µg. After addition of the activator containing solution to the assay mixture and preincubation for 5 min at 50°C, the reaction was started with 500 µmol of methanol or ethanol. Only fractions devoid of MDH activity were used for quantification of activator protein activity. The measured reaction rates (v) were corrected for activator independent MDH activities (v_o). One unit of activity is defined as the amount of activator protein (expressed in µg) that stimulates a fixed quantity of purified Bacillus sp. C1 MDH (2.68 µg MDH/ml assay mixture) to 50% of V_{max}-v_o under the reaction conditions described. Double reciprocal plots of reaction rates versus activator concentration (S) resulted in straight lines, indicating Michaelis-Menten type kinetics. The following equation was applied:

$$v - v_o = \{V_{max} - v_o\} * S / (K_a + S)$$

One unit of activity therefore corresponds to the activation constant (K_a) of the reaction, which was determined from titration curves as shown in Fig. 5.

Formaldehyde reductase activity (the activity of NAD-dependent methanol dehydrogenase in the reverse direction) was measured by following the formaldehyde-dependent oxidation of NADH at 340 nm, using the procedure described before (8). CoASH-linked NAD-dependent acetaldehyde dehydrogenase [EC 1.2.1.10] was assayed using a slightly modified version of the spectrophotometric assay described by Hillmer and Gottschalk (14). The reaction mixture (1 ml) contained: glycine/KOH buffer, pH 9.0, 100 µmol; $MgSO_4$, 5 µmol; DTT, 5 µmol; NAD, 1.5 µmol; CoASH, 150 nmol and acetaldehyde, 50 µmol. The following enzymes were assayed according to previously published methods; hexulose-6-phosphate synthase (spectrophotometric assay) (13), NADH oxidase and NADH dehydrogenase (8).

MDH activity was also measured polarographically using a Clark-type oxygen electrode by coupling NADH production to oxygen consumption via PMS. The reaction mixture (3 ml) contained: glycine/KOH, pH 9.5, 300 µmol; $MgSO_4$, 15 µmol; NAD, 3 µmol; PMS, 0.3 µmol, low speed extract or high speed extract. After air saturation and temperature equilibration, the reaction was started with 1.5 mmol methanol. At 50°C, 100% air saturation corresponds to 0.187 mmol O_2/l H_2O.

One unit of enzyme activity is defined as the amount of enzyme catalyzing the conversion of one µmol of substrate per min under the assay conditions described.

Enzyme Purification-Purification of MDH has been described previously (8). The activator protein was purified from Bacillus sp. C1 cells grown under methanol limitation in chemostat culture at D = 0.026 h^{-1}. All steps were carried out with a System Prep 10 liquid chromatography system (Pharmacia LKB Biotechnology Inc.) and were performed at room temperature. Samples, taken after each purification step, were stored at -80°C.

Step 1: Preparation of Crude Extract-Crude extract (47 ml containing 860 mg of protein) was prepared as described above.

Step 2: 1st Hydrophobic-Interaction Chromatography-$(NH_4)_2SO_4$ was added to a final concentration of 1.7 M. After centrifugation during 10 min at 18,000 x g, the supernatant was injected onto a Phenyl-Sepharose CL-4B column (packed volume of 75 ml), equilibrated with 50 mM Tris.HCl buffer, pH 7.5, containing 5 mM $MgSO_4$, 5 mM 2-mercaptoethanol (buffer A) plus 1.7 M $(NH_4)_2SO_4$. A 450 ml linear gradient of 1.7-0 M $(NH_4)_2SO_4$ was applied at a flow rate of 3.0 ml/min. Active fractions were pooled and concentrated by $(NH_4)_2SO_4$ precipitation. The precipitate was collected by centrifugation during 30 min at 18,000 x g at 4°C and dissolved in a total volume of 16 ml 20 mM Tris.HCl buffer, pH 7.5, containing 5 mM $MgSO_4$ and 5 mM 2-mercaptoethanol (buffer B). MDH and activator activities were effectively separated by this procedure.

Step 3: 1st Anion-Exchange Chromatography-The concentrated pool was desalted in buffer B by passage through a Sephadex G25 gel filtration column (BioRad P010). The sample obtained (124 mg protein) was loaded onto a Fast Flow Q-Sepharose column (packed volume of 25 ml) equilibrated with buffer B. Proteins were eluted by applying a 270 ml linear 0-1 M KCl gradient at a flow rate of 3.0 ml/min. Peak fractions were pooled and concentrated by $(NH_4)_2SO_4$ precipitation. The precipitate was dissolved in 2.5 ml buffer B.

