Formaldehyde dismutase activities in Gram-positive bacteria oxidizing methanol

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Extracts of methanol-grown cells of Amycolatopsis methanolica and Mycobacterium gastri oxidized methanol and ethanol with concomitant reduction of N,N-dimethyl-4-nitrosoaniline (NDMA). Anion-exchange chromatography revealed the presence of a single enzyme able to catalyse this activity in methanol- or ethanol-grown cells of M. gastri. A. methanolica, however, possessed two different enzymes, one of which was similar to the single enzyme found in M. gastri. The methanol:NDMA oxidoreductases (MNO) were purified to homogeneity from methanol-grown cells of A. methanolica and M. gastri. Both enzyme preparations showed similar relative molecular masses with subunits of M, 50000 and 490000, and native enzymes of M, 268000 and 255000 (gel-filtration data for A. methanolica and M. gastri, respectively). Both enzymes also displayed a similar substrate specificity. They were active with methanol and various other primary alcohols (yielding the corresponding aldehydes), polyols and formaldehyde. In addition, the MNO enzymes produced methylformate from methanol plus formaldehyde, and catalyzed formaldehyde dismutase and NADH-dependent formaldehyde reductase reactions. They did not possess NAD(P)⁺ or dye-linked alcohol dehydrogenase or oxidase activities.

Introduction

Gram-negative, methanol-utilizing bacteria catalyse the conversion of methanol into formaldehyde with the well-known PQQ-containing methanol dehydrogenase (EC 1.1.99.8), which is located in the periplasmic space (Anthony, 1982; Duine et al., 1987). In contrast, the situation in the Gram-positive counterparts, which do not possess a periplasmic space, is in most cases still unresolved. In recent years, it has become clear that Gram-positive bacilli employ a novel NAD⁺-dependent methanol dehydrogenase, a decameric protein containing one zinc ion and one to two magnesium ions and a tightly but noncovalently bound NAD(H) cofactor per subunit (Arfman et al., 1991, 1992; Vonck et al., 1991; de Vries et al., 1992). In further studies, we have concentrated on the actinomycete Amycolatopsis methanolica (De Boer et al., 1990a), previously known as Streptomyces sp. 239 (Kato et al., 1975) and Nocardia sp. 239 (Hazew et al., 1983). In this organism, the situation with respect to methanol oxidation appears to be more complicated. Early on (Kato et al., 1975), a dye-linked methanol dehydrogenase activity, measured with DCPIP was detected in this organism. Since NAD⁺ was shown to activate the reaction (Duine et al., 1984), it was realized that the corresponding enzyme differed significantly from the typical dye-linked PQQ-containing methanol dehydrogenase present in Gram-negative bacteria, and was therefore designated novel methanol dehydrogenase, n-MDH (Duine et al., 1984). n-MDH activity, however, could not be detected reproducibly and little progress was made in the following years. Recently, we reported an alternative and reproducible assay for detection of methanol dehydrogenase activity in A. methanolica employing the tetrazolium dye MTT (TD-ADH; Van Ophem et al., 1991). However, n-MDH and TD-ADH may not represent the same enzyme system since they differ in substrate specificity and only n-MDH is activated by NAD⁺. The data available for n-MDH (Duine et al., 1984) and TD-ADH (Van Ophem et al.,

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Abbreviations: MNO, methanol:NDMA oxidoreductase; PQQ, pyrroloquinoline quinone; NDMA, N,N-dimethyl-4-nitrosoaniline; DCPIP, dichlorophenol-indophenol; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; PMS, phenazine methosulphate; n-MDH, novel (DCPIP-, NAD⁺-linked) methanol dehydrogenase; TD-ADH, tetrazolium dye (MTT)-dependent alcohol dehydrogenase.
correspond to a single protein, but are part of a rather loose enzyme complex (L. V. Bystrykh, unpublished data). In the case of N-MDH, the complex may constitute an unusual methanol oxidizing enzyme with a bound NAD(P)\(^+\) cofactor, which transfers its reducing equivalents directly to a dedicated NADH dehydrogenase (Duine et al., 1984). The same methanol oxidizing enzyme may also function within a TD-ADH enzyme complex, but most likely is associated in a different way or with alternative dye-reducing components.

