STELLINGEN behorende bij het proefschrift van Peter W. van Ophem.


10. Bij de bepaling van de $K_{m}$-waarde van een substraat voor een enzym is het noodzakelijk deze waarde te testen met substraatconcentraties die er ver verwijderd van liggen. Dit proefschrift.

11. Amycolatopsis methanolica gebruikt waarschijnlijk een nieuwe route voor de oxidatie van formaldehyde tot CO_{2}. Dit proefschrift.
12. Het motto van ECB-6: "Biotechnology in a Europe without frontiers", is door de huidige politieke ontwikkelingen achterhaald.


14. De tijd die aan de lay-out van een proefschrift besteed wordt, staat niet in verhouding met de aandacht die de diverse hoofdstukken van de lezers krijgen.

15. Als het het parlement ernst is met de aanpak van de gokverslaving, moet zij de z.g. krasloterij niet toestaan.
Enzymes involved in methanol dissimilation in *Amycolatopsis methanolica* and some other Gram-positive bacteria

Proefschrift

ter verkrijging van de graad van doctor aan de Technische Universiteit Delft, op gezag van de Rector Magnificus, prof. drs. P.A. Schenck, in het openbaar te verdedigen ten overstaan van een commissie aangewezen door het College van Dekanen op 26 januari 1993 te 10.00 uur door

Petrus Wilhelmus van Ophem, geboren te Hoorn (NH), scheikundig doctorandus.
Dit proefschrift is goedgekeurd door de promotor: prof.dr.ir. J.A. Duine.

This research was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO).
aan mijn ouders
Dankwoord.

Het voor-/na-/dankwoord, een van de meest gelezen onderdelen van een proefschrift; immers, iedel als men is, wil de lezer graag weten: "word ik genoemd?" En terecht, want het is al vaak genoeg gememoreerd, het produceren van zo'n boekje doe je niet alleen. Velen hebben er, direct of indirect, een bijdrage aan geleverd en voor hen is dit stukje dan ook met name bestemd. Echter, om in dit verband allenamen (een ruwe schatting: 250) te gaan noemen voert mijns inziens te ver, maar een aantal kan ik niet onvermeld laten.

Mijn dank gaat uit naar mijn promotor, Hans Duine, voor zijn vele adviezen met betrekking tot de experimenten en zijn (vaak vlotte) beoordeling en bijschaging van de artikelen; de collega's uit Groningen onder leiding van Lubbert Dijkstra en Lönja (Lenja) Bystrykh voor de prettige samenwerking en discussies; prof. dr. Van Beeumen voor het sequenceren van de eiwitten; de stagiaires Edwin Bonefaas, Roland Blaauw, Gerda Salaroe, Marcel Kesselring, Karin Zuijderduin en Marianne Martis, die allen hun steentje bijgedragen hebben aan het binnenhalen van de resultaten; alle medewerkers, studenten en stagiaires die in al die jaren de afdeling enzymologie bevolkt hebben en die met hun ideeën, adviezen en hulp de resultaten van dit proefschrift medevorm hebben gegeven. Hierbij mogen de sociale aspecten die bijgedragen hebben aan de prettige werksfeer (praat, taart en koek, ergens een hapje eten, schaken, dia-avonden, tour- en andere borrels, andere bezigheden en, last, but not least, 't Keldertje) zeker niet onvermeld blijven. Tevens dank aan alle andere medewerkers (zo vergeet ik er geen een) van het Kluyverlaboratorium, zonder wiens assistentie en gezelligheid (o.a. feesten, (weer) 't Keldertje, Ben en de Johnsons) een aantal zaken zeker minder (of helemaal niet) waren verlopen. Ook dank aan alle mensen van buiten het Kluyverlab, die met hun ideeën en werkzaamheden een bijdrage aan de inhoud van dit boekje hebben geleverd.

Als laatste wil ik de familie danken voor de steun die ze altijd geweest zijn. Met name mijn ouders wil ik in dit verband noemen, omdat ze me altijd gesteund hebben en mij alle kans hebben gegeven om dit doel te bereiken, ook al moesten ze de vraag: "Zeg, die zoon van jullie, wat doet-ie nou eigenlijk?" beantwoorden met: "Ja, precies weet ik het niet, maar het is iets met enzymen en bacteriën."
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Abbreviations used:

ADH: alcohol dehydrogenase
DCPIP: 2,6-dichlorophenol-indophenol
DL-A1DH: aldehyde dehydrogenase (dye-linked)
DTT: dithiothreitol
FA1DH: formaldehyde dehydrogenase
FA1DM: formaldehyde dismutase
FD-FA1DH: factor-dependent formaldehyde dehydrogenase
FDH: formate dehydrogenase
FEDH: formate ester dehydrogenase
GD-FA1DH: GSH-dependent formaldehyde dehydrogenase
GSH: (reduced) glutathione
MDH: methanol dehydrogenase (EC 1.1.99.8)
MFF: methanol-oxidizing, formaldehyde-reducing, and formaldehyde dismutating enzyme
NDMA: p-nitroso-N,N-dimethylaniline
NDMA-ADH: NDMA-linked alcohol dehydrogenase
n-MDH: methanol dehydrogenase (novel type)
PMS: phenazine methosulfate
PQQ: pyrroloquinoline quinone
RuMP: ribulose monophosphate
TD-ADH: tetrazolium dye-dependent alcohol dehydrogenase
Chapter 1

General introduction.

1. METHYLOTROPHIC BACTERIA.

A wide variety of micro-organisms is able to grow on \( C_1 \)-compounds as their sole carbon and energy source. \( C_1 \)-compounds lack a carbon-carbon bond, but according to the definition of Colby and Zatman [1], multi-carbon substrates like pectin, containing methyl ester moieties which yield methanol upon hydrolysis, are included. Both bacteria and yeasts have representatives in this group of so-called methylotrophs, that is organisms able to grow on reduced \( C_1 \)-compounds (anaerobic bacteria, methanogens included, will not be considered here and neither are the methylotrophic yeasts). The physiology and enzymology of methylotrophic growth have been frequently reviewed during the past decade [2 - 9].

Aerobic, methylotrophic bacteria can be subdivided into two groups [2]: 1. obligate methylotrophs, bacteria only able to grow on \( C_1 \)-compounds; 2. facultative methylotrophs, organisms which do not only grow on \( C_1 \)-substrates, but also on non-\( C_1 \)-substrates. In addition, non-methylotrophs with the ability to convert \( C_1 \)-compounds as an additional carbon and/or energy source, will be considered here since several enzyme activities for dissimilation of these compounds are found in these organisms as well. Methane-utilizers are usually called methanotrophs. Not all methanotrophs are able to grow on methanol, although this compound is an intermediate in the pathway of \( CH_4 \) dissimilation.

Methylotrophic bacteria assimilate \( C_1 \)-units and convert them into compounds containing three carbon atoms. The \( C_3 \)-compounds serve as building blocks for the synthesis of cell constituents such as proteins, nucleic acids and lipids. Three different assimilation routes have been described [2, 3]: 1. the serine pathway; 2. the ribulose monophosphate (RuMP) pathway; 3. the ribulose biphosphate pathway or Calvin cycle. The latter pathway, typical for autotrophs (bacteria using \( CO_2 \) as their major carbon source), is present in methylotrophic bacteria which use \( C_1 \)-compounds to generate \( CO_2 \). The RuMP pathway has formaldehyde as a building block, while in the serine pathway formaldehyde and \( CO_2 \) are assimilated.

Energy generation from formaldehyde oxidation can occur in two ways: either by a cyclic route or by a direct linear pathway. The first
possibility consists of the RuMP pathway in which formaldehyde is oxidized to CO₂ and H₂O, thereby generating NAD(P)H. In the direct linear pathway, formaldehyde is oxidized to these products by formaldehyde and formate dehydrogenases (Figure 1), which are either coupled to the respiratory chain (dye-linked enzymes) or are present in the cytoplasm (NAD(P)-dependent enzymes). The results described in this thesis even point to a third possibility in which factor-dependent formaldehyde dehydrogenase and formate ester dehydrogenase (Chapter 9; Fig. 1) play a role. The site of attachment of the dye-linked dehydrogenases to the respiratory chain is presently unknown. Energy generation from the methanol oxidation step occurs via dehydrogenases. Electron transfer from quinoprotein methanol dehydrogenases (EC 1.1.99.8; MDH) to O₂ occurs via a chain consisting of two special cytochromes, namely cytochrome c₅₅₃ and cytochrome c₅₅₄ and a cytochrome c oxidase [10]. Whether the NAD(P)-containing methanol dehydrogenases are coupled to the respiratory chain or transfer the reduction equivalents to the NAD/NADH pool, is presently unknown (a special NADH dehydrogenase is associated with the Amycolatopsis methanolica methanol dehydrogenase [11], but the Bacillus Cl enzyme is able to reduce external NAD [12], suggesting that both possibilities may occur).

Methanotrophic bacteria convert CH₄ into methanol by means of methane mono-oxygenase of which two types exist, the soluble and the particulate type [2, 3, 13]. The concentration of copper in the growth medium determines which type is present. The soluble type requires NADH as electron donor [13], this being generated by NAD-dependent formaldehyde and formate dehydrogenases [13]. It has been suggested [14, 15] that reducing equivalents for the particulate type can be provided by the methanol oxidation step. Since methanotrophs contain the classical quinoprotein MDH, it has been proposed [14, 15] that reversed electron flow occurs from this
enzyme. However, the mechanism of this or the involvement of an alternative (NAD-dependent) methanol dehydrogenase has not been considered so far.

Transport of $C_1$-compounds into the cell (reviewed in [16]) has been investigated for methane, methanol, formaldehyde and methylamine, but only for the latter compound an active transport system has been reported. This was demonstrated in Arthrobacter P1 [17], for other organisms the occurrence of a methylamine transport system has only been suggested. Gram-negative bacteria convert methanol and methylamine into formaldehyde by dehydrogenases located in the periplasm [2, 18 - 20]. Transport of formaldehyde from the periplasm to the cytosol for assimilation probably occurs by means of an energy-driven formaldehyde transport system [21, 22] using a specific carrier [22]. The dye-linked (form)aldehyde dehydrogenase activity detected in extracts of Methylobacillus flagellatum, seems to be located in the periplasm too [23].

Fourty years ago only little was known about biological conversions of $C_1$-compounds. Since then an increasing interest developed, mainly driven by attempts to produce single-cell protein and the potentials for application of methanotrophs. This is well illustrated by the drastic increase in research papers and reviews on this subject [2 - 9]. Methylo trophic organisms can be used for the production of enzymes [2, 24], of single cell protein [2, 3, 25, 26], of useful metabolites such as glycerol, aldehydes [3, 27, 28], and amino acids [2, 25, 28 - 30], but also for the removal of pollutants from waste water [31].

The number of known Gram-negative methylo trophs far exceeds that of their Gram-positive counterparts. At the start of this project knowledge on the enzymology of $C_1$-conversions concerned the Gram-negative ones. From the limited information available at that time for the Gram-positive ones [11, 32 - 34], it could be concluded that at least the methanol oxidizing enzyme was different. As shown in this thesis for A. methanolicus, Mycobacterium gastri, Rhodococcus species, and by others [35 - 38] for Bacillus species, this is indeed the case. Furthermore, as also shown in this thesis, special enzymes exist in A. methanolicus and R. erythropolis for conversion of formaldehyde into $CO_2$ and $H_2O$.

2. METHANOL DEHYDROGENASES.

The nucleotinamide-coenzyme-dependent alcohol dehydrogenases are widespread in Nature, occurring from bacteria to man [39, 40]. Especially the NAD-dependent enzymes from horse liver and baker's yeast (EC 1.1.1.1) have been well studied with respect to mechanism and structure [39]. Although these enzymes have a very wide specificity for primary alcohols,
Table 1: Alcohol dehydrogenases in prokaryotes.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>EC number</th>
<th>Coenzyme</th>
<th>Cofactor</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADH</td>
<td></td>
<td>NAD(P)</td>
<td></td>
<td>Prokaryotes</td>
<td>39</td>
</tr>
<tr>
<td>(enzymes structurally related and those non-related to EC 1.1.1.1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADH</td>
<td></td>
<td>NAD</td>
<td></td>
<td>Zymomonas mobilis</td>
<td>42</td>
</tr>
<tr>
<td>(Fe-containing)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzylalco.-DH</td>
<td></td>
<td>NAD</td>
<td></td>
<td>Acinetobacter</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>calcoaceticus</td>
<td></td>
</tr>
<tr>
<td>EDH</td>
<td></td>
<td>PQQ</td>
<td></td>
<td>Pseudomonas sp.</td>
<td>44, 45</td>
</tr>
<tr>
<td>QH-EDH (type I)</td>
<td></td>
<td>Haem c/PQQ</td>
<td></td>
<td>Comamonas testosteroni</td>
<td>46</td>
</tr>
<tr>
<td>QH-EDH (type II)</td>
<td></td>
<td>Haem c/PQQ</td>
<td></td>
<td>Acetobacter sp.</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Gluconobacter sp.</td>
<td>48</td>
</tr>
<tr>
<td>PVA-DH</td>
<td>1.1.95.23</td>
<td>Haem c/PQQ</td>
<td></td>
<td>Pseudomonas sp.</td>
<td>49</td>
</tr>
<tr>
<td>PEG-DH</td>
<td>1.1.95.20</td>
<td>Quinone</td>
<td></td>
<td>Flavobacterium</td>
<td>50</td>
</tr>
<tr>
<td>NDMA-ADH</td>
<td></td>
<td>NAD(H)</td>
<td></td>
<td>A. methanolic</td>
<td>Chapter 5</td>
</tr>
</tbody>
</table>

1ADH: alcohol dehydrogenase; EDH: quinoprotein ethanol dehydrogenase; QH-EDH: quinohaemoprotein ethanol dehydrogenase; PVA-DH: polyvinylalcohol dehydrogenase; PEG-DH: polyethylene glycol dehydrogenase; NDMA-ADH: p-nitroso-N,N-dimethylaniline-linked nicotinoprotein alcohol dehydrogenase.