Step 4: 2nd Anion-Exchange Chromatography-The sample was desalted in buffer B as described in step 3 and injected onto a Mono-Q HR 5/5 anion-exchange column equilibrated in buffer B (flow rate 1 ml/min). Bound protein was eluted by applying a 30 ml linear 0-1 M KCl gradient. Active fractions were pooled.

Step 5: 2nd Hydrophobic-Interaction Chromatography-After addition of $(NH_4)_2SO_4$ to a final concentration of 1.7 M and centrifugation (10 min, 18,000 x g), the supernatant was applied onto a Phenyl-Superose HR 5/5 hydrophobic-interaction column equilibrated with buffer A. Proteins were eluted with a 30 ml linear 1.7-0 M $(NH_4)_2SO_4$ gradient at a flow rate 0.5 ml/min. Active fractions were pooled.

Step 6: Gel Filtration Chromatography-Samples of 200 µl, containing approximately 200 µg of protein, were injected onto a Superose 12 HR 10/30 gel filtration column equilibrated with 100 mM Tris.HCl buffer, pH 7.5, containing 5 mM $MgSO_4$ and 5 mM 2-mercaptoethanol at a flow rate 0.5 ml/min. Peak fractions of five separate runs were combined and stored at -80°C.

Molecular Weight Estimation-The native molecular weights of activator protein, and of MDH in the presence or absence of activator proteins were estimated by gel filtration chromatography. Samples, containing 0.2-2 mg of protein, were applied onto a Superose 12 HR 10/30 column and eluted as described above. A calibration curve was prepared with thyroglobulin (M_r 670,000), gamma globulin (M_r 158,000), ovalbumin (M_r 44,000), myoglobin (M_r 17,000) and cyanocobalamin (M_r 1,350).

Electrophoresis-SDS-polyacrylamide gel electrophoresis was performed using 15% polyacrylamide gels (15). The molecular weight of the denatured activator protein was estimated using the following standards: phosphorylase A (M_r 94,000), transferrin (M_r 80,000), albumin (M_r 68,000), catalase (M_r 58,000), fumarase (M_r 50,000), citrate synthase (M_r 46,000), carbonic anhydrase (M_r 31,000) and ribonuclease A (M_r 16,000). Gels were stained for protein with Coomassie Blue G-250.

Stoichiometry of the MDH Reaction-The stoichiometry of the methanol dehydrogenase reaction was studied by comparing the methanol dependent formation of NADH and formaldehyde under standard conditions. The formaldehyde concentration was determined using the method described by Nash (16). Samples obtained at equilibrium were used to calculate the equilibrium constant of the reaction. Controls containing all reactants except MDH, or all reactants except methanol, showed no color production.

Studies on the Interaction between MDH and Activator Protein with Immobilized MDH-Purified MDH (10 mg of protein) was immobilized on CH-Sepharose 4B (1.25 g freeze dried powder) according to the manufacturer's specifications, with 1-ethyl-3(3-dimethyl-aminopropyl)-carbodiimide hydrochloride as coupling agent. Immobilized MDH was packed in a column (packed volume of 5 ml) and subsequently connected to a System Prep 10 liquid chromatography system (Pharmacia LKB Biotechnology Inc.). The column was equilibrated with 100 mM glycine/KOH buffer, pH 9.0, containing 5 mM $MgSO_4$, 1 mM NAD and 500 mM methanol (buffer C) at a flow rate of 0.6 ml/min at 50°C. The NADH concentration in the eluent was measured by following the absorbance at 340 nm. The applied flow rate was sufficiently high to establish a steady state NADH concentration which was considerably lower than the reaction equilibrium. At zero time, 1.6 µg of purified activator protein in buffer C was injected onto the column. Fractions of 0.5 ml were collected and screened for the presence of activator protein as described above. As a control, the experiment was carried out at 10°C.

Kinetics-All kinetic data were obtained under the same experimental conditions as used for activity measurements. In case of biphasic kinetics, the K_m- and V_{max} values of the two segments could be approximated from primary data using the kinetic analysis procedure described by Neal (17). The k_{cat} values of MDH were calculated on the basis of a subunit M_r of 43,000 (2). Each subunit possesses one zinc ion, and is therefore assumed to contain one active site (8, 11).

Protein Determination-Protein concentrations were determined by the method of Bradford (18) using the Bio-Rad protein assay kit and bovine serum albumin as a standard.