In view of the above, we decided to develop an assay for the putative methanol oxidizing enzyme itself so that it could be purified and characterized. The method used here was initially designed for assaying alcohol dehydrogenases with artificially and covalently bound NAD(P)(Dunn & Bernhard, 1971; Kovář et al., 1984). The same method was also applied in the study of formaldehyde dismutase (EC 1.2.99.4), a natural example of an enzyme containing a bound NAD\(^+\), catalysing the dismutation of formaldehyde to methanol and formate (Kato et al., 1986). The above mentioned enzymes displayed alcohol dehydrogenase activity when coupling the oxidation of alcohols to reduction of a specific dye, \(N,N\)-dimethyl-4-nitrosoaniline (NDMA), but were not active with free NAD\(^+\). Based on this assay system, we found NDMA-linked alcohol oxidizing activities in methanol-grown cells of the Gram-positive bacteria \(A.\ methanolica\) and \(Mycobacterium\ gastri\), but not in \(Corynebacterium\ sp. XG\) or \(Bacillus\ methanolicus\) sp. C1.

**Methods**

**Cultivation.** \(Amycolatopsis\ methanolica\) NCIB 11946 was grown in chemostat culture \((D = 0.075\ h^{-1}, 37^\circ\ C,\ air\ supply\ 11\ min^{-1}\) in a fermenter with a working volume of 1200 ml, using the 2-fold concentrated mineral medium with 2% \((v/v)\) methanol described previously (de Boer et al., 1990b). \(Mycobacterium\ gastri\) MB19 was grown both in batch and chemostat cultures using 1% \((v/v)\) methanol in mineral medium (de Boer et al., 1990b) with the following modifications: 1 g yeast extract \(1\) and 1 ml stock trace elements solution (Vishniac & Santer, 1957) \(1\) were added. \(Corynebacterium\ sp. XG\) (Bastide et al., 1989) and \(B.\ methanolicus\ sp. C1\) (Arfman et al., 1992) were grown in batch flasks as described earlier.

Batch cultivation of the bacteria was done in Erlemeyer flasks (total volume 500 ml) with 200 ml medium at the following temperatures: \(M.\ gastri\), \(Corynebacterium\ sp. XG,\ 30^\circ\ C;\ A.\ methanolicus,\ 37^\circ\ C;\ B.\ methanolicus\ sp. C1,\ 50^\circ\ C.\ Cells\ were\ harvested\ at\ the\ end\ of\ the\ exponential\ growth\ phase.

**Preparation of cell-free extracts.** Cells were harvested by centrifugation for 10 min at 18000 \(g\) (\(r_p\), 698 cm), 4\(^\circ\ C,\ washed\ once\ with\ 0.1\ \text{m}-\text{potassium\ phosphate\ buffer,\ pH}\ 7.0,\ repeatedly\ centrifuged\ and\ stored\ frozen\ at\ \text{--75}\ ^\circ\ C.\\ Crude\ extracts\ were\ prepared\ by\ resuspension\ of\ freshly\ harvested\ or\ frozen\ cells\ in\ the\ buffer\ mentioned\ above\ \(2\ ml\ per\ g\ of\ cells)\), then disintegrated by passage (three to five times) through a French pressure cell, 4\(^\circ\ C,\ 1.5 \times 10^8\ \text{kN} \text{m}^{-2}\). The homogenate was centrifuged at 4\(^\circ\ C,\ 25000 \(g\) (\(r_p\), 698 cm), 30 min. Crude extract was used for enzyme assays and enzyme purification.

**Enzyme assays.** Methanol:NDMA oxidoreductase (MNO) activity was assayed in a double beam recording spectrophotometer (Hitachi 100-60) at 30\(^\circ\ C,\ using\ the\ following\ mixture: potassium\ phosphate\ buffer,\ pH\ 6.3, 0.1 m; NDMA, 0.01 m; methanol, 25 m; enzyme preparation. The assay was performed at 440 nm and the molar extinction coefficient of NDMA was taken as 35400 \(\text{m}^{-1} \text{cm}^{-1}\) (Duine & Bernhard, 1971). The reaction was started by addition of methanol. Ethanol: NDMA oxidoreductase reaction was assayed as above except that 20 m-ethanol was used instead of methanol. Formaldehyde dismutase activity was assayed in a 5 ml working volume, at 30\(^\circ\ C,\ pH\ 6.3,\ by\ following\ formic\ acid\ production\ using\ a\ \text{pH}-\text{stat\ as\ described\ by\ Kato\ et\ al.\ (1983). The reaction was started by addition of 20 m-formaldehyde. Formaldehyde reductase activity was measured spectrophotometrically by following the oxidation of NADH at 340 nm. The assay mixture (1 ml) contained: 0.1 m-potassium phosphate buffer, pH 5.0; NADH, 0.2 m; enzyme preparation. The reaction was started by addition of 0.2 m-formaldehyde. NAD\(^+\) or NAD\(^+\)/DCPIP-dependent alcohol dehydrogenase activities were assayed with methanol or ethanol as described earlier (Duine et al., 1984). One unit of the enzyme activity corresponds to the conversion of 1 \(\mu\)mol of substrate min\(^{-1}\).

