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

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 (M₀ = 50,000) is a dimer of M₁ = 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 Mg²⁺ 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 (versus substrate concentration) toward C₁-C₄ primary alcohols and NAD. The data suggest that in the presence of activator protein plus Mg²⁺ ions, MDH possesses a high affinity active site for alcohols and NAD, in addition to an activator- and Mg²⁺-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 pyrroloquinolone quinone (PQQ) 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

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 stea throatophilus 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 C₁-C₄ 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 M₀ = 50,000 protein plus Mg²⁺ 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. stea throatophilus 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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† The abbreviations used are: PQQ, pyrroloquinolone quinone; MDH, NAD-dependent methanol dehydrogenase; ADH, alcohol dehydrogenase; nMDH, NAD-dependent PQQ-containing methanol dehydrogenase; SDS, sodium dodecyl sulfate; PMS, phenazine methosulfate.

1 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 over decameric MDH with a concomitant slight decrease in $K_m$. Rather, the biphasic kinetics could be resolved in two catalytic mechanisms which possess a common Michaelis-Menten type affinity site.

The data therefore suggest that a high affinity site appears over decameric MDH with a concomitant slight decrease in $K_m$. Rather, the biphasic kinetics could be resolved in two catalytic mechanisms which possess a common Michaelis-Menten type affinity site.

Regulatory mechanisms controlling enzyme activity and involving interactions between separate proteins may either be based on product channeling (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 ($M_a = 50,000$) over decameric MDH ($M_m = 430,000$) (11) required for maximal stimulation of MDH activity in *vivo*, 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 *vivo* 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 methylo trophs require a high NH$_4^+$ concentration in *vivo* 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.

Acknowledgment—We are grateful to Leonid V. Bystrykhh (Institute of Biochemistry and Physiology of Microorganisms, U.S.S.R. Academy of Science, Pushchino, Moscow Region, U.S.S.R.) for valuable discussions.

REFERENCES


Continued on next page.
**EXPERIMENTAL PROCEDURES**

**Growth Conditions**

The organism used, *Bacillus sp. C1, P1, M2, and X* were obtained from the DSMZ Collection and grown in DSMZ medium 124 supplemented with 0.05% [2].

**Purification of Activator Protein**

3.957 g of lyophilized cells was washed with distilled water and suspended in 5 ml of 10 mM Tris buffer, pH 7.0. The activity was determined by its ability to stimulate the MDH activity, as described below. After incubation at 50°C for 10 min, the samples were centrifuged at 10,000 g at 4°C for 10 min. The supernatant was then used for the subsequent purification steps.

**Protein Concentration**

Protein concentrations were determined by the method of Bradford [3], using the Bio-Rad protein assay kit and bovine serum albumin as a standard.

**Determination of MW**

The MW of the activator protein was determined by gel filtration chromatography using a Sephadex G-200 column (Pharmacia Biotech AB) and a Pharmacia LKB Ultrofier II electrophoresis system (Pharmacia Biotech AB) and using bovine serum albumin, ovalbumin, and myoglobin as standards. The MW of the activator protein was determined to be approximately 20 kDa.

**RESULTS**

**Chromatography of Extracts of Methanol Grown *Bacillus sp. C1**

Chromatography of the methanol grown *Bacillus sp. C1* resulted in a stimulated 3-fold increase in the MDH activity, while the MDH activity was not restored when the activating protein was omitted. The activating protein was purified by ion exchange chromatography and gel filtration chromatography as described below. The MDH activity could be restored when the activating protein was added together with the purified MDH component, but not when only the purified MDH component was added. The activating protein was further purified by Sephadex G-200 chromatography and gel filtration chromatography. The activating protein was then subjected to SDS-PAGE and analyzed by Western blotting using antibodies raised against the activating protein.

**DISCUSSION**

The activating protein was further purified by ion exchange chromatography and gel filtration chromatography. The activating protein was then subjected to SDS-PAGE and analyzed by Western blotting using antibodies raised against the activating protein. The results were in agreement with the literature, indicating that the activating protein was a 20 kDa protein.

**REFERENCES**

Fig. 1. Effect of protein concentration, Mg<sup>2+</sup> ions and activating fraction on MDH activity in crude extract of *Bacillus* sp. CI. Crude extract (10 mg protein/mg) was used for the assays. MDH activity was measured in the presence of 1 mM Mg<sup>2+</sup> ions (squares), in the presence of a saturating amount of activating fraction (3.6 units) plus 5 mM Mg<sup>2+</sup> ions (circles) or without Mg<sup>2+</sup> ions (triangles). Reactions were started with 500 nM methanol.

Dissociation of Mg<sup>2+</sup>-Mg<sup>2+</sup> filtration of purified MDH gave rise to a small symmetric peak of about 0.05 30,000, adjacent to the native 180,000 peak (Fig. 1, panel B). Fractions of this minor peak did not display MDH activity of MDH-stimulating activity in the purified enzyme assay. The MDH-g-tion fraction was resolved from the same figure, which evidenced complete dissociation of MDH into individual subunits was observed during storage at -20 °C for 48 h, as indicated by gel filtration chromatography and electron microscopy (C). The monomers could not be reactivated by the activating fraction. MDH dissociation was not responsible for the disappearance of MDH activity with respect to protein concentration in crude extracts, since purified MDH displayed normal proportional activity, both in the absence of activating fraction or Mg<sup>2+</sup> ions, or in the presence of a saturating amount of activating fraction plus Mg<sup>2+</sup> ions (data not shown).