Analytical-Purified activator protein (60 µg) was dialysed thoroughly against 10 mM Tris.HCl buffer, pH 7.0. Hydrolysis was carried out in the gas phase for 24 h at 106°C with 6 N HCl. The amino acid composition of the purified activator protein was analyzed on a 420 A Derivatizer (Applied Biosystems) linked to a 130 A Separation Systems (Applied Biosystems). Half-cystine was determined as cysteic acid after performic acid oxidation and hydrolysis. The N-terminal amino acid sequence of the activator protein was analyzed as described in the accompanying paper, using 0.5 nmol of the purified protein (11).

Materials-All chemicals were reagent grade, except those used in the derivatizer and the sequenator, which were sequence grade. Phenyl-Sepharose, Fast Flow Q-Sepharose, Phenyl-Superose HR 5/5, Mono-Q HR 5/5 and Superose 12 HR 10/30 were purchased from Pharmacia LKB Biotechnology Inc. The gel filtration standards, electrophoresis reagents, protein dye reagent and bovine serum albumin were purchased from Bio-Rad. DNase I, NAD, NADH, CoASH and electrophoresis standards (except for human transferrin, Serva), were obtained from Boehringer. PMS was purchased from Sigma. The Clark-type oxygen electrode was purchased from Yellow Springs Instruments Co. Formaldehyde was prepared from paraformaldehyde by incubating aqueous solutions in closed bottles at 110°C for 10 h.

RESULTS

Stimulation of MDH Activity by a M_r 50,000 Gel Filtration Fraction-Gel filtration chromatography of crude extracts of methanol grown Bacillus sp. C1 invariably resulted in a severe reduction of NAD-dependent methanol dehydrogenase activity, while more than 90% of NADH-dependent formaldehyde reductase activity was recovered. MDH activity could be restored, however, by combining the fraction which exhibited formaldehyde reductase activity (approximate M_r 280,000) with a separate fraction of M_r 50,000 (see Fig. 1, panel A). The activating fraction itself was completely devoid of MDH or formaldehyde reductase activity and did not stimulate the formaldehyde reductase reaction under the conditions used. Stimulating activity required the presence of Mg^{2+} and was completely lost following incubation for 5 min at 90°C. The molecular weight and heat lability of the activating component indicated that the factor was of a proteinaceous nature.

Methanol dehydrogenase assays with preparations devoid of the activator protein fraction, or lacking Mg^{2+} ions, invariably displayed linear initial reaction rates. In the presence of the activating fraction plus Mg^{2+} ions, however, initial reaction rates were either linear or accelerating, depending on the pretreatment of the assay components. Linear reaction rates invariably were observed when the activating fraction was preincubated with Mg^{2+} for 5 min at 50°C, before starting the reaction with NAD, methanol or MDH preparations. In contrast, accelerating reaction rates were observed when assays were started with crude extract (containing both MDH and activating fraction), or when temperature equilibrated (50°C) assay mixtures with (partially) purified MDH were stimulated by addition of activating fraction or Mg^{2+} (kept at room temperature).

When using optimal assay conditions (preincubation of activating fraction with Mg^{2+}), MDH activity could be titrated reproducibly by varying the concentration of the activating fraction in the assay mixture. In the presence of a saturating amount of activating fraction, MDH activity in crude extract was proportional with respect to the MDH concentration (Fig. 2). Proportional MDH activities were also observed in the absence of Mg^{2+}, albeit that the activities were approximately tenfold lower. In contrast, without additional activating fraction, the specific MDH activity in crude extracts increased with increasing protein concentrations. Prolonged preincubation (above 5 min) of MDH at this sub-optimal concentration of activating fraction did not result in a further increase in MDH activity, although the two proteins retained more than 90% of their activity for at least 30 min at 50°C. Disproportional activity and accelerating reactions described above were not specific for methanol as a substrate but were observed with all C_1-C_4 primary alcohols.