**Enzyme purification.** Purification of the MNO enzymes from both \(A.\ methanolica\) and \(M.\ gastri\) was performed via a two-step FPLC procedure. Crude extract prepared from 3 g of frozen cells was applied on a Q-Sepharose column (1 x 10 cm) (Pharmacia) in 0.1 m-potassium phosphate buffer \(\text{pH}\ 7.5. A\ gradient\ of\ KCl\ \(0-1\ m)\ was\ applied\ at\ a\ flow\ rate\ of\ 0.5\ \text{ml}\ min^{-1}\ over a period of 1 h. Active fractions were collected, dialysed for 1 h against 1 m-potassium phosphate, \(\text{pH}\ 7.5,\ then\ applied\ onto\ a\ phenyl-Superose\ HR\ 5/5\ column, equilibrated\ with\ the\ same\ buffer\ as\ used\ for\ dialysis. Finally, the enzyme was eluted in a gradient \(0-0.65\ m,\ \text{pH}\ 7.5\) of potassium phosphate, over a period of 30 min at a flow rate of 0.5 ml min\(^{-1}\). Active fractions were pooled and stored frozen at \(-80^\circ\ C.

**Estimation of kinetic constants.** In most cases the single-substrate Michaelis–Menten equation (with competitive substrate inhibition if detected) was applied to calculate apparent \(V_m, K_i,\ and\ K_v\ values. Concentrations of the second substrates were kept constant as described under enzyme assays. For comparison a two-substrate ping-pong-type equation with competitive inhibition by both substrates was used to estimate true kinetic constants. Fitting of the experimental data was achieved via linear regression analysis using primary plots of reaction rates versus substrate concentrations.

**Relative molecular mass estimation.** The relative molecular masses of the purified native enzymes were estimated by gel-filtration chromatography using a calibrated Superose 12 HR 10/30 column. A calibration curve was prepared with thyroglobulin \((M, 670000)\) gamma globulin \((M, 158000), ovalbumin \((M, 44000),\ and\ myoglobin\ \((M, 17000).

**Assay of methyloformate.** MNO enzyme samples were incubated with 40 m-methanol and 40 m-formaldehyde at 20\(^\circ\ C\) for 1 h in 10 m-potassium phosphate buffer, \(\text{pH}\ 7.0,\ in\ a\ final\ volume\ of 1 ml. The assay mixture was filtered through a membrane with a cut-off of 10 kDa; 1 \(\mu l\) of the filtrate was applied onto an HP-1 column (cross-linked methyl-silicone; 30 m x 0.53 mm; layer thickness 2-65 \(\mu\)m) attached to a Hewlett-Packard HP 5890 series II gas chromatograph. Injector, column and detector temperatures were 250, 40 and 250\(^\circ\ C,\ respectively. A flame ionization detector was used for the assay.

**Electrophoresis.** Denaturing polyacrylamide (12%) gel electrophoresis (SDS-PAGE; Laemmli & Favre, 1973) was performed in combination with a PhastSystem electrophoresis unit (Pharmacia-LKB). The following prestained protein markers (Sigma, USA) were used: \(\beta\)-m-acroglobulin \((M, 180000),\ \gamma\)-galactosidase \((M, 116000),\ fructose-6-phosphate\ kinase \((M, 84000),\ pyruvate\ kinase\ \((M, 58000),\ isomerase \((M, 48500),\ lactic\ acid\ dehydrogenase\ \((M, 36500),\ tro-
Table 1. Methanol: NDMA and ethanol: NDMA oxido-reductase activities in crude extracts of A. methanolicus and M. gastri cells grown in batch flasks with different substrates