The smallest representative of this series, methanol, is a very bad one or not a substrate at all. The explanation given is that the polarity of methanol is too high to afford adequate binding in the hydrophobic active site of these enzymes [41]. Also other quite different types of alcohol dehydrogenases (Table 1) show the same behaviour. As will be discussed, during the past decades it has become clear that methanol oxidation in bacteria requires unique dehydrogenases (Table 2).

Already in the early days of methylothrophy, it was found that methylothrophic yeasts use flavoprotein methanol or alcohol oxidase (EC 1.1.3.13) [51] and Gram-negative bacteria a dye-linked alcohol dehydrogenase, later identified as a quinoprotein (MDH: EC 1.1.99.8), for converting methanol into formaldehyde (Table 2). As is already clear from the names, the enzymes have also a very wide substrate specificity, but in contrast to the ones classified as EC 1.1.1.1, they have excellent kinetic
Table 2: Bacterial dehydrogenases able to or involved in methanol oxidation.

<table>
<thead>
<tr>
<th>Enzyme (activity)</th>
<th>Source</th>
<th>EC number</th>
<th>Cofactor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDH</td>
<td>Facultative and obligate methylotrophs</td>
<td>1.1.99.8</td>
<td>PQQ</td>
<td>2 - 9</td>
</tr>
<tr>
<td>GRAM-NEGATIVE BACTERIA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAD-MDH</td>
<td>Thermophilic <em>Bacillus</em> strains</td>
<td>-</td>
<td>NAD</td>
<td>12, 86 - 88</td>
</tr>
<tr>
<td>n-MDH</td>
<td><em>Brevibacterium methylicum</em></td>
<td>-</td>
<td>?</td>
<td>38</td>
</tr>
<tr>
<td>Amycolatopsis methanolica</td>
<td>-</td>
<td>PQQ/NAD</td>
<td>11, 82</td>
<td></td>
</tr>
<tr>
<td>Thermaactinomyxete strain 381</td>
<td>-</td>
<td>PQQ?/NAD?</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>TD-ADH</td>
<td>Amycolatopsis methanolica</td>
<td>-</td>
<td>NAD?</td>
<td>84</td>
</tr>
<tr>
<td>Rhodococcus species</td>
<td>-</td>
<td>NAD?</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>d1-MDH</td>
<td><em>Clostridium thermoautotrophicum</em></td>
<td>-</td>
<td>PQQ</td>
<td>90</td>
</tr>
<tr>
<td>Corynebacterium XG</td>
<td>-</td>
<td>?</td>
<td>91</td>
<td></td>
</tr>
<tr>
<td>MFF</td>
<td>Amycolatopsis methanolica</td>
<td>-</td>
<td>NADP</td>
<td>Chapter 4</td>
</tr>
<tr>
<td>Mycobacterium gastri MB19</td>
<td>-</td>
<td>NADP</td>
<td>Chapter 4</td>
<td></td>
</tr>
</tbody>
</table>

1 Abbreviations used: MDH, quinoprotein methanol dehydrogenase (EC 1.1.99.8); NAD-MDH, methanol dehydrogenase with NAD as a cofactor; n-MDH, novel methanol dehydrogenase, requiring NAD plus DCPIP in the assay; TD-ADH, tetrazolium dye-dependent alcohol dehydrogenase; d1-MDH: dye-linked methanol dehydrogenase; MFF: methanol-oxidizing, formaldehyde reducing, and formaldehyde-dismutating enzyme.

Parameters for methanol oxidation. The characteristics of MDH are discussed in section 2.1, those of the methanol-oxidizing enzymes of Gram-positives in section 2.2.

2.1 Methanol dehydrogenase of Gram-negative bacteria.

MDH is present in Gram-negative bacteria capable of utilizing methane or methanol. The well-characterized [2 - 6, 9] enzyme, which constitutes about 10 - 15 % of the total soluble protein of the cell, is located in the periplasmic space [18 - 20]. Until recently, it was considered to be a
dimeric enzyme consisting of two identical subunits of 60 - 70 kDa (in some organisms a monomeric form is found [2, 52]). Evidence has now been presented that MDH from Methylobacterium extorquens AM1 has an $\alpha_2\beta_2$ configuration [53] and it is very likely that the same applies to all other MDH's. Another recent finding is that MDH is a metalloprotein, containing one atom of Ca per enzyme molecule [54].

The cofactor of MDH is PQQ (pyrroloquinoline quinone, the semi-systematic name for 2,7,9-tricarboxy-1H-pyrrolo-(2,3f)-quinoline-4,5-dione (Figure 2)) [55, 56]. MDH contains 2 moles of PQQ per mole of enzyme. It is not clear to which subunit the cofactor is bound. Growth on methanol (and sometimes also on other substrates [57 - 59]) induces the production of the cofactor, which has been frequently detected in the extracellular medium in micromolar concentrations [60, 61]. PQQ is the cofactor of several alcohol and glucose dehydrogenases [5, 9, 62, 63]. The term quinoproteins comprises PQQ-containing enzymes and enzymes containing the integrated quinone cofactors 4-(2'-tryptophyl)-tryptophan-6,7-dione (tryptophyl tryptophane quinone) and 6-hydroxy-phenylalanine-3,4-dione (topa quinone) [63].

The mechanism of action of MDH and the involvement of PQQ in this has been reviewed [9, 64]. Methanol binds to the oxidized form of MDH at the C$_5$-position of PQQ and this complex decomposes into reduced MDH and formaldehyde. In the *in vitro* assay, this step is accelerated enormously by an activator, most probably NH$_3$, formed from the added NH$_4^+$-salts at the high pH required for optimal activity. MDH is then re-oxidized in two steps via its semiquinone form by artificial one-electron acceptors such as the cationic dyes Wursters' Blue or phenazine methosulfate (PMS). Anionic dyes like ferricyanide or DCPIP, although having a similar or even higher redox potential, are inactive as well as the coenzymes NAD(P) [2, 65]. For the "in
"vivo assay" with cytochrome cL as electron acceptor, the pH optimum is 7, much lower than that of the in vitro assay. This is caused by the fact that the reaction between reduced MDH and ferricytochrome cL is optimal at this pH [65, 66]. The methanol oxidation step in the pH 7.0 assay can be stimulated by an as yet unidentified, low molecular weight activator. This so-called factor X, which is not ammonia or an ammonium salt, becomes inactivated in the presence of oxygen and reduced cytochrome c [67]. Different opinions exist as to whether the autoreduction mechanism of cytochrome cL is relevant for the mechanism of reoxidation of reduced MDH by oxidized cytochrome cL. Thus, Beadmore-Gray et al. found at pH 9 high autoreduction rates of cytochrome cL from Methylphilus methylotrophus (approx. 100 times higher than at pH 7), while comparable rates were observed at pH 7 in the presence of MDH during methanol conversion. They concluded that both reaction mechanisms are similar [68]. Dijkstra et al. found insignificant autoreduction rates of cytochrome cL of Hyphomicrobium X while much higher rates of reoxidation of reduced MDH with that cytochrome were observed than that reported by Anthony et al. [68]. Therefore, the Delft group concluded that different mechanisms operate in the autoreduction of cytochrome cL and in its reduction by reduced MDH [69]. It seems that there exists agreement now on the latter point since in a recent review, Anthony found it more probable that the autoreduction might be due to endogenous reductant [70].

MDH has a wide substrate specificity: not only methanol is oxidized, but many primary alcohols as well [71, 72]. Methanol is the best substrate since it has the highest affinity (a $K_m$ of 10 - 20 μM). Although most MDH's are only able to oxidize primary alcohols, exceptions exist which use also secondary alcohols as substrates [2]. However, a strict differentiation seems artificial since the secondary alcohol, cyclopropanol, is an effective inhibitor for enzymes specific for primary alcohols [73 - 75]. Irreversible inactivation occurs by adduct formation with PQQ at the C5-position [64].

MDH is also able to oxidize formaldehyde in the in vitro assay but it is questionable whether formaldehyde oxidation in vivo occurs via MDH. Thus it has been found that MDH-mutants [76] or cells in which MDH has been inactivated with cyclopropanol [73], oxidize formaldehyde at an unchanged rate. On the other hand, it has been proposed that cellular regulation of formaldehyde oxidation by MDH might occur by way of a modifier protein [66, 77, 78], which increases the affinity for methanol and decreases it for
formaldehyde. Surprisingly, the modifier protein also facilitates oxidation of a non-C_1 substrate, 1,2-propanediol [77].

Since so far no other methanol-oxidizing enzyme has been detected in Gram-negative methanol- or methane utilizers, it is clear that the physiological role of MDH concerns the conversion of this compound. In view of the broad substrate specificity, one would expect that MDH is also used for growth on other primary alcohols in those facultative methylotrophs able to grow on these compounds. However, this seems not to be the case, at least in certain organisms. For instance, NAD-dependent alcohol dehydrogenase, is used for growth on ethanol in the methylotroph Diplodoccus PAR [79] and a dye-linked alcohol dehydrogenase is used for that purpose in the MDH-producing Acetobacter methanolicus [80]. The dye-linked alcohol dehydrogenase might be a quinoprotein alcohol dehydrogenase as has been found in Pseudomonas aeruginosa [44, 45]. Although the quaternary structure and the overall properties of this enzyme are the same as for MDH [44, 45, J.M.J. Schrover, unpublished results], it is unable to oxidize methanol. Hence, PQQ is not the sole factor responsible for the ability of MDH to oxidize methanol. Most probably a special configuration of the active site provides MDH with the ability to bind the polar methanol besides the other more apolar primary alcohols.

2.2 Methanol oxidoreductases of Gram-positive bacteria.

At present, no Gram-positive methanotrophs are known and only a limited number of methanol utilizers have been found in this group of bacteria [32, 81]. Although first attempts to identify the methanol oxidizing enzyme failed [32, 33], in subsequent studies with A. methanolicus progress was made with detection of methanol dehydrogenase activity by applying the unusual combination of NAD and DCPIP as electron acceptor in the assay, leading to methanol-dependent DCPIP reduction [11]. The partly purified dehydrogenase, indicated as novel methanol dehydrogenase (n-MDH), appeared to be specific for methanol since ethanol could not act as substrate and cyclopropanol did not inactivate. Results from purification revealed that n-MDH activity eluted together with an NAD-dependent (form)aldehyde dehydrogenase activity and an NADH dehydrogenase (or diaphorase) activity. NADH dehydrogenase activity could be removed and the remaining fraction showed no activity in the assay, although an increase in absorbance at 340 nm to a limited extent was observed upon methanol addition. Upon reconstitution of this fraction with the NADH dehydrogenase fraction (which took place in a time-dependent process) activity reappeared, but only when the dehydrogenase from A. methanolicus was used, not with NADH dehydrogenase from Bacillus subtilis.
This clearly showed that the rise in absorbance at 340 nm is not due to (free) NADH production. Therefore, Duine et al. proposed n-MDH to consist of a multi-enzyme complex in which the reducing equivalents are directly transferred from the methanol dehydrogenase to a NADH dehydrogenase (Figure 3) [11, 82]. It can be imagined that, subsequently NADH dehydrogenase becomes reoxidized by DCPIP \textit{(in vitro)} or by the respiratory chain \textit{(in vivo)}. Since a linear relationship existed between n-MDH activity and PQQ content of the fractions obtained by applying chromatographic steps, it was speculated that n-MDH is a quinoprotein. However, although it was assumed that both NAD and PQQ are involved in the electron transfer, real evidence for this was not provided.