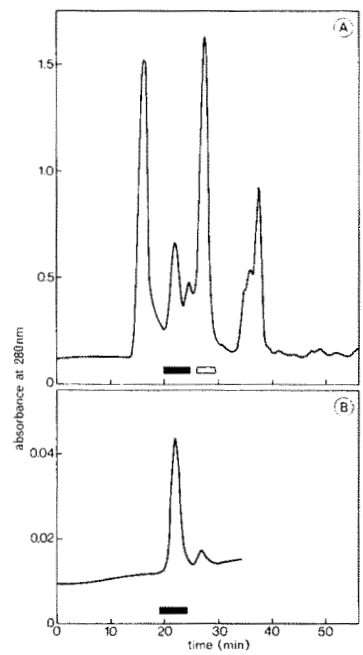

Fig. 1. **Gel filtration profiles of crude extract and purified MDH of Bacillus sp. C1.** Panel A; 200 µl freshly prepared extract (2 mg protein) of cells grown under methanol limitation in chemostat culture (D = 0.1 h^{-1}). Panel B; 200 µl of purified MDH (200 µg protein). The Superose 12 column (Pharmacia) was equilibrated at room temperature with 100 mM Tris.HCl buffer, pH 7.5, containing 5 mM $MgSO_4$ and 5 mM 2-mercaptoethanol at a flow rate of 0.5 ml/min. Samples were injected at zero time. Closed horizontal bars indicate fractions containing MDH activity. The open horizontal bar indicates fractions that show MDH-stimulating activity.

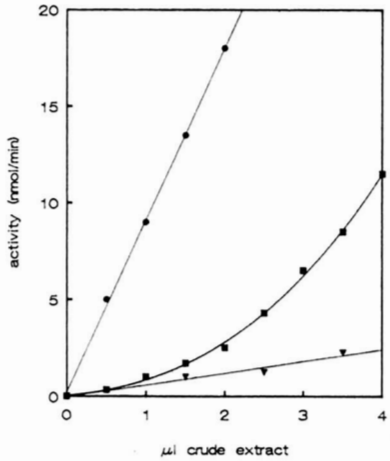

Fig. 2. **Effect of protein concentration, Mg^{2+} ions and activating fraction on MDH activity in crude extract of Bacillus sp. C1.** Crude extract (2.1 mg protein/ml) was prepared from cells grown in a methanol limited chemostat culture at $D = 0.1\ h^{-1}$. MDH activity was measured in the presence of 5 mM Mg^{2+} ions (squares), in the presence of a saturating amount of activating fraction (3.5 units) plus 5 mM Mg^{2+} ions (circles) or without Mg^{2+} ions (triangles). Reactions were started with 500 mM methanol.

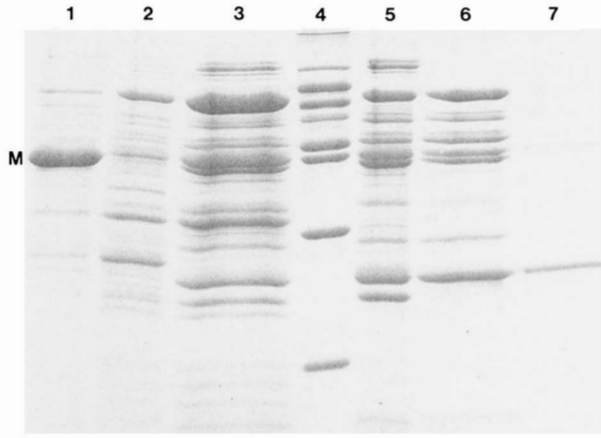

Fig. 3. **Denaturing polyacrylamide gel electrophoresis of samples obtained during purification of MDH activator protein of Bacillus sp. C1.** The activator protein was purified from cells grown in chemostat culture under methanol-limiting conditions ($D = 0.026\ h^{-1}$). The amounts of protein loaded on the gel are shown in parentheses. Lane 1, crude extract (10 µg); lane 2, Phenyl-Sepharose pool (7 µg); lane 3, Q-Sepharose pool (20 µg); lane 4, marker proteins (see methods); lane 5, Mono-Q pool (11 µg); lane 6, Phenyl-Superose pool (8 µg); lane 7, Superose 12 pool (1 µg). The gel (15% polyacrylamide) was stained for protein with Coomassie Blue G-250. M indicates the position of MDH.

Dissociation of MDH-Gel filtration of purified MDH gave rise to a small symmetric peak of about M_r 50,000 adjacent to the native M_r 280,000 peak (Fig. 1, panel B). Fractions of this minor peak did not display MDH activity or MDH-stimulating activity. As the purified enzyme preparation was shown to be homogeneous (11), the small peak could only result from enzyme dissociation. Complete dissociation of MDH into individual subunits was observed during storage at 4°C for 48 h, as indicated by gel filtration chromatography and electron microscopy (11). The monomers could not be reactivated by the activating fraction. MDH dissociation was not responsible for the disproportionality of MDH activity with respect to protein concentration observed in crude extracts, since purified MDH displayed normal proportional activity, both in the absence of activating fraction or Mg^{2+} ions, or in the presence of a saturating amount of activating fraction plus Mg^{2+} ions (data not shown).