<table>
<thead>
<tr>
<th>Growth substrate (1%, w/v, final concn)</th>
<th>Specific activities (mU mg⁻¹) of extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tested with...</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
</tr>
<tr>
<td>Methanol</td>
<td>3</td>
</tr>
<tr>
<td>Ethanol</td>
<td>3</td>
</tr>
<tr>
<td>Propan-1-ol</td>
<td>1</td>
</tr>
<tr>
<td>Propan-2-ol</td>
<td>NG</td>
</tr>
<tr>
<td>Butanol</td>
<td>2</td>
</tr>
<tr>
<td>Ethyleneglycol</td>
<td>NG</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0</td>
</tr>
<tr>
<td>Glucose</td>
<td>0</td>
</tr>
</tbody>
</table>

NG, No growth.

methanolicus and M. gastri, respectively. At the same time the NDMA-dependent ethanol oxidizing activities decreased in M. gastri (Table 1). These results may be taken to suggest that A. methanolicus and M. gastri employ NAD⁺-dependent alcohol dehydrogenases for ethanol oxidation, but not for methanol oxidation. We therefore decided to study the methanol:NDMA oxido-reductases in these organisms in more detail.

Detection of alcohol: NDMA oxido-reductase activities in crude extracts of Gram-positive bacteria

Reduction of NDMA coupled to oxidation of methanol and ethanol was detected in crude extracts of Amycolatopsis methanolicus and Mycobacterium gastri cells grown on various substrates (Table 1). No NDMA-dependent alcohol-oxidizing activities were found in cells (methanol-grown) of Corynebacterium sp. XG, or B. methanolicus sp. Cl. The specific alcohol:NDMA oxido-reductase activities detected were relatively low but could be measured reproducibly mainly because of the high molar extinction coefficient of NDMA. Methanol and ethanol:NDMA oxido-reductase activities were detected only in cells of A. methanolicus, grown on primary alcohols (Table 1). A different and more complicated pattern was observed with M. gastri. NDMA-dependent alcohol oxidizing activities could be detected in cells grown on, for instance, methanol or glycerol but not with ethanol or butanol.

Unlike the situation in methylo trophic bacilli (Arfman et al., 1991), only trace activities of an NAD⁺-dependent methanol dehydrogenase were detected in methanol- or ethanol-grown cells of A. methanolicus and M. gastri (< 1 mU mg⁻¹). Transition from methanol- to ethanol-containing medium caused induction of NAD⁺-dependent ethanol dehydrogenase activities, increasing from less than 1 mU mg⁻¹ to 20 and 200 mU mg⁻¹ in A. methanolicus and M. gastri, respectively. At the same time the NDMA-dependent ethanol oxidizing activities decreased in M. gastri (Table 1). These results may be taken to suggest that A. methanolicus and M. gastri employ NAD⁺-dependent alcohol dehydrogenases for ethanol oxidation, but not for methanol oxidation. We therefore decided to study the methanol:NDMA oxido-reductases in these organisms in more detail.

FPLC analysis of NDMA-linked alcohol oxidizing activities

Fractionation of crude extracts of methanol-limited chemostat-grown cells of A. methanolicus on a Q-Sepharose column revealed the presence of two proteins active with NDMA and alcohols. One of these was active with both methanol and ethanol, whereas the other one was specific for ethanol (Van Ophem et al., 1993). In cells of M. gastri grown on methanol, only one enzyme was found, catalysing both NDMA-linked ethanol- and methanol-oxidizing reactions. However, when the strain was grown on propan-1-ol, FPLC analysis indicated the presence of an additional protein active with ethanol but not with methanol. This protein eluted at the same position as the ethanol:NDMA oxidizing enzyme from A. methanolicus. None of these fractions contained any NAD⁺-dependent alcohol dehydrogenase activity.

A further purification step on phenyl-Superose yielded homogeneous preparations of the NDMA-linked methanol oxidizing enzymes from both A. methanolicus and M. gastri (Table 2). This is the first instance of the purification to homogeneity of an enzyme of A. methanolicus with methanol-oxidizing activity, proving its existence as an individual protein. At present, NDMA is the only known artificial electron acceptor for methanol oxidation by both these enzymes: no methanol oxidizing

sephosphate isomerase (Mr 26,600). Gels were stained for protein with Coomassie brilliant blue G-250.

Protein concentration: Protein concentration was measured by using the direct spectrophotometric assay (Kalb & Bernlohr, 1977) or the Bio-Rad protein assay (Bradford method) using BSA (Bio-Rad) for a calibration curve.