In contrast to the earlier studies (thereby using the assay as described previously [11]), n-MDH activity could only be demonstrated now and then in anaerobically prepared cell free extract preparations (Chapter 2). In addition, when activity was detected it appeared to be unstable. Two quinoproteins could be purified from these extracts, but no activity was found for them. Recently, n-MDH activity was demonstrated in crude extracts of the thermoactinomyces strain 381 [83], but no further data have been presented so far. Therefore, it seems that conditions for the assay and/or for preparation of the extract are very critical in n-MDH detection.

A tetrazolium dye-dependent alcohol dehydrogenase (TD-ADH) activity able to convert methanol and requiring high phosphate or sulfate salt concentrations could be reproducibly detected in extracts of \textit{A. methanolicus} [84 and Chapter 3]. Attempts to purify TD-ADH revealed that it consists of at least two components (unpublished results), indicating a complexity as has been found for n-MDH. However, differences exist when comparing the two complexes [Chapter 3, 84]: TD-ADH activity is reproducibly detected, n-MDH
activity is not; n-MDH activity is observed with DCPIP, TD-ADH only with specific tetrazolium dyes; n-MDH activity is stimulated by addition of NH₄⁺ salts, TD-ADH activity by high phosphate or sulfate salt concentrations; n-MDH is specific for methanol, TD-ADH prefers long-chain aliphatic primary alcohols; n-MDH has been detected in methanol-grown cells, TD-ADH is also present in cells grown on other alcohols; PQQ is present in n-MDH-containing fractions, not in those containing TD-ADH. However, despite these differences, it can not be excluded that n-MDH and TD-ADH are somehow related (Chapter 9).

Since difficulties were met in attempts to reconstitute TD-ADH from fractions obtained by chromatography, a search was made for the methanol-oxidizing component in it. When using p-nitroso-N,N-dimethylaniline (NDMA), alcohol dehydrogenase activity could be demonstrated in cell free extracts (Chapter 4 and 5). Purification revealed that two different enzymes are responsible for this (see also section 3.3): methanol-oxidizing, formaldehyde-reducing, and formaldehyde-dismutating enzyme (MFF), able to oxidize methanol and other alcohols; NDMA-dependent alcohol dehydrogenase (NDMA-ADH) able to oxidize primary alcohols, except methanol.

MFF has been purified and characterized (Chapter 4): it is a decameric protein consisting of identical subunits of 50 kDa; it contains tightly-bound NADP as cofactor; it shows structural relatedness with NAD-dependent methanol dehydrogenase from *Bacillus* C1 [35]. Since MFF is able to oxidize methanol in the assay, it could be responsible for methanol oxidation in vivo. However, since the enzyme is also able to perform the formaldehyde dismutase reaction and is able to produce methylformate on incubation with methanol and formaldehyde, other roles could be envisaged. The likeliness of that and the exclusivity of MFF in methanol oxidation are discussed in Chapter 9. MFF is also present in methanol-grown *Mycobacterium gasti* (an organism which contains the RuMP pathway for formaldehyde fixation [85], just as A. methanolicum). Since TD-ADH activity has not been found for this organism and PQQ (and, therefore, probably also n-MDH activity) is absent during growth on methanol [84, Chapter 3], despite the fact that both organisms have MFF, the in vivo methanol-oxidizing systems could be different.

Purification and characterization of NDMA-ADH has been achieved (Chapter 5): it is a trimeric enzyme with a Mr of 110 kDa, containing tightly-bound NAD. Comparison of the N-terminal amino acid sequence (21 amino acids) with that of MFF showed no similarity, but substantial homology (40% identical amino acids) exist between NDMA-ADH and NAD-dependent alcohol dehydrogenase (EC 1.1.1.1) from horse liver. From this it is clear that NDMA-ADH is
structurally different from MFP. No physiological role of the enzyme can be indicated yet (Chapter 5).

Several thermotolerant *Bacillus* strains able to grow on methanol have been isolated [81, 86 - 88]. The enzyme responsible for methanol oxidation in *Bacillus methanolicus C1* [88a] has been purified and characterized [12, 35, 81]. It is an NAD-dependent dehydrogenase (NAD-MDH) catalyzing the oxidation of various alcohols, including methanol. The purified enzyme is hardly active, but the addition of Mg$^{2+}$ and an activator protein restore the activity. NAD-MDH is a decameric protein composed of identical subunits of 43 kDa. It contains 10 Zn and 20 Mg atoms and 10 tightly-bound NAD(H) molecules per enzyme molecule [35], but no PQ [12]. Although it is able to convert short chain primary alcohols, its kinetic and structural properties are quite different from the long-chain, zinc-containing NAD-dependent alcohol dehydrogenase (ADH) (EC 1.1.1.1). Sequence analysis of a number of peptides revealed similarity with a special group of alcohol dehydrogenases: ADH2 from *Zymomonas mobilis*, ADH4 from *Saccharomyces cerevisiae*, propanediol dehydrogenase from *Escherichia coli*, and butanol dehydrogenase from *Clostridium acetobutylicum* [12, 35]. The activator protein is a dimeric protein with subunits of 27 kDa [36], and neither alcohol dehydrogenase, acetaldehyde dehydrogenase, NADH dehydrogenase nor hexulose-6-phosphate synthase activity is connected with this protein [81]. NAD-MDH possesses in the presence of activator protein and Mg$^{2+}$ a high affinity active site for alcohols and NAD as well as a low affinity site for these components, while in the absence of Mg$^{2+}$ and activator protein only the low affinity site is present [36]. NAD-MDH and the activator protein are also responsible for ethanol oxidation in ethanol-grown cells, since these contain high levels of NAD-MDH and the activator protein (just as in methanol-grown cells) and no additional NAD-dependent or dye-linked alcohol converting activity could be found [89]. From *B. stearothermophilus* an NAD-dependent ADH, able to convert methanol, was purified [37], but since this enzyme has a low affinity for methanol ($K_m$ of 20 mM) and was isolated from cells grown on glucose, its role in methanol oxidation is questionable. *Brevibacterium methyllicum* possesses NAD-dependent methanol dehydrogenase activity [38], which was claimed to be similar to that found in thermophilic *Bacilli*, but it has not been studied in detail.

Two dye-linked methanol dehydrogenase activities have been detected in other Gram-positive bacteria. In a brief communication, it was reported that the acetogenic *Clostridium thermoautotrophicum* contains a PQQ-dependent methanol dehydrogenase [90]. The oxygen-sensitive enzyme was able to convert
other primary alcohols and aldehydes as well. Activity could be demonstrated using DCPIP or Wurster's Blue as electron acceptor. In contrast to MDH from Gram-negative bacteria, ammonia was not an activator, on the contrary, it appeared to be an inhibitor. No evidence has been provided so far for PQQ functioning as cofactor in this enzyme. Cell free extracts of the facultative methylotroph, Corynebacterium sp. XG, show methanol dehydrogenase activity when assayed at pH 10.0 in the presence of NH$_4^+$-salts with the PMS/DCPIP couple [91], but no MFF activity has been found in this organism (Chapter 4). Also in this case, further characterization of the enzyme is required in order to be able to compare it with other methanol oxidoreductases.

In view of the dissimilarities mentioned above, it seems that no universal enzyme exists for conversion of methanol in Gram-positive methanol-utilizers. The fact that n-MDH, TD-ADH and MFF are different, could indicate that these activities derive from different enzymes, all present in A. methanolicus, and the physiological role in methanol oxidation for some of them seems questionable. Thus, the fact that MFF activity has been found in extracts of Rhodococcus erythropolis, an organism unable to grow on methanol but able to convert formaldehyde, could be interpreted in favor of unrelatedness of MFF to methanol conversion. On the other hand, as long as the complexes have not been purified, it cannot be excluded that MFF forms a part of the n-MDH and/or TD-ADH complexes, so that it could be the sole methanol-converted enzyme in this Gram-positive methanol utilizer (Chapter 9). Finally, since MFF as well as NDMA-ADH have a bound nicotinamide nucleotide as cofactor, this property can not be the sole prerequisite for methanol conversion. Just as in the case of quinoproteins, it seems that the configuration of the active site in the "nicotinoproteins" is the decisive factor in this.

3. FORMALDEHYDE OXIDOREDUCTASES

Oxidation of formaldehyde in vitro is catalyzed by specific (e.g. NAD-linked, glutathione (GSH)-dependent formaldehyde dehydrogenase, EC 1.2.1.1) and non-specific (e.g. NAD-dependent aldehyde dehydrogenases, EC 1.2.1.3 and dye-linked aldehyde dehydrogenases, EC 1.2.99.3) enzymes (Tables 3 and 4). With respect to the latter category, it should be noted that aldehyde dehydrogenases exist which are unable to oxidize formaldehyde [40]. On the other hand, most alcohol dehydrogenases and alcohol oxidase are able to catalyze formaldehyde oxidation, although the physiological significance of this is doubtful. For the latter reason, attention will be paid only to
Table 3: Bacterial dye-linked dehydrogenases able to oxidize formaldehyde.

<table>
<thead>
<tr>
<th>Organism</th>
<th>EC number</th>
<th>Cofactor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyphomicrobium X</td>
<td>-</td>
<td>?</td>
<td>94</td>
</tr>
<tr>
<td>Hyphomicrobium ZV</td>
<td>580(^1)</td>
<td>quinone</td>
<td>95</td>
</tr>
<tr>
<td>Hyphomicrobium ZV</td>
<td>580(^2)</td>
<td>Fe/S</td>
<td>95</td>
</tr>
<tr>
<td>Ms. trichosporium</td>
<td>1.2.99.3</td>
<td>haem c</td>
<td>96</td>
</tr>
<tr>
<td>C. testosteroni</td>
<td>-</td>
<td>Mo/pterin/FAD/Fe/S</td>
<td>101, chapter 8</td>
</tr>
<tr>
<td>A. methanolica</td>
<td>-</td>
<td>Mo/pterin(?)/FAD/Fe/S</td>
<td>chapter 8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
\(^1\) induced during growth on methylamine; \(^2\) induced during growth on methanol.

Enzymes classified as (form)aldehyde oxidoreductases, that is (form)aldehyde dehydrogenases (NAD(P)-dependent or dye-linked) and formaldehyde dismutase (EC 1.2.99.4).

Specific as well as non-specific activities sometimes occur in the same organism [92, 93]. Non-specific activities are also present in bacteria grown on non-C\(_1\)-compounds. Therefore, it seems unlikely that the task of the non-specific aldehyde dehydrogenases is solely the oxidation of formaldehyde.

3.1 Dye-linked aldehyde dehydrogenases able to oxidize formaldehyde.

The presence of dye-linked aldehyde dehydrogenases (DL-ALDH) is usually detected with compounds like PMS, Wurster's Blue, or DCPIP [2]. DL-ALDH from Hyphomicrobium X is able to donate its electrons to (horse heart) cytochrome c (in vitro) [94], a combination which might reflect the in vivo situation. Dye-linked formaldehyde-oxidizing activities (compiled in Table 3) have been found both in Gram-negative as well as in Gram-positive methylo trophs. Generally, these enzymes prefer long chain aldehydes so that they can in fact be named aldehyde dehydrogenases.

Dye-linked aldehyde dehydrogenases able to oxidize formaldehyde have been partly characterized from several Gram-negative methylo trophs (Table 3). H-
Table 4: Bacterial dehydrogenases (NAD-dependent) able to oxidize formaldehyde.

<table>
<thead>
<tr>
<th>Organism</th>
<th>EC number</th>
<th>Additional factor</th>
<th>Other substrates used</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>General aldehyde dehydrogenases</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyphomicrobium X</td>
<td>-</td>
<td>no</td>
<td>aldehydes</td>
<td>92</td>
</tr>
<tr>
<td>A. methanolina</td>
<td>-</td>
<td>no</td>
<td>aldehydes</td>
<td>93, Chapter 6</td>
</tr>
<tr>
<td><strong>Formaldehyde dehydrogenases oxidizing some other aldehydes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ps. putida</em> C-83</td>
<td>1.2.1.46</td>
<td>no</td>
<td>acetaldehyde</td>
<td>106 - 108</td>
</tr>
<tr>
<td><strong>Formaldehyde dehydrogenases requiring an additional factor</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Several Gram-negative methanol-utilizers</td>
<td>1.2.1.1</td>
<td>GSH(^1)</td>
<td>higher aliphatic alcohols</td>
<td>2, 106</td>
</tr>
<tr>
<td><em>Ps. putida</em> F61</td>
<td>1.2.1.1</td>
<td>GSH(^1)</td>
<td>?</td>
<td>111</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>1.2.1.1</td>
<td>GSH(^1)</td>
<td>higher aliphatic alcohols</td>
<td>112</td>
</tr>
<tr>
<td><em>Ms. capsulatus</em></td>
<td>-</td>
<td>HSTS(^2)</td>
<td>?</td>
<td>103</td>
</tr>
<tr>
<td><em>R. erythropolis</em></td>
<td>-</td>
<td>F(^3)</td>
<td>higher aliphatic alcohols</td>
<td>104, 105</td>
</tr>
<tr>
<td><em>A. methanolina</em></td>
<td>F(^3)</td>
<td></td>
<td>higher aliphatic alcohols</td>
<td>Chapter 7</td>
</tr>
</tbody>
</table>

\(^1\) Measured with cell free extracts (the dependency on GSH or NAD(P) has not always been verified); \(^2\) heat stable, trypsin sensitive factor, treatment with a thiol compound is not required; \(^3\) heat stable factor requiring DTT-treatment upon aging.