Purification of Activator-MDH activities in low- and high-speed extracts of Bacillus sp. C1 were identical. Moreover, MDH activity was not stimulated by washed membrane fragments (high speed pellet), indicating that the activator is a soluble protein. Hydrophobic interaction chromatography was used as a first purification step as it caused a complete separation between activator protein [eluting at 1.2 M $(NH_4)_2SO_4$] and the more hydrophobic MDH protein [eluting at 0.3 M $(NH_4)_2SO_4$]. This simple separation procedure enabled quantification in further purifications steps. The activator pool was subsequently subjected to anion-exchange chromatography, which resulted in a separation between activator protein (eluting at 0.3 M KCl) and CoASH-linked NAD-dependent acetaldehyde dehydrogenase (see Discussion). High resolution anion-exchange chromatography and hydrophobic interaction chromatography, followed by a gel filtration step finally resulted in at least 85-fold purification of the activator protein, with a 15% final yield (Table I). The activator protein was judged to be (nearly) homogeneous on the basis of SDS-polyacrylamide gel electrophoresis (Fig. 3) and unambiguous N-terminal amino acid sequence analysis.

Table I

Purification of the MDH activator protein from Bacillus sp. C1.

Purification step	Total protein (mg)	Total activity (units)[a]	Specific activity (units/mg)	Recovery (%)	Fold purification
1. Crude extract	860	[b]	[b]	[b]	[b]
2. Phenyl-Sepharose pool	124	5230	42	100	1
3. Q-Sepharose pool	15	2010	134	38	3.2
4 Mono-Q pool	3.2	1090	341	21	8.1
5. Phenyl-Superose pool	1.1	890	809	17	19
6. Superose 12 pool	0.22	790	3590	15	85

Cells were grown in a methanol limited chemostat at $D=0.026h^{-1}$. [a]One unit of MDH-stimulating activity, defined as described in the Experimental Procedures, corresponds to 0.28 µg of the purified activator protein. [b]The activator could not be quantified in crude extract because of the presence of MDH activity.

Properties of Purified Activator-Denaturing gel electrophoresis revealed the presence of a single subunit with a M_r of 26,000 (Fig. 3). The M_r of the native activator protein was estimated as 50,000 by gel filtration chromatography on a calibrated Superose 12 column. An N-terminal sequence of 36 amino acid residues was determined for the purified activator protein (Fig. 4). Only one major PTH amino acid derivative was found in each cycle of degradation. These results indicate that the activator protein is composed of two identical polypeptide chains. Sequence comparison was made against the Swiss-Protein Database rel.11, but no significant similarities were found. The amino acid composition of the purified activator is shown in Table II. The predominant residues are Glx, Leu, Gly, Ala and Asx. The total of integral numbers of each amino acid residue (not including Trp) resulted in a calculated molecular weight of about 28,000 which is in close agreement with the subunit M_r of 26,000 determined by denaturing gel electrophoresis. The activator protein showed a single absorption peak at 280 nm. No significant absorption was observed at wavelengths greater than 320 nm. The activator was stable for at least 6 months when frozen at -80°C and was virtually unaffected by repeated freezing and thawing.

				5					10
Gly	Lys	Leu	Phe	Glu	Glu	Lys	Thr	Ile	Lys
Thr	Glu	Gln	Ile	15 Phe	Ser	Gly	Arg	Val	20 Val
Lys	Leu	Gln	Val	25 Asp	(Asp)	Arg	Glu	Tyr	30 Pro
Asn	Gly	Gln	Thr	35 Val	Lys				

Fig. 4. **N-Terminal amino acid sequence of purified activator protein of Bacillus sp. C1.** The sequence analysis was carried out twice with samples of 150 pmoles and 350 pmoles of protein.

Table II

Amino acid composition of purified MDH activator protein from Bacillus sp. C1.

Amino acid	Residues (mol/mol of subunit)	Amino acid	Residues (mol/mol of subunit)
Asx	19.6	Ile	13.6
Thr	13.6	Leu	23.6
Ser	14.0	Tyr	10.0
Glx	52.8	Phe	5.9
Pro	10.0	His	4.7
Gly	20.5	Lys	11.7
Ala	20.5	Arg	10.0
Cys[a]	1.0	Trp[b]	-
Val	14.7	total no. of residues	251.2
Met	5.0		

The analysis was carried out on 22 pmoles of purified activator protein. The data presented are average values of two separate runs. [a]Determined as cysteic acid. [b]Tryptophan was decomposed during acid hydrolysis.