Results

Detection of alcohol: NDMA oxido-reductase activities in crude extracts of Gram-positive bacteria

Reduction of NDMA coupled to oxidation of methanol and ethanol was detected in crude extracts of Amycolatopsis methanolicus and Mycobacterium gastri cells grown on various substrates (Table 1). No NDMA-dependent alcohol-oxidizing activities were found in cells (methanol-grown) of Corynebacterium sp. XG, or B. methanolicus sp. Cl. The specific alcohol:NDMA oxido-reductase activities detected were relatively low but could be measured reproducibly mainly because of the high molar extinction coefficient of NDMA. Methanol and ethanol:NDMA oxido-reductase activities were detected only in cells of A. methanolicus, grown on primary alcohols (Table 1). A different and more complicated pattern was observed with M. gastri. NDMA-dependent alcohol oxidizing activities could be detected in cells grown on, for instance, methanol or glycerol but not with ethanol or butanol.

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activity was observed with pyridine dinucleotides and with common artificial electron acceptors such as PMS, PMS/DCPIP and tetrazolium salts. The in vivo electron acceptor for these enzymes remains to be identified.

Relative molecular mass and quaternary structure of MNO

The relative molecular masses of the purified native MNO enzymes from *A. methanolica* and *M. gastri*, as studied by gel-filtration chromatography, were estimated as *M*., 268,000 and *M*., 225,000, respectively. SDS gel electrophoresis revealed the presence of a single subunit species in both enzyme preparations, with estimated subunit relative molecular masses of *M*, 50,000 and *M*, 49,000 for the enzymes from *A. methanolica* and *M. gastri*, respectively. Both the native enzymes thus might contain four or five identical subunits. However, electron microscopic studies have revealed that both enzymes have decameric structures (Bystrykh et al., 1993), similar to the NAD+-dependent methanol dehydrogenase in *B. methanolicus* sp. C1 (Vonck et al., 1991). These three enzymes also show a high degree of similarity in their primary amino acid sequences (Bystrykh et al., 1993).

Catalytic properties

Both purified MNO enzymes are able to catalyse three reactions: NDMA-coupled oxidation of methanol, dismutation of formaldehyde to methanol and formic acid, and NADH-dependent reduction of formaldehyde. Reduction of NADH could also be coupled to oxidation of NADH. The ability of MNO to catalyse NDMA-linked reactions as well as the formaldehyde dismutase activity strongly suggested the presence of a bound pyridine dinucleotide (Dunn & Bernhard, 1971; Kato et al., 1986). Further studies revealed the presence of tightly but noncovalently bound NADPH (Bystrykh et al., 1993).

When preparations of MNO from both *A. methanolica* and *M. gastri* were incubated with equal amounts of methanol and formaldehyde, a product with the same retention time as methylformate was detected by gas chromatographic analysis. The methylformate, however, partially hydrolysed under the assay conditions used, interfering with attempts to measure the stoichiometry of the reaction.

Effects of pH, temperature, and protein concentration

The pH optima for the methanol:NDMA oxidoreductase and formaldehyde dismutase reactions catalysed by both MNO preparations are comparable, namely pH 6.3. The formaldehyde reductase reaction displayed a lower pH optimum, namely pH 4.5-5.0.

MNO enzyme activity increased with temperature up to 55°C. Above this temperature, both enzymes inactivated within a few min. Both enzymes remained completely stable during incubation for 1 h at 30°C and pH 6.3.

Plots of the methanol:NDMA oxidoreductase (Fig. 1) and formaldehyde reductase activities of the purified MNO enzymes versus protein concentration are not linear. Fixed protein concentrations (about 20 μg of protein per ml of assay mixture) were therefore used for the further kinetic analysis of these enzymes.

Inhibitors

Addition of azide and hydrazine (final concentrations of 1 mM) to the assay mixture completely inhibited the *A. methanolica* MNO activity. Pyrazol (1 mM) caused 59% inhibition. EDTA, Mg²⁺, DTT and mercaptoethanol used at the same concentrations had no effect. Similar results were obtained with the formaldehyde dismutase assay, except that EDTA caused complete inhibition and pyrazol did not inhibit.