**Hyphomicrobium X** possesses such an enzyme (\(M_r\) of 83.5 kDa) when grown on methanol or ethanol [94]. However, growth of strain ZV 580 on methylamine induces a quite different dehydrogenase with a high affinity for formaldehyde [95]. The tetrameric enzyme (\(M_r\) of 210 kDa) has an absorption spectrum which resembles that of quinoproteins. Also other properties suggest that the cofactor might be a covalently bound quinone cofactor (A.C. Schwartz, private communication). Curiously, when this strain is grown on
methanol, a different DL-ALDH (Mr of 76 kDa) is induced which preferably oxidizes long-chain aliphatic aldehydes, and contains an iron-sulfur centre [95]. DL-ALDH (EC 1.2.99.3) from Methylosinus trichosporium is a dimeric enzyme (Mr of 43 kDa) and it contains haem as judged from the spectral properties of the purified enzyme [96]. The authors suggested that this enzyme is also present in other Gram-negative methyloptrophs since cross-reaction was observed of antisera against this enzyme with DL-ALDH's from other bacteria. DL-ALDH's have also been found in acetic acid bacteria [97-99] and from spectral properties the authors suggested that these enzymes contain PQQ. From this statement, dye-linked aldehyde dehydrogenase is now classified as a PQQ-containing protein [100], although the original DL-ALDH, rubricated as EC 1.2.99.3, was clearly a haemoprotein and the enzyme from acetic acid bacteria does not oxidize formaldehyde. Comamonas testosterone grown on ethanol possesses an aldehyde dehydrogenase able to oxidize formaldehyde [101]. The dimeric enzyme (Mr of 186 kDa) is a molybdoprotein with 1 Mo, 1 pterin, 1 FAD, 4 Fe, and 4 S per enzyme molecule. Since this organism is not able to grow on methanol, a role in formaldehyde oxidation seems questionable (however, the presence of an O-demethylase activity, yielding formaldehyde, has been demonstrated [102] in this organism so that it may have a function during growth on methoxylated aromatic compounds). Surprisingly, such an enzyme was also obtained from methanol-grown A. methanolicum (Chapter 8). Since both enzymes oxidize formate esters and this property might have physiological significance in A. methanolicum, in the latter case the enzyme has been named formate ester dehydrogenase (FEDH). Since FEDH is also induced during growth on long-chain aliphatic alcohols and the corresponding aldehydes are excellent substrates for the enzyme, the role of FEDH seems not restricted to that of formate ester conversion, and thus in formaldehyde dissimulation.

From the examples mentioned here, it appears that formaldehyde oxidation in vitro is catalyzed with a bewildering variety of DL-ALDH's with respect to quaternary structure and cofactor identity. Moreover, several of these enzymes are found in the same organism, as shown here for A. methanolicum (Chapter 6 and 8). If it is assumed that these enzymes are also effective in the in vivo situation, apparently to get rid of the toxic formaldehyde, this is best accomplished by a variety of enzymes, not by producing one of these enzymes at a high level. Curiously, the nature of the growth substrate (having formaldehyde as intermediate in its dissimulation pathway) determines which DL-ALDH is induced, as shown in the case of Hyphomicrobium ZV580 [95]. The subtleties of this and the significance of each type of aldehyde
dehydrogenase in overall formaldehyde oxidation, are completely unknown at the moment.

3.2 NAD(P)-linked dehydrogenases able to oxidize formaldehyde.

NAD(P)-dependent formaldehyde-oxidizing activities, as detected in cell free extracts, may originate either from a general aldehyde dehydrogenase (section 3.2.1), a formaldehyde dehydrogenase showing activity for a few other aldehydes (section 3.2.2), a specific formaldehyde dehydrogenase requiring the presence of a cosubstrate or activator (section 3.2.3) or from the presence of a mixture of these enzymes. The existence of this diversity and other pitfalls in detecting these enzymes has not always been realized. For instance, it has been found that certain aldehyde dehydrogenases become easily inactivated under aerobic conditions and that the sequence of addition of aldehyde and coenzyme to the enzyme is crucial for activity, implying that a conclusion on the absence of formaldehyde dehydrogenase is unjustified if the appropriate precautions are not taken [92]. Addition of glutathione (GSH) can prevent this inactivation or restore it, a role which is quite different from its role as cosubstrate in GSH-dependent formaldehyde dehydrogenase or in restoration of factor required for factor-dependent formaldehyde dehydrogenase. As (partial) purifications have already shown, dependency on an unidentified factor may exist [103 - 105] and addition of thiol compounds is in several cases necessary to keep the factor in a reduced state. Since GSH has so many roles, the conclusion that GSH-dependent formaldehyde dehydrogenase is widespread may be incorrect (in this connection it should be noted that several reports even do not mention the control experiments without GSH addition).

3.2.1 General aldehyde dehydrogenases.

The presence of an aldehyde dehydrogenase activity with the ability to oxidize formaldehyde has been demonstrated for several organisms [2, 106], but only in a few cases: the enzymes have been (partially) purified and characterized (Table 4). The activity of the enzyme from the Gram-negative methylotroph, *Hyphomicrobium X*, was stimulated by the addition of K+ ions to the assay mixture. A further increase was observed in the presence of thiol compounds. This was not due to participation in the reaction, but to prevention of inactivation as demonstrated by the adequate activity observed under anaerobic conditions in the absence of thiol compounds. It was concluded that this enzyme resembles the NAD-dependent enzyme from baker’s yeast (EC 1.2.1.5). A different enzyme is found in *A. methanolicus* when grown
batch-wise on methanol (it is almost absent under methanol-limited growth conditions). The enzyme has been purified and characterized [Chapter 6 and 91]. It has a $M_r$ of 200 kDa and is composed of 4 subunits; GSH, $K^+$, or $NH_4^+$ addition do not stimulate formaldehyde oxidation. In view of these characteristics, the enzyme seems similar to the eukaryotic NAD-dependent ALDH (EC 1.2.1.3). Since general aldehyde dehydrogenases are also induced during growth on ethanol it seems, the enzymes may have a general role in aldehyde oxidation, including that of formaldehyde.

3.2.2 Formaldehyde dehydrogenases able to oxidize other aldehydes.

A formaldehyde dehydrogenase (EC 1.2.1.46) has been found with a rather narrow substrate specificity and which does not require a cosubstrate or activator in the in vitro assay (Table 4). The dimeric enzyme ($M_r$ of 150 kDa) containing four Zn atoms per enzyme molecule has been purified from betaine-grown Pseudomonas putida C-83 [107 - 109]. It showed also activity for acetaldehyde (although the affinity for this substrate is lower that that for formaldehyde) and several higher aliphatic alcohols (except methanol and ethanol), but not for the corresponding higher aliphatic aldehydes. Since formaldehyde is hydrated to a large extent under the assay conditions (and acetaldehyde partly), this could mean that the enzyme is in fact an alcohol dehydrogenase with a preference for substrates having a certain hydrophobicity. In view of the differences in structure and catalytic behaviour, this FAldH seems different from the aldehyde dehydrogenases discussed in section 3.2.1. FAldH has also been purified from Ps. putida C1 [110] and a first characterization suggested that the $M_r$ of the enzyme is 250 kDa. However, the authors did not mention whether other aldehydes or long-chain alcohols were substrates so that a comparison is impossible.

3.2.3 Formaldehyde dehydrogenases requiring an extra factor or cosubstrate.

The most well-known enzyme in this group (Table 4) is GSH-dependent formaldehyde dehydrogenase (EC 1.2.1.1; GD-FAldH) the presence of which has been well established in eukaryotes and some Gram-negative bacteria [106, 111, 112]. This enzyme does not use formaldehyde as substrate, but S-hydroxymethylglutathione, which is formed non-enzymatically from formaldehyde and reduced glutathione. It is also active with higher aliphatic alcohols (it is in fact identical to class III alcohol dehydrogenase, as was recently discovered [113 - 117]). Thus, also this
enzyme can be considered to be an alcohol dehydrogenase, the GSH moiety in the case of formaldehyde functioning as a tag for recognition, in line with the high specificity of the enzyme for this moiety. GD-FAIDH produces S-formylglutathione rather than formate as reaction product. It is generally assumed that S-formylglutathione is further hydrolyzed to GSH and formate by S-formylglutathione hydrolase (EC 3.1.2.12) [118]. However, the presence of this enzyme has only been demonstrated in a restricted number of organisms [118 - 120]. Alternative candidates for the conversion of S-formylglutathione are formate dehydrogenase, as has been suggested for Achromobacter paryulus [121], or formate ester dehydrogenase (FEDH), as discussed in section 4.2.

GD-FAIDH has been purified from formaldehyde-grown Ps. putida F61 [111] and from acetate-grown Escherichia coli [112]. Both enzymes are dimers (subunits $M_r$ of approx. 40 kDa), the last one containing 4 Zn atoms per enzyme molecule. Alignment of the N-terminal amino acid sequence of the E. coli enzyme with mammalian GD-FAIDH (Class III ADH) shows more than 50% identical amino acids and both have the capability to oxidize long-chain aliphatic alcohols [112].

Methane-grown Methylococcus capsulatus strain Bath possesses an NAD(P)-dependent FAIDH, which requires a dialysable factor [103]. This factor is heat and trypsin-sensitive, while inclusion of GSH or another thiol compound in the assay mixture is not required. The enzyme has a $M_r$ of 115 kDa and is composed of two subunits of equal size.

The Gram-positive bacterium, Rhodococcus erythropolis is unable to grow on methanol, but when grown on 3,4-dimethoxybenzoic acid, it contains an NAD-, and factor-dependent formaldehyde dehydrogenase (FD-FAIDH) [104, 105]. Such an enzyme was also found in methanol-grown A. methanolicus [Chapter 6 and 92]. The FD-FAIDH's are very similar (Chapter 7): they contain zinc and consist of three identical subunits of 40 kDa; of the aldehydes tested, only formaldehyde is active. The factor has the following characteristics: it has a low molecular weight; it is heat stable; it has to be in a reduced state so that dithiothreitol (DTT) is routinely included in the assay [105]; it cannot be replaced by GSH, coenzyme A or tetrahydrofolate [104]; the factors from both organisms are interchangeable; the R. erythropolis factor is a weak acid with a $pK_a$ of about 6.5. High concentrations of methanol can replace the factor and addition of DTT is not required in that case. Therefore, the authors suggested [104] that not formaldehyde itself, but the hemiacetal of formaldehyde and methanol, $\text{CH}_2\text{O-CH}_2\text{OH}$, might be the substrate. In a similar reasoning, not formaldehyde but the hydroxymethyl derivative of the factor would be the substrate. Thus, also FD-FAIDH seems
to act as an alcohol dehydrogenase, in agreement with the finding that primary alcohols are substrates, these not requiring the presence of factor [105]. Since growth of the organisms on ethanol induced no FD-FAldH activity [104, Chapter 6], this suggests that the role of FD-FAldH is restricted to \( \text{C}_4 \)-dissimilation. The NAD-dependent, factor-independent FAldH activity found in cell free extracts of ethanol-grown cells [104] might originate from the presence of an NAD-dependent ALDH. On the other hand, FD-FAldH is present (although at a low level) in \textit{A. methanolica} cells grown on butan-1-ol or hexan-1-ol (Chapter 7). Therefore, FD-FAldH might have a dual role: in methanol-grown cells acting as FD-FAldH, in cells grown on higher primary alcohols as an alcohol dehydrogenase.

Since GSH has never been detected in Gram-positive bacteria [122, 123], the factor might be the counterpart of GSH with respect to its role in GD-FAldH present in Gram-negatives. The similarity in structure (30 % amino acid identity of the N-terminal part; subunits of 40 kDa) of FD-FAldH (Chapter 6) and GD-FAldH [113 - 117] supports this view.

As a consequence of the proposed mechanism, factor-formyl adduct should be the product of the reaction. This might be further hydrolysed by a special enzyme, in analogy with the assumed situation for the product of GD-FAldH. However, when screening for such an activity in cell free extracts of \textit{A. methanolica}, no formate-producing activity could be observed. As discussed in Chapter 8, oxidation of the factor-formyl adduct via FEDH seems a realistic possibility.