Interaction between Purified Activator Protein and Purified MDH-Phenomena described for crude extracts (accelerating reactions, the requirement for Mg^{2+}, disproportional MDH activity) were also observed in studies with purified MDH and activator protein, suggesting that stimulation of MDH activity does not require other cellular components. Moreover, it indicates that the disproportional MDH activity observed in crude extracts (Fig. 2) is due to a dilution sensitivity of the interaction between MDH and activator protein. The activator protein concentration required for maximal stimulation of MDH activity in vitro was determined in studies with the purified proteins (Fig. 5). Under optimal assay conditions, MDH activity of 2.68 μg/ml of the enzyme was stimulated 8-fold by addition of 1.0 μg/ml activator protein. Native decameric MDH (M_r 430,000; 11) thus becomes saturated in the presence of three molecules of dimeric activator protein (M_r 50,000). One unit of stimulating activity, defined as the affinity constant (K_s, see methods section), corresponds to 0.28 μg of purified activator protein. The activator protein showed a specific requirement for Mg^{2+}; no stimulation of MDH activity was found when other divalent cations (e.g. Zn^{2+}, Mn^{2+} or Ca^{2+}) were used. Its stimulating effect was not only observed with the standard assay buffer (glycine/KOH), but also in 100 mM Tris.HCl, pH 9.0, and 100 mM sodium carbonate buffer, pH 9.0.

The pH dependence of the MDH reaction (optimal pH of 9.5) and formaldehyde reductase reaction (optimal pH of 6.7) were not altered by the presence of activator protein. Activation was also maximal at pH 9.5. Subsequent measurements on the effects of temperature were performed at the optimal pH values using standard assay buffers. The activities of MDH, formaldehyde reductase and activator protein displayed a sharp temperature optimum at 57-59°C. The thermostability of MDH saturated with activator was studied by measuring the residual activity of the purified enzyme after different preincubation periods at various temperatures. The enzyme rapidly inactivated at temperatures above 60°C. The half-inactivation times ($t_{0.5}$) at different temperatures for MDH and formaldehyde reductase activity were identical). The following $t_{0.5}$-values were found; 50°C, $t_{0.5}$>2h; 60°C, $t_{0.5}$ = 6 min; 70°C, $t_{0.5}$ = 2 min. Temperature-inactivated MDH could not be reactivated by the addition of untreated activator protein which suggested that MDH itself had lost activity.

The substrate specificity and relative activities of purified methanol dehydrogenase with various alcohols remained unchanged in the presence of the activator protein. The following relative activities were measured at saturating substrate concentrations: methanol, 33%; ethanol, 100%; n-propanol, 73%; n-butanol, 90%; 1,3-propanediol, 6%. No activity was observed with 2-propanol, 2,3-butanediol, mannitol, and glycerol.

Studies on the stoichiometry of the methanol dehydrogenase reaction showed that, both in presence or absence of activator, equimolar amounts of NADH and formaldehyde were produced from NAD and methanol. At reaction equilibrium (50°C, pH 9.5), 450 nmol formaldehyde and NADH had been formed from 500 μmol methanol and 2 μmol NAD. The equilibrium constant was calculated to be 8.3×10^{-14} M.

Various procedures were used to demonstrate a possible physical interaction between MDH and activator protein. Gel filtration studies at room temperature with purified MDH as well as freshly prepared extracts (containing MDH and activator protein) gave no evidence for the existence of a protein complex. The native molecular weight of MDH was not affected by the presence of activator protein and the two activities became completely separated (Fig. 1). Inclusion of methanol, sucrose or sorbitol in the gel filtration buffer also had no effect. It remained possible, however, that a physical association between MDH and activator protein in the gel filtration step went unnoticed because the interaction is weak and/or because binding only occurs at the elevated temperatures also required for activity. Evidence for a physical interaction was therefore sought under MDH-activating conditions. CH-Sepharose 4B immobilized MDH retained activity, as indicated by the continuous methanol dependent production of NADH at 50°C (Fig. 6). Injection of purified activator protein onto the column resulted in a sharp increase in NADH concentration, which showed that immobilized MDH could still be stimulated. Screening of eluting fractions for the presence of MDH-stimulating activity revealed that the activator coeluted with the NADH peak. Moreover, the retention time of activator protein was identical at 50°C and 10°C, although activation only occurred at 50°C. These data thus clearly demonstrate that the activator was not specifically retained by immobilized MDH. After elution of the activator protein, the NADH concentration immediately returned to the original level, indicating that no significant amounts of (active) activator protein remained bound to the column. Activation of MDH is a reversible process as the enzyme could be activated repeatedly by subsequent injections of activator protein.