Kinetics of NDMA-linked oxidation of alcohols

The MNO enzymes from both *A. methanolica* and *M. gastri* showed a high affinity for NDMA. The alcohol:
NDMA oxidoreductase reaction obeyed Michaelis-Menten kinetics. The substrate specificity of both enzymes to alcohol substrates was very broad (Table 3). Methanol, glycerol and ethyleneglycol did not inhibit the enzymes, whereas most of the other substrates did. The kinetics of NDMA-linked oxidation of methanol was found to obey a ping-pong mechanism (Fig. 2). Comparison of the apparent kinetic parameters for primary alcohols with the true parameters revealed insignificant differences, suggesting that the apparent kinetic parameters in Table 3 are very close to the true ones.

**Formaldehyde reductase reaction**

When using the formaldehyde reductase assay, activity was found with NADH but not with NADPH as an electron donor. Both enzymes were active with all primary aldehydes tested with a carbon-chain length from C₁ to at least C₆ (Table 4). However, the progress curve of the reaction became increasingly nonlinear with increasing carbon-chain length of the aldehyde substrate. Affinity for the substrates decreased so dramatically, that saturation of the enzyme by substrate could not be attained. Finally, the enzyme gradually deviated from Michaelis-Menten function with increasing carbon-chain length of the substrate.

**Table 3. Substrate specificities of the methanol:NDMA oxidoreductases from A. methanolica and M. gaseri**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$V'_\text{max}$ (mU mg⁻¹)</th>
<th>$K'_m$ (mM)</th>
<th>$K'_i$ (mM)</th>
<th>$V'_\text{max}$ (mU mg⁻¹)</th>
<th>$K'_m$ (mM)</th>
<th>$K'_i$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDMA (+ methanol)</td>
<td>65</td>
<td>0.018</td>
<td>∞</td>
<td>162</td>
<td>0.023</td>
<td>∞</td>
</tr>
<tr>
<td>Methanol</td>
<td>57</td>
<td>2.65</td>
<td>∞</td>
<td>79.2</td>
<td>3.4</td>
<td>∞</td>
</tr>
<tr>
<td>Ethanol</td>
<td>90</td>
<td>0.057</td>
<td>16.5–6</td>
<td>82</td>
<td>0.003</td>
<td>14.6–6</td>
</tr>
<tr>
<td>Propanol</td>
<td>77</td>
<td>0.002</td>
<td>104</td>
<td>199</td>
<td>0.001</td>
<td>46.4–6</td>
</tr>
<tr>
<td>Butanol</td>
<td>76</td>
<td>0.002</td>
<td>20.1</td>
<td>154</td>
<td>0.001</td>
<td>43.8</td>
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<tr>
<td>Propan-2-ol</td>
<td>73</td>
<td>0.110</td>
<td>29.7</td>
<td>73</td>
<td>0.071</td>
<td>235.0</td>
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<tr>
<td>Isonicbutanol</td>
<td>73</td>
<td>0.001</td>
<td>36.9</td>
<td>72</td>
<td>0.001</td>
<td>181.2</td>
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<tr>
<td>2-Methoxyethanol</td>
<td>63</td>
<td>0.010</td>
<td>12</td>
<td>66</td>
<td>0.58</td>
<td>39.8</td>
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<tr>
<td>Formaldehyde</td>
<td>51</td>
<td>0.007</td>
<td>1.5</td>
<td>75.8</td>
<td>0.29</td>
<td>82.8</td>
</tr>
<tr>
<td>Ethyleneglycol</td>
<td>66</td>
<td>0.047</td>
<td>∞</td>
<td>167.6</td>
<td>0.85</td>
<td>∞</td>
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<tr>
<td>Glycerol</td>
<td>72</td>
<td>0.970</td>
<td>∞</td>
<td>146.4</td>
<td>8.04</td>
<td>∞</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td>40</td>
<td>17.0</td>
<td>220</td>
<td>28</td>
<td>40</td>
<td>∞</td>
</tr>
</tbody>
</table>

∞, No inhibition.

*The limit of the $K_m$ measurement was 0.001 mM.
value obtained for formaldehyde was rather high in this reaction, compared to the values obtained for the NDMA- or NADH-dependent activities mentioned above. However, the $V_{\text{max}}$ values for the formaldehyde dismutase reaction were hundreds of times higher than the values obtained for the formaldehyde oxidizing and formaldehyde reducing reactions measured with NDMA and NADH, respectively. Clearly, the large differences in $V_{\text{max}}$ and $K_m$ values for formaldehyde in these reactions must be a reflection of different rate limiting steps in the reaction cycles.