### 3.3 Formaldehyde dismutases.

Formaldehyde dismutase (FAldM) (EC 1.2.99.4) or cannizzarase was first described for \textit{Pseudomonas putida} F61 grown in the presence of formaldehyde [124] (this organism also contains GD-FAldH [111]). The enzyme converts formaldehyde into methanol and formate without requiring the addition of an electron acceptor or donor. FAldM is also able to convert other aldehydes by catalysing a cross-dismutation reaction between formaldehyde and another aldehyde, thereby yielding formic acid and the corresponding alcohol of the aldehyde [125]. The enzyme contains firmly bound NAD, which is reduced and then again oxidized when formaldehyde is converted into formate and methanol, respectively. The enzyme can also be assayed as alcohol dehydrogenase by using p-nitroso-N,N-dimethylaniline (NDMA) as electron acceptor [126]. \textit{Ps. putida} F61 is a formaldehyde-resistant strain being able to grow at concentrations up to 60 mM and since FAldM shows high specific activities with 20 mM formaldehyde as substrate \( V_{\text{max}} \) of the purified enzyme
is 190 μmol formic acid formed/min/mg protein) the physiological role of this enzyme is most likely the detoxification of formaldehyde. From experiments applying $^2$H-NMR to whole cells of *Staphylococcus aureus* in the presence of labelled formaldehyde, the methanol and formate detected suggested that such an enzyme is also present in this organism [127]. When the cells were incubated with $^{13}$C-methanol and $^2$H-formaldehyde, methylformate could be detected as an additional product, this probably formed by an enzymic oxidation of the hemiacetal formed from methanol and formaldehyde. However, the authors did not indicate how the methylformate could be converted. Applying such an incubation to *Ps. putida* F61 cells, only methanol and formate were detected, not methylformate [127]. Thus, differences might exist in FALDM's for methylformate production or in capacity to convert methylformate.

As has already been mentioned in section 2.2, MFF and NDMA-ADH can also be detected with NDMA as electron acceptor. However, only MFF shows FALDM activity (Chapter 4). In addition, MFF was able to produce methylformate on incubation with methanol and formaldehyde. Although FALDM and MFF have similarities, there are also differences [124, 126, L.V.Bystrykh, unpublished results]: MFF is able to oxidize methanol using NDMA, FALDM is not; FALDM is a tetramer consisting of subunits of 55 kDa, MFF is a dodecameric protein with subunits of 49 kDa; *A. methanolicus* is sensitive for formaldehyde (concentrations above 0.8 mM are lethal), suggesting that the dismutase activity of MFF is not used for that purpose in vivo, this in contrast to such a role proposed for FALDM in *Ps. putida* F61. Therefore, despite the fact that both have dismutase activity, the enzymes can be regarded as different.

In this context it should be noted that classical alcohol dehydrogenase (EC 1.1.1.1) also shows FALDM activity and methylformate formation [128 - 130]. However, the enzymes discussed in this section, containing bound NAD(P) as cofactor (rather than using NAD(P) as coenzyme), are much more effective in performing this reaction (the specific activity of FALDM from *Ps. putida* F61 is approx. 6 times higher than that of ADH from horse liver).

From the 3 examples known nowadays, it is clear that FALDM occurs in Gram-positive as well as in Gram-negative bacteria. Since an assay for FALDM is not routinely performed in methylotrophic research, the presence of the enzyme might have been overlooked, perhaps explaining the absence of (expected) formaldehyde dehydrogenases in some cases.
4. FORMATE AND FORMATE ESTER DEHYDROGENASES.

4.1 Formate dehydrogenases.

Table 5: Formate dehydrogenases from methylotrophic organisms.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Cofactor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NAD-linked formate dehydrogenases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Achromobacter parvulus</td>
<td>no</td>
<td>136</td>
</tr>
<tr>
<td>Pseudomonas sp. 101</td>
<td>?</td>
<td>140</td>
</tr>
<tr>
<td>Moraxella C-1</td>
<td>no</td>
<td>135</td>
</tr>
<tr>
<td>Mycobacterium vaccae 10</td>
<td>?</td>
<td>141</td>
</tr>
<tr>
<td><strong>NAD- and dye-linked formate dehydrogenases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudomonas oxalaticus</td>
<td>FMN/Fe/S</td>
<td>137</td>
</tr>
<tr>
<td>Pseudomonas sp. 101</td>
<td>?</td>
<td>140</td>
</tr>
<tr>
<td>Mycobacterium vaccae 10</td>
<td>?</td>
<td>141</td>
</tr>
<tr>
<td>Methylosinus trichosporium</td>
<td>FAD/Fe/S/Mo</td>
<td>139</td>
</tr>
</tbody>
</table>

\(^1\) when grown on medium containing tungsten-salts; \(^2\) when grown on medium containing molybdenum-salts.

Formate dehydrogenases are found in non-methylotrophic as well as methylotrophic bacteria [131, 132], but since the topic of this thesis deals with the enzymes of the latter group, only formate dehydrogenases participating in the methylotrophic pathway will be considered. Two types of formate dehydrogenases (FDH) have been found in cell free extracts of methylotrophs [2, 132, 133]: NAD-dependent ones and dye-linked ones. Most of the organisms possess NAD-dependent FDH, while dye-linked FDH is produced by **Hyphomicrobiurn X** and **Mb. extorquens** AM1 [2]. Some organisms seem to have both types [2], although this may be a wrong conclusion since molybdoprotein dehydrogenases frequently use NAD as well as dyes as electron acceptor [134].

Only a limited number of NAD-linked FDH's have been (partially) purified and characterized from methylotrophic bacteria. They can be subdivided into two groups: those active with only NAD and those able to use dyes as well (Table 5). The following enzymes belong to the first group: FDH from
*Moraxella* sp. strain C-1, grown on a mixture of methanol and formate [135] and that from methanol-grown *Achromobacter parvulus* [136]. The enzymes have similar properties: they consist of two identical subunits of 40 - 45 kDa and do not contain a cofactor or prosthetic group. To the latter group belong FDH's purified from *Pseudomonas oxalaticus* [137] and *Methylosinus trichosporum* OB3b [138]. They consist of four subunits and contain flavin mononucleotide and Fe/S-clusters. Recently, it was reported that FDH from *M. trichosporum* contains an unusual flavin and Mo [139], indicating that at least this enzyme is a molybdenoprotein. Evidence for a molybdenoprotein nature of FDH has also been provided in the case of enzyme from *Pseudomonas* sp. 101 [140] and *Mycobacterium vaccae* 10 [141], both grown on methanol. Curiously, the presence of Mo or W salts in the growth medium induced different FDH's: in the presence of Mo a FDH activity could be demonstrated both active with NAD as well as with artificial electron acceptors, in the presence of W an enzyme was found active with NAD, but not with artificial dyes. Also other properties were different when comparing these enzymes: in the presence of Mo salts the *M. trichosporum* FDH's are approx. 300 - 400 kDa, much higher than for those obtained from cells grown in the presence of W salts (approx. 90 kDa).

Dye-linked FDH activities, not active with NAD(P), have been found in cell free extracts of some Gram-positive bacteria, but as these enzymes were not purified, it is not clear whether they really differ from the enzymes discussed above. *M. gastri* MB19 grown on methanol contains a DCPIP-linked FDH, but the activity was also found in extracts of cells grown on glucose or on mannitol [142]. Formaldehyde dehydrogenase activities could not be demonstrated in extracts of methanol-grown cells (unpublished results). Therefore, it is questionable whether FDH is involved in a methylocotrophic pathway of this organism. Whole cells of *A. methanolicus* are able to oxidize formate, although at a low rate [34, 143]. Dye-linked FDH has been demonstrated in crude extracts of methanol-grown *A. methanolicus* [33], but this could not be confirmed by others [34, 142, 143]. The reason for this is most likely the instability and/or the (rather low) activity of the enzyme (Chapter 8). In cell free extracts of 3,4-dimethoxybenzoic acid grown *R. erythropolis*, no NAD-dependent or dye-linked (phenazine ethosulphate/DCPIP and methylene blue were tested as electron acceptors) FDH activity could be found [104]. Also in methanol-grown *Bacillus C1* these activities could not be demonstrated [12]. In the latter case, this is understandable since formaldehyde is oxidized via the RuMP pathway in this organism. In the first case, in view of the presence of FD-FAldH, an FED might be involved in the dissimilation pathway of formaldehyde (see section 4.2).
4.2 Formate ester dehydrogenases.

Since FDH activity in *A. methanolicum* is low, FD-FAldH does not yield formate, and a hydrolase was not found for the expected product (Chapter 7), an alternative route for the oxidation of the reaction product of FD-FAldH (factor-formyl adduct) might exist. A formate ester dehydrogenase (FEDH) was found indeed and the purification and characterization of this molybdoprotein are described in Chapter 8. Since the enzyme is able to oxidize methylformate, which might be produced by MFF, besides factor-formyl ester conversion methylformate oxidation could be another function of FEDH, as discussed in Chapter 9. However, further research is required to exclude the possibility that the sole role is in aldehyde oxidation (as suggested originally for the *E. testosteroni* enzyme [101]), and formate ester oxidation is just fortuitous.

5. OUTLINE OF THE THESIS.

At the start of the project little was known regarding the enzymology of methanol oxidation in Gram-positive bacteria. Just as occurs with Gram-negative methylo trophs, it has been found that the Gram-positive actinomycete, *Amycolatopsis methanolicum* NCIB 11946 [144] (previously known as Streptomyces sp. 239 [33] and Nocardia sp. 239 [34]) produces micromolar concentrations of PQQ in its growth medium when growing on methanol [34]. A novel type of MDH (n-MDH), forming part of a multi enzyme complex, had been proposed to catalyse the conversion of methanol into formaldehyde, NAD and PQQ being the cofactors of this reaction [11]. To corroborate the proposed structure and function, n-MDH was chosen as a model to elucidate the methanol oxidizing process in Gram-positive methylo trophs.

*A. methanolicum*, isolated from a soil sample of New Guinea [33], is a facultative methylothroph able to grow on a broad range of substrates. Of the C1-substrates (methanol, formaldehyde, formate, methylamine) tested only methanol sustained growth, both in batch cultures as well as in continuous cultures [34, 144]. During growth on methanol in a chemostat, formaldehyde is assimilated via the RuMP pathway since the indicatives, the key enzymes hexulose phosphate synthase and hexulose phosphate isomerase, are present in cell free extracts of methanol-grown cells [34, 143], but the key enzymes of the ribulose biphosphate cycle or the serine pathway are not or at a very low level [34].

Serious problems were met when repeating the previously developed assay for n-MDH: in many trials activity was found only a few times; the activity appeared to be very labile (Chapter 2). As this excluded purification, the
attention was directed to purification of PQQ-containing proteins since PQQ production is strictly related to growth on methanol and PQQ appeared to be present in n-MDH [11]. However, although two PQQ-containing proteins could be (partly) purified, no activity could be assigned to them (Chapter 2). Therefore, for the time being it was decided to detect and characterize the other enzymes of the methanol dissimilation route (Chapter 6 - 8).

In the search for formaldehyde-oxidizing enzymes three different activities were detected in crude extracts of methanol-grown cells (Chapter 6): 1. NAD-dependent aldehyde dehydrogenase; this enzyme was purified and characterized [93 and Chapter 6]. 2. NAD-, and factor dependent FA1DH; this enzyme was also purified and characterized (Chapter 7). 3. DCPIP-linked aldehyde dehydrogenase (no attempts were made to purify the enzyme). Since FD-FA1DH seemed to play the major role in formaldehyde oxidation and the product is a formate ester for which no hydrolase was found, a search was made for a formate ester-oxidizing enzyme as well as for formate dehydrogenase for which contradictory reports existed on its occurrence [33, 34, 142, 143]. With respect to the latter, Kato et al. had reported a dye-linked activity [33], but this could not be repeated by others [34, 142, 143]. Low levels of dye-linked FDH activity were detected but the enzyme appeared to be extremely unstable (Chapter 8). The search for a formate ester oxidoreductase was successful since a dye-linked dehydrogenase appeared to catalyse this reaction. Characterization of the purified FED made clear that it is a molybdenoprotein, being able to oxidize formate esters and aldehydes, and resembling the aldehyde dehydrogenase from C. testosteroni [101]. The physiological relevance of FD-FA1DH and FEDH is discussed in Chapter 9.

