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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_r 50000 and 49000, and native enzymes of M_r 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 concen-

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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.

trated on the actinomycete *Amycolatopsis methanolica* (De Boer *et al.*, 1990*a*), previously known as *Streptomyces* sp. 239 (Kato *et al.*, 1975) and *Nocardia* sp. 239 (Hazeu *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.*,

1991) further indicate that these activities do not 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^+ (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 °C, air supply 1 l 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.*, 1990*b*). *Mycobacterium gastri* MB19 was grown both in batch and chemostat cultures using 1% (v/v) methanol in mineral medium (de Boer *et al.*, 1990*b*) with the following modifications: 1 g yeast extract l^{-1} and 1 ml stock trace elements solution (Vishniac & Santer, 1957) l^{-1} were added. *Corynebacterium* sp. XG (Bastide *et al.*, 1989) and *B. methanolicus* sp. C1 (Arfman *et al.*, 1991, 1992) were grown in batch flasks as described earlier.

Batch cultivation of the bacteria was done in Erlenmeyer flasks (total volume 500 ml) with 200 ml medium at the following temperatures: *M. gastri*, *Corynebacterium* sp. XG, 30 °C; *A. methanolica*, 37 °C; *B. methanolicus* sp. C1, 50 °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_{av} 6·98 cm), 4 °C, washed once with 0·1 M-potassium phosphate buffer, pH 7·0, repeatedly centrifuged and stored frozen at −75 °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 °C, 1·5 × 10^5 kN m^{-2}. The homogenate was centrifuged at 4 °C, 25000 g (r_{av} 6·98 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 °C, using the following mixture: potassium phosphate buffer, pH 6·3, 0·1 M; NDMA, 0·01 mM; methanol, 25 mM; enzyme preparation. The assay was performed at 440 nm and the molar extinction coefficient of NDMA was taken as 35400 M^{-1} cm^{-1} (Dunn & Bernhard, 1971). The reaction was started by addition of methanol. Ethanol:NDMA oxidoreductase reaction was assayed as above except that 20 mM-ethanol was used instead of methanol. Formaldehyde dismutase activity was assayed in a 5 ml working volume, at 30 °C, pH 6·3, by following formic acid production using a pH-stat as described by Kato *et al.* (1983). The reaction was started by addition of 20 mM-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 mM; enzyme preparation. The reaction was started by addition of 0·2 mM-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 µmol 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 × 10 cm) (Pharmacia) in 0·1 M-potassium phosphate buffer pH 7·5. A gradient of KCl (0–1 M) was applied at a flow rate of 0·5 ml min^{-1} over a period of 1 h. Active fractions were collected, dialysed for 1 h against 1 M-potassium phosphate, 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 (1–0·05 M, 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 °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_{max}, K_m and K_i 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_r 670000) gamma globulin (M_r 158000), ovalbumin (M_r 44000) and myoglobin (M_r 17000).

Assay of methylformate. MNO enzyme samples were incubated with 40 mM-methanol and 40 mM-formaldehyde at 20 °C for 1 h in 10 mM-potassium phosphate buffer, 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 µl of the filtrate was applied onto a HP-1 column (cross-linked methyl-silicone; 30 m × 0·53 mm; layer thickness 2·65 µm) attached to a Hewlett-Packard HP 5890 series II gas chromatograph. Injector, column and detector temperatures were 250, 40 and 250 °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: α_2-macroglobulin (M_r 180000), β-galactosidase (M_r 116000), fructose-6-phosphate kinase (M_r 84000), pyruvate kinase (M_r 58000), fumarase (M_r 48500), lactic acid dehydrogenase (M_r 36500), trio-

Table 1. *Methanol:NDMA and ethanol:NDMA oxidoreductase activities in crude extracts of A. methanolica and M. gastri cells grown in batch flasks with different substrates*

Growth substrate (1%, w/v, final concn)	Tested with...	Specific activities (mU mg⁻¹) of extracts			
		A. methanolica		*M. gastri*	
		Methanol	Ethanol	Methanol	Ethanol
Methanol		3	5	9	12
Ethanol		3	4	0	0
Propan-1-ol		1	6	16	37
Propan-2-ol		NG	NG	12	12
Butanol		2	1	0	0
Ethyleneglycol		NG	NG	0	0
Glycerol		0	0	28	23
Glucose		0	0	0	0

NG, No growth.

sephosphate isomerase (M_r 26600). 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 oxidoreductase 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 methanolica* 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. C1. The specific alcohol:NDMA oxidoreductase activities detected were relatively low but could be measured reproducibly mainly because of the high molar extinction coefficient of NDMA. Methanol and ethanol:NDMA oxidoreductase activities were detected only in cells of *A. methanolica*, 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 methylotrophic bacilli (Arfman *et al.*, 1991), only trace activities of an NAD⁺-dependent methanol dehydrogenase were detected in methanol- or ethanol-grown cells of *A. methanolica* 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.*

methanolica 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. methanolica* and *M. gastri* employ NAD⁺-dependent alcohol dehydrogenases for ethanol oxidation, but not for methanol oxidation. We therefore decided to study the methanol:NDMA oxidoreductases 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. methanolica* 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. methanolica*. 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. methanolica* and *M. gastri* (Table 2). This is the first instance of the purification to homogeneity of an enzyme of *A. methanolica* 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