Table 1

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Protein (mg)</th>
<th>Total Activity (units)&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Specific Activity (units/mg)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude extract</td>
<td>860</td>
<td>1</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>2. Phenyl-Sepharose pool</td>
<td>124</td>
<td>5230</td>
<td>42</td>
<td>100</td>
</tr>
<tr>
<td>3. Q-Sepharose pool</td>
<td>15</td>
<td>2010</td>
<td>134</td>
<td>38</td>
</tr>
<tr>
<td>4. Mono-Q pool</td>
<td>3.7</td>
<td>1090</td>
<td>341</td>
<td>21</td>
</tr>
<tr>
<td>5. Phenyl-Sepharose pool</td>
<td>1.1</td>
<td>860</td>
<td>809</td>
<td>17</td>
</tr>
<tr>
<td>6. Superose 12 pool</td>
<td>0.22</td>
<td>790</td>
<td>3590</td>
<td>17</td>
</tr>
</tbody>
</table>

Cells were grown in a methanol-limited chemostat at OD<sub>430</sub> 0.05. One unit of MDH-stimulating activity, defined as described in the Experimental Procedures, corresponds to an increase in OD<sub>430</sub> of 0.01 per hour. The activating fraction could not be quantified in crude extract because of the presence of MDH activity.

Fig. 3. Denaturing polyacrylamide gel electrophoresis of samples obtained during purification of MDH activator protein of *Bacillus* sp. CI. The activator protein was purified from cells grown in chemostat culture at D<sub>0.026</sub> h<sup>-1</sup>. The amount of protein loaded in each lane is shown in parentheses. Lane 1, crude extract (10 µl); Lane 2, Phenyl-Sepharose pool (7 µl); Lane 3, Q-Sepharose pool (20 µl); Lane 5, Other proteins (see Methods); Lane 6, Mono-Q pool (10 µl); Lane 8, Phenyl-Sepharose pool (8 µl); Lane 9, Superose 12 pool (11 µl). The gel (15% polyacrylamide) was stained for proteins with Coomassie Blue G-250. M indicates the position of MDH.

Table 2

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Residues (mol/mol of protein)</th>
<th>Residues (mol/mol of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly</td>
<td>15.6</td>
<td>18.4</td>
</tr>
<tr>
<td>Lys</td>
<td>13.6</td>
<td>18.5</td>
</tr>
<tr>
<td>Ser</td>
<td>14.9</td>
<td>16.8</td>
</tr>
<tr>
<td>Thr</td>
<td>52.6</td>
<td>54.8</td>
</tr>
<tr>
<td>Pro</td>
<td>20.5</td>
<td>21.8</td>
</tr>
<tr>
<td>Asp</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Val</td>
<td>14.7</td>
<td>16.5</td>
</tr>
</tbody>
</table>

The analysis was carried out on 20% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The samples were prepared as described in the Experimental Procedures. The positions of the markers are indicated at the right of the gel.
Interaction between Purified Activator Protein and Purified Mdh-Phenomena described for crude extracts (Fig. 3) also occurred in preparations for Mdh. Similar results were also observed in studies with purified Mdh and activator protein, suggesting that stimulation of Mdh activity by the activator protein is not a result of any other enzyme activity. However, it is possible that the stimulation of Mdh activity by the activator protein is not a result of any other enzyme activity. However, it is possible that the stimulation of Mdh activity by the activator protein is not a result of any other enzyme activity.

Effects of Activator Protein on the Kinetics of Alcohol Oxidation by Mdh-Oxidation rates of Mdh with various activators were determined with purified Mdh and activator protein. The effects of activator protein on the kinetics of alcohol oxidation by Mdh were determined with purified Mdh and activator protein. The effects of activator protein on the kinetics of alcohol oxidation by Mdh were determined with purified Mdh and activator protein.

Fig. 8. Interaction studies with immobilized Mdh-Purified Mdh (10 μg of protein) was immobilized on Sepharose 4B. The immobilized Mdh was purified by ion exchange chromatography and was used in the determinations of the effect of activator protein on the kinetics of alcohol oxidation by Mdh. The immobilized Mdh was purified by ion exchange chromatography and was used in the determinations of the effect of activator protein on the kinetics of alcohol oxidation by Mdh.

Fig. 9. Effects of activator protein on the kinetics of alcohol oxidation by Mdh-Oxidation rates were measured with varying concentrations of activator protein. The effects of activator protein on the kinetics of alcohol oxidation by Mdh were determined with varying concentrations of activator protein. The effects of activator protein on the kinetics of alcohol oxidation by Mdh were determined with varying concentrations of activator protein.
Table III

<table>
<thead>
<tr>
<th>Effect of activator protein on the kinetic properties of MDH.</th>
<th></th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>METHANOL</td>
<td>ETHANOL</td>
<td>FORMALDEHYDE</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Km (mM)</td>
<td>Vmax (U/mg)</td>
<td>Km (mM)</td>
<td>Vmax (U/mg)</td>
</tr>
<tr>
<td>Whole cells (batch grown)</td>
<td>2.6</td>
<td>1.3</td>
<td>0.7</td>
<td>1.3</td>
</tr>
<tr>
<td>Purified MDH without activator</td>
<td>230</td>
<td>1.2</td>
<td>94</td>
<td>3.7</td>
</tr>
<tr>
<td>Purified MDH saturated with activator</td>
<td>40.8</td>
<td>1.2</td>
<td>57.3</td>
<td>2.1</td>
</tr>
<tr>
<td>Experimental section. The kinetics of methanol oxidation by whole cells has been described elsewhere (8). The K and V values were measured with the optimized spectrophotometric assay described elsewhere. The kinetic constants were determined at saturating NAD or NADH concentrations. In the graph of the kinetic curves, the kinetic constants were approximated from primary data using the kinetic model described by Neal (17). The low Km values of the biphasic curves were determined using fivefold higher MDH and activator protein concentrations than used for measuring the high Km values (see Fig. 7). nd: not determined.</td>
<td></td>
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</table>