At a later stage of the project a methanol oxidizing activity which could be reproducibly detected by applying special tetrazolium dyes and high phosphate or sulfate concentrations in the assay, was discovered. The properties and distribution of this tetrazolium-dye-linked alcohol dehydrogenase (TD-ADH) activity are described in Chapter 3. In attempts to purify the enzyme, indications were obtained that TD-ADH consists of several components. Unfortunately, reconstitution of the putative components to activity was unsuccessful. Therefore, it was attempted to develop an assay for detecting the methanol dehydrogenase component of the complex directly by transferring the reducing equivalents from the presumed bound NAD(P)H to an external electron acceptor. NDMA appeared to be suited for that purpose (Chapter 4). Upon purification two enzymes appeared to be responsible for the observed activity, namely MFF, able to oxidize methanol as well as primary alcohols (Chapter 4), and NDMA-ADH, oxidizing primary alcohols except methanol (Chapter 5). Although the original goal, the elucidation of
the structure and function of n-MDH, has not been achieved, the course taken has yielded a number of novel enzymes, of which the physiological significance is discussed in Chapter 9.

REFERENCES


Chapter 2

The methanol dehydrogenase multi enzyme complex and PQQ-containing proteins from *Amycolatopsis methanolica*.

SUMMARY

It has been reported that the Gram-positive methanol utilizer, *Amycolatopsis methanolica*, contains a unique methanol dehydrogenase (n-MDH) [FEBS Lett. 168, 217 - 221]. However, despite many trials and extensive variation of assay conditions, in the present work activity of this enzyme could only be demonstrated now and then and when found it disappeared within a few hours. This suggests that some unknown critical conditions exist with respect to stability of n-MDH. Growth on methanol, but not on several other substrates, induced the appearance of PQQ in the extracellular medium as well as bound to protein, supporting the view that a quinoprotein (PQQ-containing) enzyme is involved in the methanol oxidation process. Two chromatographically different PQQ-containing proteins appeared to be present in cell free extracts of methanol-grown cells and one of these was partly purified. The attempts undertaken to detect enzymatic activity for the quinoproteins were unsuccessful so that no physiological role can be assigned to the proteins.

INTRODUCTION

*Amycolatopsis methanolica* [1] (previously known as *Streptomyces* sp. 239 [2] and *Nocardia* sp. 239 [3]) excretes pyrroloquinoline quinone (PQQ) into its medium during growth on methanol [3] in amounts comparable to that for Gram-negative methanol utilizers. However, the classical quinoprotein methanol dehydrogenase (MDH), as found in Gram-negative methylotrophs [4], is absent in this organism [3]. Duine et al. were able to detect activity of a novel MDH (n-MDH) by including NAD as well as DCPIP in the assay mixture [5]. When n-MDH was partly purified, a preparation was obtained also showing NAD-dependent (form)aldehyde dehydrogenase and NADH dehydrogenase activities. After denaturating the proteins in this preparation, PQQ was detected in amounts proportional to methanol-converting activity, suggesting the participation of a quinoprotein in this process. Upon further purification, a fraction was obtained which was not active in the assay, but
which showed a rise in absorbance at 340 nm when methanol was added. This was not due to free NADH production since addition of NADH dehydrogenase from *Bacillus subtilis* did not give rise to activity. Only when a NADH dehydrogenase-exhibiting fraction obtained in the purification step was added, the original activity for methanol with NAD plus DCPIP could be restored in a time-dependent manner. Therefore, it was suggested that n-MDH forms part of a multi-enzyme complex, together with the (form)aldehyde and the NADH dehydrogenase, in which NAD functions as a tightly bound cofactor transferring the reduction equivalents derived from methanol to the NADH dehydrogenase component.

To characterize the multi enzyme complex and to provide evidence for the proposed mechanism, we embarked on its purification. However, as problems were met with respect to detecting activity and keeping the enzyme stable (*vide infra*), it was attempted to purify and to find activity for the putative quinoprotein component of the complex.

Abbreviations used: DCPIP: 2,6-dichlorophenol-indophenol; KPB: potassium phosphate buffer; MDH: (classical) methanol dehydrogenase (EC 1.1.99.8); n-MDH: novel methanol dehydrogenase; PQQ: pyrroloquinoline quinone.

**METHODS**

**Cultivation of the organism.**

*A. methanolicus* was grown aerobically at 37 °C, either batch-wise on 1 % methanol (v/v) [3] or fed-batch-wise with methanol limitation [6]. The organism was also grown batch-wise on the following substrates: 1 % (v/v) ethanol; 0.2 % (v/v) 1-butanol; 0.1 % (v/v) 1-hexanol; 0.5 % (w/v) sodium acetate; 0.5 % (w/v) glucose.

**Preparation of cell free extract.**

Cell free extract was prepared as described [6]. Anaerobic disruption of cells was performed in the following way: all solutions were degassed and subsequently flushed with N₂. Frozen cells were resuspended in 20 mM potassium phosphate buffer (KPB), pH 7.2, in an anaerobic cabinet under an N₂/H₂ (97.5 %/2.5 %) atmosphere. The cell suspension, to which DNase was added, was transferred to a French Pressure Cell. Cell disruption occurred at 110 MPa and the broken cell suspension was collected in a vessel kept under N₂. This was brought into the anaerobic cabinet, and the suspension was poured into centrifuge tubes. The tightly capped tubes were centrifuged
for 30 min at 48,000 x g and the supernatant was immediately used for activity measurements.

**Enzyme assays.**

Methanol dehydrogenase activity was measured using several assays and with several additions (see Results section). Activity was determined either by following DCPIP reduction at 600 nm or NADH production at 340 nm. Measurements were performed of samples with and without the presence of 0.1 M NH₄⁺. n-MDH activity was measured (aerobically) as described [5] and anaerobically as follows: all the solutions were degassed and flushed with N₂. Cuvettes were filled in the anaerobic cabinet and stoppered with Suba seals. After transfer to the spectrofotometer, the reaction was started by adding an anaerobic methanol solution via a syringe provided with a hypodermic needle. Dye-linked MDH activity was also measured using the assays as described by Kato et al. [2] and by Bastide et al. [7]. NAD-dependent MDH activity was determined according to Arfman et al. [8], as well as with the assay described for alcohol dehydrogenase (EC 1.1.1.1) [9] using methanol instead of ethanol at a concentration of 10 mM. Glucose dehydrogenase was determined with Wursters' Blue as electron acceptor according to Dokter et al. [10]. NAD-dependent (form)aldehyde dehydrogenase activity and NADH dehydrogenase activity were measured as described [5], and NAD-/factor-dependent formaldehyde dehydrogenase activity as in [6]. Oxygen consumption was measured at 37 °C in an oxygraph using a Clark-type electrode.

**Analytical methods.**

PQQ was determined with a biological assay, using quinohaemoprotein alcohol dehydrogenase apo-enzyme [11]. To detect free as well as protein-bound PQQ, measurements occurred of undenatured and denatured samples (3 min at 100 °C and removal of the denatured protein by centrifugation for 2 min at 16,000 x g), respectively. Protein concentrations were determined by using the Bradford assay with bovine serum albumine as a standard [12]. Purification was determined by the method of Lang and Lang [13].

**Purification of PQQ-proteins.**

Cell free extract from methanol-grown cells (batch-wise) was applied to a DEAE-Sepharose column CL-6B (12.2 x 2.2 cm), equilibrated with 20 mM KPB, pH 7.2. The column was washed with 3 volumes of the same buffer, and proteins were eluted in 16 h with a linear gradient of 0 to 1 M KCl in 20 mM KPB, pH 7.2, at a flow rate of 0.9 ml/min. Fractions were tested for the presence of bound and free PQQ, yielding indications for a quinoprotein at an elution
volume of approx. 210 ml (quinoprotein I) and approx. 330 ml (quinoprotein II) as indicated in Fig. 1. Fractions containing PQQ-protein I (Fig. 1) were pooled and the protein was concentrated by pressure filtration (using a membrane with a cutoff of 10,000). Buffer exchange of the concentrate was carried out on a PD-10 (Pharmacia) column, equilibrated with 20 mM sodium pyrophosphate buffer (NaPPB), pH 8.0. The preparation was applied to a Mono Q 10/10 column, equilibrated with 20 mM NaPPB, pH 8.0. Elution occurred in 30 min with a linear gradient from 0 to 0.3 M KCl in 20 mM NaPPB, pH 8.0, at a flow rate of 2 ml/min. The protein eluted at 0.16 M KCl. After concentrating as described above, the sample was applied to a Superose-12 gel filtration, equilibrated with 0.1 M KPB, pH 7.0, and operated at a flow rate of 0.5 ml/min. Manipulations were carried out at room temperature, except DEAE-Sephadex chromatography which occurred at 4°C.

Polyacrylamide gel electrophoresis.

The homogeneity of the preparations was checked by using polyacrylamide gel electrophoresis (PAGE) on Pharmacia 4/30 gradient gels in a Pharmacia GE-411 electrophoresis apparatus cooled with tap water. Electrophoresis was conducted in 50 mM Tris/385 mM glycine, pH 8.3, for 16 h at 150 V. The gel was cut in two parts and one part was stained with Coomassie Brilliant Blue R-250 for 1 h. The corresponding protein band in the other part was cut out, and after addition of 100 µl 20 mM KPB, pH 7.2, the gel was desintegrated with a glass rod. Subsequently, the suspension was incubated for 10 min at 100°C. After centrifugation for 1 min at 16,000 x g, the supernatant was tested for the presence of PQQ.

RESULTS

n-MDH activity.

Despite many attempts, it was not possible to detect n-MDH activity according to the procedure originally prescribed [5]. Also all other assays for MDH gave negative results. Neither cell free extracts of cells (fresh or frozen paste) grown batch-wise nor from cells grown fed-batch-wise showed activity. The inclusion of the following compounds in the assay mixture gave no effect: 0.1 % or 1 % (v/v) Triton X-100 or Tween-20; 1 mM Ca²⁺ or Mg²⁺ salts; 0.2 M K⁺ salts; 1 mM dithiothreitol; 10 % (w/v) polyethylene glycol; 2 % (w/v) sorbitol; 0.5 mM KCN. Also when these compounds (KCN was not tested) were added to the cell suspension before disruption was performed, no effect was seen. When DCPIP was replaced by other electron acceptors (100
Table 1: Stability of n-MDH activity in cell free extracts.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>n-MDH activity (nmol DCPIP/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>45</td>
</tr>
<tr>
<td>1.5</td>
<td>5.4</td>
</tr>
<tr>
<td>3.5</td>
<td>0.6</td>
</tr>
</tbody>
</table>

The cell free extract was prepared anaerobically and was kept under a N₂ atmosphere at room temperature.

µM Wursters' Blue; 50 µM cytochrome c, 0.2 mM O₂, 0.1 mM phenazine methosulfate (PMS), 0.1 mM PMS/40 µM DCPIP), no success was met. Variation of pH (tested were 0.1 M KPB, pH 6.5, 7.1, and 8.0, respectively), of temperature (room temperature and 37 °C were tested), of methanol concentration in the assay (tested were 10 mM, 0.1 M and 0.3 M) and of type of buffer (50 mM Tris/HCl, 25 mM CHES/NaOH, 0.1 M borate, 0.1 M NH₄OH, and 0.1 M sodium carbonate, all at pH 9.0) was also unsuccessful. Changing the order of addition of the constituents of the assay did not result in activity. To check interference due to the presence of an endogenous electron acceptor, the putative production of formate was determined. However, no formate could be detected after 10 - 30 min incubation. Finally, also the other assays for MDH activity mentioned in the Methods section gave negative results.

n-MDH activity was only detected a few times when the preparation of cell free extract and the assay were performed anaerobically, thereby using cells grown fed-batch-wise. The activity was very unstable, disappearing within few hours (Table 1). Since the results were not reproducible, it is unclear whether the anaerobic conditions are really essential.

Induction of POQ and of POQ-containing proteins.

A. methanolicus was grown on methanol, ethanol, 1-butanol, 1-hexanol, acetate and glucose (all batch-wise). Only cells growing on methanol produced POQ, as judged from its presence in the growth medium. Typically, 1 - 3 µM POQ was found in the medium after batch-wise growth and 4 - 10 µM at
The PQQ content in the fractions was measured for denatured and non-denatured samples. The difference between these two values is the "PQQ-content". ● = PQQ content; ▲ = formaldehyde dehydrogenase activity (arbitrary units); ■ = NADH dehydrogenase activity (arbitrary units); I = PQQ-protein I; II = PQQ-protein II; $V_e$ = elution volume.

fed-batch-wise growth. Also when PQQ was measured in cell free extracts after protein denaturation, only those of methanol-grown cells showed the presence of the cofactor. In this case no differences were found when comparing cells grown batch-wise and fed-batch-wise. It is concluded therefore that synthesis of PQQ is induced at growth on methanol. Since the PQQ content of denatured cell free extract was always higher than that of the non-denatured ones, this indicated the presence of bound PQQ.

Detection of PQQ-containing proteins.