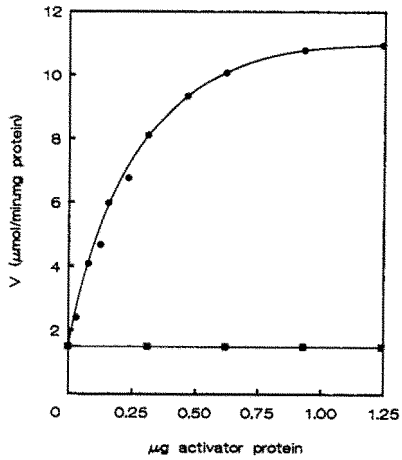

Fig. 5. **Titration of MDH activity with activator protein.** The assays were performed with purified MDH and activator protein under standard conditions in the absence (squares) or presence of Mg^{2+} ions (circles). The reaction mixtures contained 2.68 μg/ml of purified MDH.

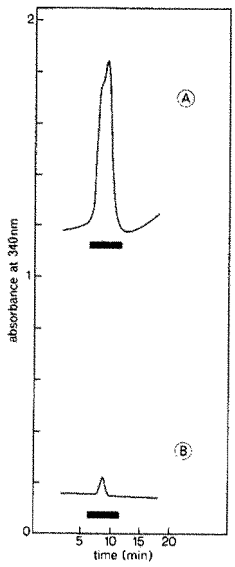

Fig. 6. **Interaction studies with immobilized MDH-**Purified MDH (10 mg of protein) was immobilized on CH-Sepharose 4B. The column was equilibrated with 100 mM glycine/KOH buffer, pH 9.0, containing 5 mM $MgSO_4$, 1 mM NAD and 500 mM methanol and was run at a flow rate of 0.6 ml/min at 50°C (A) and at 10°C (B). At zero time, 1.6 μg of purified activator protein was injected. The closed bars indicate fractions containing activator protein.

Effects of Activator Protein on the Kinetics of Alcohol Oxidation by MDH-Oxidation rates of washed cell suspensions of Bacillus sp. C1 at various methanol concentrations followed normal Michaelis-Menten kinetics. Single K_s values of 2.6 mM for methanol and 0.7 mM for ethanol were calculated from Eadie-Hofstee plots. In contrast, MDH activity in crude extracts displayed biphasic kinetics towards methanol and NAD (8). The residual MDH activity after purification, which is no longer dependent on Mg^{2+}, was sufficiently high to allow a determination of the affinity constants for methanol and ethanol (Fig. 7, Table III). Unlike crude extracts, purified MDH displayed Michaelis-Menten kinetics towards methanol and ethanol, albeit that the affinity for these substrates was extremely low (230 mM and 94 mM, respectively). Saturation of purified MDH with purified activator protein, however, resulted again in biphasic kinetics with respect to the alcohol substrates (Fig. 7) and NAD (see below). Studies with intermediate activator/MDH ratios showed that low levels of activator protein (molar ratio 1:6) already resulted in a strongly increased V_{max} and a slightly increased affinity for methanol. At higher ratios, also a second kinetic phase became apparent with an approximately 20-fold lower K_m for methanol and a 10-fold lower V_{max} when compared to the first (low affinity) phase (Fig. 7, Table III). The K_m- and V_{max} values of the two segments could be approximated when it was assumed that they result from the superposition of two independent catalytic mechanisms, both displaying Michaelis-Menten kinetics but with different kinetic constants (17). The theoretical v/s-curves derived from the approximated kinetic constants showed a good fit to the experimental data (insets Fig. 7). Hill plots of kinetic data of methanol or ethanol oxidation by MDH saturated with activator yielded Hill coefficients of approximately unity. The activator protein had no effect on the kinetic properties of formaldehyde reductase. The kinetic data of MDH are summarized in Table III. At a saturating methanol concentration (500 mM), purified MDH saturated with activator protein also displayed biphasic kinetics towards NAD with K_m values of 190 and 15 μM, whereas the K_m for NAD of purified enzyme without activator protein was 90 μM. MDH saturated with activator protein displayed Michaelis-Menten type kinetics towards Mg^{2+}, with a K_m-value of 80 μM. The K_m of the purified enzyme for NADH was below 15 μM, which could not be measured accurately because at lower NADH concentrations the reaction rates leveled off rapidly.