**Discussion**

The results presented show that several Gram-positive bacteria able to oxidize methanol (*A. methanolica*, *M. gastri*) contain a highly active formaldehyde dismutase. The enzymes studied also display alcohol:NDMA oxidoreductase activity with a very broad substrate specificity towards methanol (albeit with a poor affinity) and various other primary alcohols, and formaldehyde. Both these MNO enzymes possess a firmly bound pyridine dinucleotide (NADPH) cofactor which is catalytically active (Bystrykh et al., 1993). The kinetics of this type of enzyme is generally of the ping-pong type (Kovář et al., 1984; Kato et al., 1986; Frey, 1987), as was shown to be the case for MNO (Fig. 2).

From the various reactions catalysed by MNO, the highest activity by far was observed with the formaldehyde dismutase assay (compare Tables 3 and 4). This may reflect the fact that neither NDMA (in the methanol:NDMA oxidoreductase reaction) nor NADH (in the aldehyde reductase reaction) are physiological substrates for the MNO enzymes. The information currently available for other oxidoreductases with tightly bound pyridine nucleotide cofactors, such as glucose-fructose oxidoreductase from *Zymomonas mobilis* (Zachariou & Scopes, 1984), lactate–oxaloacetate trans-

hydrogenase from *Veillonella alcalescens* (Allen, 1966, 1982) and formaldehyde dismutase from *Pseudomonas putida* (Kato et al., 1986), shows that under physiological conditions these enzymes also do not use free pyridine dinucleotides as substrates.

The MNO enzymes and formaldehyde dismutase from *P. putida* share the ability to catalyse the NDMA-linked oxidation of alcohols, and to dismutate formaldehyde (Kato et al., 1986). A further comparison of properties, however, reveals significant differences between these enzymes as well. *P. putida* F61 is a non-methylotrophic formaldehyde-resistant strain. In this organism, formaldehyde dismutase functions as a formaldehyde detoxifying enzyme, the synthesis of which is induced by addition of formaldehyde to the medium. This enables *P. putida* to resist formaldehyde concentrations up to 60 mM (Kato et al., 1983). Unlike *P. putida*, both *A. methanolica* and *M. gastri* are methylotrophic bacteria and still sensitive to formaldehyde. *A. methanolica* is unable to tolerate formaldehyde levels above 0.8 mM (de Boer et al., 1990b). Moreover, when gradually increasing the methanol concentration in the feed of a chemostat culture of *A. methanolica*, we observed formaldehyde accumulation in the culture and a decrease in MNO activity (data not shown). It thus appears doubtful that MNO fulfills the same in vivo function as formaldehyde dismutase from *P. putida*. Further differences are that the *P. putida* formaldehyde dismutase (tetramer with subunits of $M$, 55000) does not display methanol:NDMA oxidoreductase activity, unlike the MNO enzymes (decamers with subunits of $M$, 49–50000) from *A. methanolica* and *M. gastri*. The prosthetic groups of these enzymes are also different, with NADPH in MNO (Bystrykh et al., 1993) and NADH in formaldehyde dismutase (Kato et al., 1986). No similarity is apparent between the N-terminal amino acid sequences of formaldehyde dismutase of *P. putida* (N. Kato, personal communication) and the MNO enzymes (Bystrykh et al., 1993). Instead, amino acid sequencing data and computer image analysis revealed rather strong similarities between the MNO enzymes and methanol dehydrogenase from *Bacillus methanolicus* sp. C1 (Vonck et al., 1991; Bystrykh et al., 1993). All three enzymes appear to belong to a recently established third family of alcohol dehydrogenases (Bystrykh et al., 1993; de Vries et al., 1992). The *Bacillus* methanol dehydrogenase is the enzyme responsible for methanol oxidation in this Gram-positive bacterium. It thus appears likely that the MNO enzymes have a similar function in the Gram-positive bacteria *A. methanolica* and *M. gastri*. Recently, we have been able to isolate a number of methanol-negative mutants of *A. methanolica* and the various classes are being further characterized at the moment. Nevertheless, it is already clear that mutants blocked in MNO have
completely lost the ability to oxidize methanol and no longer grow with methanol as sole carbon and energy source. Further work is certainly required to elucidate the full in vivo role of the MNO enzymes. The enzyme in *A. methanolica* may be an essential component of a multi-enzyme methanol dehydrogenase complex, resulting in the n-MDH or TD-ADH activities reported earlier (Duine et al., 1984; van Ophem et al., 1991).

References