Besides free PQQ eluting at 0.55 M KCl (not shown), PQQ was detected in fractions eluting at 0.20 M KCl (PQQ-protein I) and at 0.30 M KCl (PQQ-protein II) (Fig. 1). The PQQ was not free since it was only detected after denaturation, suggesting that it is bound to proteins. The amount of PQQ detected in proteins eluting at 0.20 M KCl was always higher than in those eluting at 0.30 M KCl, the ratio varying from 3 to 10 times. No correlation of this ratio could be detected with growth conditions (batch-wise or fed-batch-wise).

Attempts to purify and characterize the PQQ-proteins.

As shown in Table 2, PQQ-protein I was purified 45-fold with a yield of 6.4% (with respect to PQQ). Addition of PQQ to the PQQ-containing protein
samples did not result in reconstitution, suggesting that no apo-protein was obtained during purification. Also addition of 1 mM concentrations of Ca$^{2+}$ or Mg$^{2+}$-salts to the elution buffers (to prevent dissociation of PQQ) did not improve the yield. Thus, the tentative conclusion is that the low yield is due to protein denaturation. PAGE followed by protein staining revealed a main band and 6 - 7 minor bands. PQQ was only detected in the main band. PQQ-protein I has a $M_r$ of 80,000 as judged by gel filtration on a Superose-12.

Table 2: Purification of PQQ-protein I.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Total Protein (mg)</th>
<th>Total PQQ$^1$ (nmol)</th>
<th>Yield (%)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell free extract</td>
<td>1700</td>
<td>185</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>DEAE-Sepharose</td>
<td>191</td>
<td>129</td>
<td>70</td>
<td>6.2</td>
</tr>
<tr>
<td>Mono-Q</td>
<td>5.9</td>
<td>24</td>
<td>13</td>
<td>37</td>
</tr>
<tr>
<td>Superose-12</td>
<td>2.4</td>
<td>12</td>
<td>6.4</td>
<td>46</td>
</tr>
</tbody>
</table>

$^1$ = protein-bound PQQ.

![Absorption spectrum](image)

Figure 2: Absorption spectrum of PQQ-protein I.

The absorption spectrum of the final preparation of PQQ-protein I (0.7 mg protein/ml) was measured in 0.1 M KPB, pH 7.0.
column. Its absorption spectrum showed maxima at 280 and 345 nm (Figure 2). Neither n-MDH, NAD-dependent (form)aldehyde dehydrogenase (both factor-dependent as well as factor-independent), NADH dehydrogenase nor glucose dehydrogenase activity was detected in the preparation.

The fraction eluting at 0.3 M KCl (Fig. 1) with the putative PQQ-protein II showed NAD-/factor-dependent formaldehyde dehydrogenase and NADH dehydrogenase activities. Attempts to separate the activities from each other (by applying hydroxylapatite, anion exchange (Mono-Q), and gelfiltration chromatography) were without success. However, hydrophobic interaction chromatography on a Phenyl-Sepharose column, using a linear gradient from 1.5 M ammonium sulfate to 0 M in 10 mM KPB, pH 7.0, was successful since PQQ-protein II eluted at 1.5 M (NH4)2SO4 and the factor-dependent formaldehyde dehydrogenase and the NADH dehydrogenase eluted at a lower ammonium sulfate concentration (approx. 0.2 M). Apparently the combination of a high salt concentration and a hydrophobic surface is able to disrupt a complex. Although this behaviour is reminiscent to the multi enzyme complex observed previously [5], no n-MDH activity was detected neither in the fraction eluting at 0.3 M KCl nor in fractions obtained with hydrophobic interaction chromatography. This result discouraged attempts to purify and characterize PQQ-protein II.

DISCUSSION

Despite many trials and variations applied in the assay, it was not possible to detect (stable) n-MDH activity in a reproducible way, these problems precluding purification of the enzyme. Since others have also reported negative results [14], the question can be posed whether the observations made in the past [5] could be due to an artefact. However, no other MDH activity in this organism has been reported or was found in the present work. Moreover, activity was found now and then in the present work and a preliminary report [15] mentions n-MDH activity to occur in cell free extracts of methanol-grown thermoactinomycete strain 381. This suggests that some critical condition is required which was missed in the present work. The recent success (L.V. Bystrykh, personal communication) in finding such a condition confirms this view.

Growth of A. methylotica on methanol, but not on ethanol, induces PQQ production [3]. This was confirmed in the present work and it was also shown that growth on 1-butanol, 1-hexanol, acetate or glucose is similarly ineffective with respect to PQQ-induction. Growth on methanol induces n-MDH activity residing in a complex containing a PQQ-protein [5]. In the present
work, two PQQ-proteins, indicated as I and II, were detected. Protein I was purified to near homogeneity. Although its absorption spectrum is similar to that of glucose dehydrogenase (soluble type) [10], no dehydrogenase activity for glucose was found and neither for methanol or formaldehyde. Assuming that this protein is involved in methanol oxidation, its involvement seems not to be related to the first step, in accordance with the observation that methanol addition did not change its absorption spectrum.

PQQ-protein II, formaldehyde and NADH dehydrogenase eluted together from the DEAE-Sepharose column with a KCl containing buffer, just as observed previously for the n-MDH complex [5]. However, despite this similarity, no n-MDH activity was detected. Since most of the time activity was already absent in cell free extract, presumably some unknown essential component becomes inactivated during preparation of the extract. The relatedness of protein I and II is presently unknown.

REFERENCES

A novel dye-linked alcohol dehydrogenase activity present in some Gram-positive bacteria

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1. SUMMARY

An assay was developed which allowed reproducible detection of methanol oxidation by cell free extracts of methanol-grown Amycolatopsis methanolica. The dye-linked activity was only observed when high concentrations of phosphate or sulphate salts were applied in the assay. The specific activity strongly increased when raising the amount of cell free extract in the assay. From the large number of electron acceptors tested only the tetrazolium dyes MTT and INT showed significant activity. Activity was observed with primary as well as secondary alcohols. A similar activity was found in ethanol-grown A. methanolica, Rhodococcus erythropolis and Rhodococcus rhodochrous, but not in other bacteria tested. The identity and possible involvement in alcohol oxidation of this novel dye-linked activity is discussed.

2. INTRODUCTION

Methanol oxidation in Gram-negative bacteria proceeds via a periplasm-located dehydrogenase that uses a cytochrome c as electron acceptor [1]. This so-called methanol dehydrogenase (MDH) is a quinoprotein as pyrroloquinoline quinone (PQQ) is the cofactor. Since Gram-positive bacteria do not have a periplasmic space, the methanol oxidizing system must be different. This has been shown indeed to be the case for the Gram-positive methanol-utilizer, Amycolatopsis methanolica (previously known as Nocardia sp. 239 [2]). From this work, a novel methanol dehydrogenase (n-MDH) was postulated and a tentative characterization revealed that it forms part of a multi-enzyme complex, together with a NAD-dependent formaldehyde dehydrogenase and a NADH dehydrogenase [3]. Partly purified complex contained PQQ, but in the assay the presence of NAD was re-
quired for activity, although NADH production did not occur.

Meanwhile methanol oxidizing enzymes have been observed in other Gram-positive methylo-
trophs. Thus, a NAD-dependent alcohol dehydro-
genase with the capacity to oxidize methanol is present in thermotolerant and thermophilic Bacil-
lus species [4,5]. The enzyme is not a quinoprotein since extraction did not reveal any PQQ [6]. Corynebacterium sp. XG has been reported to contain a dye-linked (dichlorophenol-indophenol) (DCPIP) activity for methanol oxidation [7]. The properties of this enzyme are unknown.

Progress in further work, aiming to characterize n-MDH in more detail, was severely limited, because difficulties were experienced with respect to reproducibly detecting activity in crude extracts. While attempting to establish improved assay conditions, one of us (G-J.E.) discovered methanol-
oxidizing activity in extracts of A. methanolaica using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl-
tetrazolium bromide (MTT) as an electron acceptor. Subsequent studies on optimization of assay conditions, distribution and possible physiological role of this novel tetrazolium dye-linked al-
cohol dehydrogenase (TD-ADH) activity are re-
ported in this paper.

3. METHODS

3.1. Cultivation of the organisms

Amycolatopsis methanolaica was grown according to Hazeu et al. [8] on one of the following substrates: 1% (v/v) methanol; 1% (v/v) ethanol; 0.5% (w/v) methylvamine; 0.5% (w/v) sodium formate; 1% (w/v) glucose. Rhodococcus rhodochrous LMD 89,129 and Rhodococcus erythropolis DSM 1069 were grown according to Eggeling and Sahm [9] on 0.5% (v/v) ethanol and the latter organism also on 2 mM 3,4-dimethoxybenzoic acid. The following organisms were grown on media according to published procedures: Mycobacteri-
um gastri MBI9 on 1% (v/v) methanol or on 1% (v/v) ethanol [10]; Hyphomicrobiurn X on 0.4% (v/v) methanol [11]; Comamonas testosteronei on 1% (v/v) ethanol [12]; Pseudomonas aeruginosa ATCC 17933 on 1% (v/v) ethanol [13]; Pseudo-
monas BB1 on 0.5% (v/v) ethanol [14]; Acetobacter peroxidans on 0.5% (v/v) ethanol [15]. All the organisms were grown in batch cultures.

3.2. Preparation of cell free extracts

Cells were harvested, washed twice with 20 mM potassium phosphate buffer (KPB), pH 7.2, and disrupted as described by Duine et al. [3]. For anaerobic experiments, the suspension and the buffer were sparged with nitrogen, the French Pressure Cell was filled with cell suspension in an anaerobic cabinet under an N₂/H₂ (97.5%/2.5%) atmosphere and the disruption was performed un-
der a nitrogen atmosphere.

3.3. Enzyme assays

n-MDH activity was measured using NAD and DCPIP as described by Duine et al. [3], except using anaerobic conditions. For that purpose, all the solutions were degassed and flushed with nitrogen. Cuvettes were filled in an anaerobic cabinet and stoppered with Suba seals. The reaction was started by adding anaerobic methanol solution via a syringe provided with a hypodermic needle.

TD-ADH activity was measured at 37°C by following the formation of MTT-formazan at 550 nm in 1 M potassium phosphate buffer (KPB), pH 7.6, containing 50 μM MTT. After addition of cell free extract the reaction was continued, until all endogenous substrate was removed (usually 2–3 min). After that the assay was carried out by adding methanol (50 mM final concentration). For other alcohols tested, much lower concentrations (1 mM) were used.

NAD-dependent alcohol dehydrogenase activity was measured in 0.12 M sodium pyrophos-
phate buffer, pH 9.0, containing 2.5 mM NAD. The reaction was started by adding methanol (50 mM) or ethanol (1 mM). An alternative procedure [4] was also used. MDH activity was measured as described [7], using the phenazine methosulfate (PMS)/DCPIP dye couple.

Specific activities were calculated by using a molar extinction coefficient for MTT-formazan at 550 nm of 8100 M⁻¹ cm⁻¹ [16], for DCPIP at 600 nm of 22.10³ M⁻¹ cm⁻¹ and for NADH at 340 nm of 6220 M⁻¹ cm⁻¹ [17].
3.4. Oxygen consumption

Oxygen consumption by whole cells was measured using a Clark-type oxygen electrode at 37°C. Cells were washed twice with 10 mM KPB, pH 7.0, and resuspended in the same buffer. After recording the endogenous oxygen consumption rate, substrate was added at the concentrations mentioned in the text.

3.5. Analytical methods

Protein determinations were performed according to Bradford with bovine serum albumin as a standard [18]. The cofactor PQQ was determined with a biological assay [12]. Formate production was determined according to Höpner and Knappe [19].

4. RESULTS AND DISCUSSION

4.1. n-MDH activity in A. methanolicum

In contrast to the earlier studies [3], n-MDH activity could not be reproducibly demonstrated in cell free extracts of *A. methanolicum*. Only when cell disruption and the assay were performed anaerobically was n-MDH activity occasionally observed. Moreover, the enzyme system was extremely unstable, since 90% of the activity was lost within 1.5 h. Efforts to improve stability have been unsuccessful so far. Assays for other methanol dehydrogenase activities recently described [4,7] gave negative results. In the search of conditions for a reproducible n-MDH assay, methanol oxidizing activity was found with MTT as electron acceptor. This TD-ADH activity was reproducibly detected in cell free extracts, allowing studies on the optimization of assay conditions, and a primary characterization of the TD-ADH system and its distribution.