Table 2. *Purification of methanol:NDMA oxidoreductases from*
A. methanolica and M. gastri

Step	Protein (mg ml^{-1})	Specific activity (mU mg^{-1})	Total activity (mU)	Yield (%)	Purifi- cation (-fold)
(a) *A. methanolica*					
1. Crude extract	15·9	7	457	100	1
2. Q-Sepharose	0·3	61	158	35	9·1
3. Phenyl-Superose	0·2	73	68	15	10·9
(b) *M. gastri*					
1. Crude extract	3·5	28	1943	100	1
2. Q-Sepharose	1·5	60	1479	76	2·1
3. Phenyl-Superose	2·8	96	1352	68	3·4

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_r 268000 and M_r 255000, respectively. SDS gel electrophoresis revealed the presence of a single subunit species in both enzyme preparations, with estimated subunit relative molecular masses of M_r 50000 and M_r 49000 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 NDMA 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, Mg^{2+}, 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

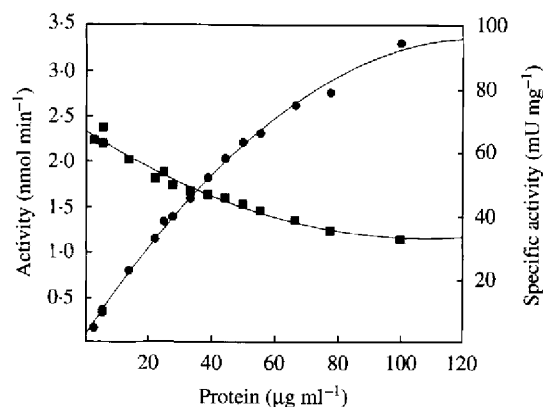The MNO enzymes from both *A. methanolica* and *M. gastri* showed a high affinity for NDMA. The alcohol:

Fig. 1. Effect of MNO protein (from *M. gastri*) concentrations on methanol:NDMA oxidoreductase activities. ■, Specific activity; ●, total activity.
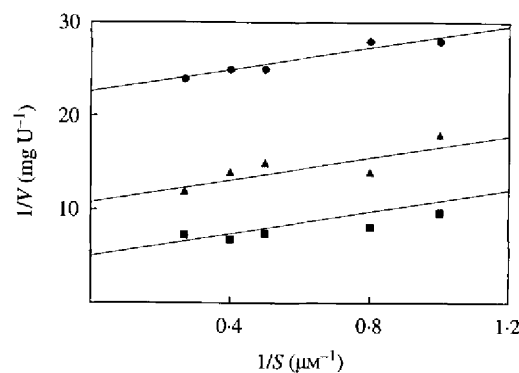

Fig. 2. Double reciprocal plots of methanol:NDMA oxidoreductase activity of MNO from *A. methanolica*. Concentrations of NDMA: 12 μM (●); 28 μM (▲); 42 μM (■).

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 C_1 to at least C_4 (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.

Formaldehyde dismutase reaction

Plots of the formaldehyde dismutase activities of MNO from *A. methanolica* and *M. gastri* versus protein concentration were linear. The formaldehyde dismutase reaction obeyed Michaelis–Menten kinetics with respect to formaldehyde. No activity was found with acetaldehyde, propionaldehyde or butyraldehyde. The K_m

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

Substrate	A. methanolica			M. gastri		
	V'_{max} (mU mg^{-1})	K'_m* (mM)	K'_i (mM)	V'_{max} (mU mg^{-1})	K'_m* (mM)	K'_i (mM)
NDMA (+methanol)	65	0·018	∞	162	0·023	∞
Methanol	57	2·65	∞	79·2	3·4	∞
Ethanol	90	0·057	165·6	82	0·003	14·6
Propanol	77	0·002	104	199	0·001	46·4
Butanol	76	0·002	20·1	154	0·001	43·8
Propan-2-ol	73	0·110	29·7	73	0·071	255·0
Isobutanol	73	0·001	36·9	72	0·001	181·2
2-Methoxyethanol	63	0·010	12	66	0·58	39·8
Formaldehyde	51	0·007	1·5	75·8	0·29	82·8
Ethyleneglycol	66	0·047	∞	167·6	0·85	∞
Glycerol	72	0·970	∞	146·4	8·04	∞
Ethanolamine	40	17·0	220	28	40	∞

∞, No inhibition.
*The limit of the K_m measurement was 0·001 mM.

Table 4. *Aldehyde reductase* (*NADH-dependent*) *and formaldehyde dismutase reactions catalysed by the methanol:NDMA oxidoreductases from A. methanolica and M. gastri*

Substrate	A. methanolica		M. gastri	
	V'_{max} (mU mg^{-1})	K'_m (mM)	V'_{max} (mU mg^{-1})	K'_m (mM)
Aldehyde reductase reaction				
NADH (with CH$_2$O)	100	0·6	19·8	0·07
Formaldehyde	14	0·01	14·5	0·26
Acetaldehyde	15	0·7	90·5	35·5
Propionaldehyde	72	246	71	68
Butyraldehyde	–	–	50	75
Formaldehyde dismutase reaction				
Formaldehyde	85000	19·5	132000	12·4

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_{max} of the formaldehyde dismutase reaction was 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_{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.*, 1990*b*). 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 fulfils 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_r 55000) does not display methanol:NDMA oxidoreductase activity, unlike the MNO enzymes (decamers with subunits of M_r 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).

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