A comparison of the kinetic properties (K_m- and k_{cat}/K_m values) of Bacillus sp. C1 MDH and two other methanol converting alcohol dehydrogenases, ADH from Bacillus stearothermophilus strain 2334 and horse liver ADH, is shown in Table IV. The k_{cat}/K_m values for the latter two enzymes, calculated per ADH subunit, were derived from published data (19).

Stimulation of methanol dehydrogenase activity by the activator protein was not strain specific. The purified activator protein from Bacillus sp. C1 stimulated NAD-dependent MDH activity in extracts of all methylotrophic Bacillus strains tested: TS1, TS2, TS4, PB1, AR2, C1, TF, WM5.1, S2, 4(55) and KA.

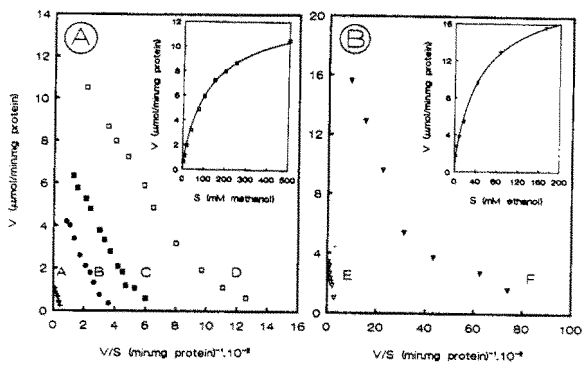

Fig. 7. **Kinetics of methanol and ethanol oxidation by purified MDH.** Eadie-Hofstee plots of methanol oxidation (panel A, curves A-D) and ethanol oxidation (panel B, curves E and F) by MDH. Panel A; the reaction mixtures (1 ml) contained: A, 26.8 μg MDH (no activator); B, 6.42 μg MDH and 0.124 μg activator (molar ratio 6:1); C, 5.35 μg MDH and 0.205 μg activator (molar ratio 3:1); D, 2.68 μg MDH and 0.93 μg activator (molar ratio 1:3). Panel B; the reaction mixtures (1 ml) contained: E, 10.7 μg MDH (no activator); F, 2.68 μg MDH and 0.93 μg activator (molar ratio 1:3). The kinetic constants for curves A, D, E and F, which were approximated from primary data using the kinetic analysis procedure described by Neal (17), are shown in Table III. The insets show the fits of the primary data of curves D and F to the theoretical v/s plots (solid lines) corresponding to the approximated kinetic constants.

Table III

Effect of activator protein on the kinetic properties of MDH.

	METHANOL		ETHANOL		FORMALDEHYDE	
	Km1,2 (mM)	Vmax1,2 (U/mg)	Km1,2 (mM)	Vmax1,2 (U/mg)	Km (mM)	Vmax (U/mg)
Whole cells (batch grown)	2.6	1.3	0.7	1.3	nd	nd
Purified MDH without activator	230	1.3	94	3.7	0.7	19.6
Purified MDH saturated with activator	140, 8	12, 1.2	57, 3	18, 2.1	2.0	19.6

The values were measured with the optimized spectrophotometric assay described in the experimental section. The kinetics of methanol oxidation by whole cells has been described elsewhere (8). The K_m and V_{max} for each substrate were determined at saturating NAD or NADH concentrations. In case of biphasic kinetics, the kinetic constants were approximated from primary data using the kinetic analysis procedure described by Neal (17). The low K_m segments of the biphasic curves were determined using five fold higher MDH and activator concentrations (12.5 µg MDH and 4.65 µg activator protein) than used for measuring the high K_m values (see Fig. 7). nd: not determined.

Table IV

Kinetic constants of methanol converting alcohol dehydrogenases.

	METHANOL		ETHANOL	
	K_m (mM)	k_{cat}/K_m $(M.s)^{-1}$	K_m (mM)	k_{cat}/K_m $(M.s)^{-1}$
Horse liver ADH	30	1.6	0.18	15×10^3
B. stearothermophilus strain 2334 ADH	20	3.0	0.08	16×10^3
Bacillus sp. C1 MDH				
- activator protein	230	4.1	94	28
+ activator protein	140, 8	61, 108	57, 3	226, 502

The kinetic constants for horse liver ADH (subunit M_r 40,000) and ADH from *Bacillus stearothermophilus* 2334 (subunit M_r 35,000) were derived from published data (19). The k_{cat}-values are calculated on the basis of subunit molecular weights. MDH from *Bacillus* sp. C1 has a subunit M_r of 43,000.