4.2. Assay conditions for TD-ADH

The observations on the assay were in accordance with what is expected for an enzymic reaction (no activity with heat-denatured cell free extract; straight lines in Lineweaver-Burk plots, indicating Michaelis-Menten behaviour of the enzyme—data not shown). Activity increased when the potassium phosphate concentration in the assay was raised (Fig. 1). A comparable stimulation was observed with 1 M sodium phosphate buffer, but 2 M NaCl, KCl or NH₄Cl (all in 20 mM KPB, pH 7.6) had no effect. Sulfate-salts stimulated the activity in a similar way: 1 M Na₂SO₄ (in 20 mM KPB, pH 7.6) was just as effective as 1 M KPB. The specific activity also increased when increasing amounts of cell free extract were used (Fig. 2). Since both stimulating effects could not be optimized for practical reasons, the maximal specific activity of TD-ADH may in fact be much higher than achieved here. The stimulation produced by the ('salting out') ions, phosphate and sulfate, and by increasing amounts of cell free extract, suggest that activity depends on association of some essential components.

TD-ADH activity has an optimal temperature for activity of 45°C and a pH optimum between 7.5 and 8.0 (in 1 M KPB) and of 7.0 in 10 mM MOPS-buffers supplemented with 1 M Na₂SO₄. The following additions had no effect on the activity: 1 mM concentrations of Ba²⁺, Ca²⁺, Co²⁺, Cu²⁺, Fe²⁺, Mg²⁺, Mn²⁺, Mo⁶⁺, Ni²⁺, Sn²⁺, Zn²⁺ salts, 10 mM concentrations of Ca²⁺, Mg²⁺ salts or 10 mM EDTA; 2.5 mM NAD or NADP; 50 μM FMN, FAD or PQQ; bovine serum albumin.
Primary alcohols (tested from methanol up to 1-butanol) were adequate substrates. As shown by the apparent kinetic parameters (Table 1), ethanol and propanol are better substrates than methanol. Also secondary alcohols (2-propanol and 2-butanol were tested) showed activity, but the tertiary alcohol, 2-methyl-2-propanol, did not. The same variation of the specific activity upon changing the phosphate concentration or the amount of cell free extract was observed with each of the alcohols that functioned as a substrate.

Using methanol as a substrate, formate was detected as the product instead of formaldehyde. This can be explained from the fact that formaldehyde was a substrate in the assay, although it did not show the typical characteristics observed for the alcohols. Therefore, it is oxidized most probably via one of the (formaldehyde dehydrogenases present in this organism [20].

4.4. Induction and distribution of TD-ADH

The presence of TD-ADH activity could also be demonstrated in cell free extracts of ethanol-grown A. methanolicus, but not in that from cells grown on methylamine, sodium formate or glucose. The typical dependency on the phosphate concentration and the amount of cell free extract added was also found in this case. Furthermore, the substrate specificity and kinetic parameters

Table 1 Substrate specificity of TD-ADH

Experiments were performed with 50-μl aliquots of cell free extract (containing 26 mg protein/ml) of A. methanolicus. Apparent kinetic parameters were determined with Lineweaver-Burk plots.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Cell free extract of cells grown on:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Methanol</td>
</tr>
<tr>
<td></td>
<td>1100 35</td>
</tr>
<tr>
<td></td>
<td>800 44</td>
</tr>
<tr>
<td>Ethanol</td>
<td>&lt;10 43</td>
</tr>
<tr>
<td>1-Propanol</td>
<td>&lt;10 50</td>
</tr>
<tr>
<td>2-Propanol</td>
<td>200 25</td>
</tr>
<tr>
<td></td>
<td>&lt;10 65</td>
</tr>
<tr>
<td></td>
<td>200 40</td>
</tr>
</tbody>
</table>
Table 2
TD-ADH activities in some other Gram-positive bacteria

50-µl aliquots of cell free extract of the following organisms were used: Rhodococcus erythropolis (Rh.er.) grown on ethanol (EtOH) and 3,4-dimethoxybenzoic acid (MBA); Rhodococcus rhodochrous (Rh.rr) grown on ethanol; Mycobacterium gastrri MB19 (M.gr) grown on methanol (MeOH) or ethanol. The specific activities are expressed in nmol MTT/min./mg protein.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Specific activities of organisms (grown on):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rh.er. (EtOH)</td>
</tr>
<tr>
<td>Dependency on +:</td>
<td></td>
</tr>
<tr>
<td>phosphate concentration</td>
<td>+</td>
</tr>
<tr>
<td>amount of cell free extract</td>
<td>+</td>
</tr>
<tr>
<td>Specific activity with:</td>
<td></td>
</tr>
<tr>
<td>50 mM methanol</td>
<td>19</td>
</tr>
<tr>
<td>1 mM ethanol</td>
<td>39</td>
</tr>
<tr>
<td>1 mM 1-propanol</td>
<td>45</td>
</tr>
<tr>
<td>1 mM 2-propanol</td>
<td>31</td>
</tr>
</tbody>
</table>

+= strong dependency; ± = weak dependency; = no dependency.

were similar (Table 1). It appears, therefore, that ethanol-grown cells also possess TD-ADH.

In view of the special conditions required for the detection of TD-ADH activity, the presence of the enzyme might have been overlooked in other alcohol-utilizing bacteria. Therefore, cell free extracts of a number of Gram-positive and Gram-negative bacteria grown on alcohols were tested. In case any activity was found, dependency on the phosphate concentration and on the amount of cell free extract added was checked. All the Gram-negative bacteria investigated here showed the previously reported activities related to the presence of quinoprotein methanol or alcohol dehydrogenases [11–15], but not to MTT-reducing activity (except for Acetobacter peroxidans). As a dependency on phosphate concentration and amount of cell free extract was not found, MTT-formazan production most probably does not derive from TD-ADH.

Rhodococcus erythropolis and A. methanolica both have a factor-dependent formaldehyde dehydrogenase [9,20]. Therefore, it was interesting to look for similarity in enzymes for alcohol oxidation. As it appears from Table 2, cell free extracts from R. erythropolis and R. rhodochrous clearly showed TD-ADH activity with the characteristics found for the A. methanolica enzyme. Since these Rhodococci do not grow on methanol, formaldehyde, produced by demethylation of 3,4-dimethoxybenzoic acid, was tested for its ability to induce the enzyme. However very low activity was detected (Table 2), and it was assumed that alcohols were the inducer for the synthesis of the enzyme activity.

Mycobacterium gastrri and A. methanolica both use the ribulose monophosphate (RuMP) pathway for formaldehyde fixation [8,10]. Although activity was detected, stimulation by phosphate was very low (a factor 1.5 while typically a value of 6.5 is observed going from 0.1 M KPB to 1 M KPB), suggesting that TD-ADH activity is absent in M. gastrri. Thermophilic Bacillus species, grown on methanol, contain an NAD-dependent methanol dehydrogenase activity [6], but not TD-ADH. Ethanol-grown cells also do not contain this enzyme (N. Arfman, personal communication). However, NAD-dependent methanol dehydrogenase was not detected in methanol-grown M. gastrri (although the activity for ethanol was 96 nmol NADH produced/min./mg protein). Also PQQ (indicative for n-MDH) and PMS/DCPIP-linked activity, as observed for Corynebacterium sp. XG [7], were absent. Therefore, methanol oxidation in M. gastrri seems to proceed via a still unknown enzyme.

4.5. Identity and function of TD-ADH

The assay conditions for TD-ADH activity preclude the involvement of known NAD-dependent
or quinoprotein alcohol dehydrogenases. The preliminary characterization of TD-ADH suggests that it resides in a multienzyme complex, just as has been suggested for n-MDH. Comparison shows some differences: n-MDH is specific for methanol, ethanol shows higher activity for TD-ADH than methanol; n-MDH is detectable with DCPIP; TD-ADH with the tetrazolium dyes MTT and INT; TD-ADH is reproducibly detected in cell free extracts, n-MDH is not; n-MDH is stimulated by the presence of NH₄⁺-salts, TD-ADH is not, but requires the presence of salting out anions; PQQ has been detected in n-MDH, while this is absent in extracts containing TD-ADH. However, despite these differences, it cannot be excluded that both activities derive from some basic structure consisting of identical components, the dissimilarities being related to additional components.

Cell free extracts of ethanol-grown *A. methanolic* and the *Rhodococcus* strains have TD-ADH activity. Other ethanol-oxidizing activities were absent or had a very low specific activity (Hazeu et al. [8] reported a NAD-dependent ADH activity of 5 nmol NADH produced/min/mg protein for ethanol-grown *A. methanolic*). Therefore, growth of these organisms on ethanol seems to proceed via TD-ADH.

In growth experiments of *A. methanolic* on a mixture of methanol and ethanol, diauxic growth has been observed [8]. However, although the organism had a preference for ethanol to the point where all the ethanol had been consumed, 40% of the methanol had also disappeared. In view of the fact that TD-ADH is the sole activity detectable for ethanol in ethanol-grown cells and its capacity to convert methanol, TD-ADH could also be responsible for the disappearance of methanol. This view is supported by the observation that cell free extracts of ethanol-grown *Rhodococcus* strains oxidize methanol, although they are unable to grow on this substrate. After a lag phase, as soon as growth of *A. methanolic* on methanol started, PQQ was detected in the culture fluid [8]. Whether this is related to induction of a novel enzyme specific for methanol (for instance n-MDH), or to another enzyme in C₃-dissimilation or modification of TD-ADH (to a variant with n-MDH like properties), is presently unknown.

### Table 3

Affinity constants of *A. methanolic* for methanol and ethanol

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Methanol</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>4–6 mM</td>
<td>19–25 mM</td>
</tr>
<tr>
<td>Ethanol</td>
<td>33 μM</td>
<td>31 μM</td>
</tr>
</tbody>
</table>

As has been reported already [8], and was confirmed in this study (Table 3), methanol-grown *A. methanolic* cells are able to oxidize ethanol with a high affinity for this substrate. Reversibly, ethanol-grown cells were able to oxidize methanol (Table 3). However, it was consistently found that methanol-grown cells had a higher affinity for methanol (compared to ethanol) than ethanol-grown cells. This is not reflected in the activities of TD-ADH observed in the corresponding cell free extracts since the ratio for methanol and ethanol was nearly constant. An explanation could be that growth on methanol induces a specific enzyme for methanol conversion (n-MDH or a modified TD-ADH), undetectable with the TD-ADH assay, and due to its lability, unreproducibly detected with the n-MDH assay. Although in this interpretation the observed TD-ADH activity has no functional role at growth on methanol, its unusual properties and role in ethanol oxidation by Gram-positive bacteria justify further characterization.

### ACKNOWLEDGEMENTS

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Methanol-oxidizing, formaldehyde-reducing, and formaldehyde dismutase activities in Gram-positive bacteria oxidizing methanol.

Summary

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 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-oxidizing, formaldehyde-reducing and formaldehyde-dismutating enzymes (MFF's) were purified to homogeneity from methanol-grown cells of A. methanolica and M. gastri. Both enzyme preparations showed similar molecular weights, with 50 kD and 49 kD subunits, and native enzymes of 268 kD and 255 kD (gel-filtration data for A. methanolica and M. gastri, respectively). These oxidoreductases also displayed a similar substrate specificity. They were active with methanol and various other primary alcohols (yielding the corresponding aldehydes), and polyols. 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 catalyze 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 (Arfman et al., 1991; 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 (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 designated therefore 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 appear not to 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 case of n-MDH the complex may constitute an unusual methanol oxidoreductase with a bound NAD(P) cofactor, which transfers its reducing equivalents directly to a dedicated NADH dehydrogenase (Duine et al., 1984). The same methanol oxidoreductase 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 oxidoreductase 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; Kovar et al., 1984). It was also applied in the study of formaldehyde dismutase (EC 1.2.99.4), which is the natural example of an alcohol oxidoreductase with a bound NAD⁺, capable of catalyzing dismutation of formaldehyde to methanol and formate (Kato et al., 1986). Both types of enzyme were not active with free NAD⁺ but could be successfully assayed as an alcohol oxidoreductase by coupling the oxidation of alcohols to reduction of a specific dye, N,N'-dimethyl-4-nitrosoaniline (NDMA). Based on this assay system, we found NDMA-linked alcohol oxidizing activities in methanol-grown cells of the Gram-positive bacteria A. methanolicola, Mycobacterium gasti, but not in Corynebacterium sp. XG or Bacillus methanolicus C1.

Abbreviations used: MFF, methanol-oxidizing, formaldehyde-reducing and formaldehyde-dismutating enzyme; PQQ, pyrroloquinoline quinone; NDMA, N,N'-dimethyl-4- nitrosoaniline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; PMS, phenazine methosulfate; SDS, sodium dodecylsulfate; n-MDH, novel (DCPIP-, NAD⁺-linked) methanol dehydrogenase; TD-ADH, tetrazolium dye (MTT-) dependent alcohol dehydrogenase

Methods

Cultivation.

Amycolatopsis methanolicola NCIB 11946 was grown in chemostat culture (D = 0.0075 h⁻¹, 37°C, air supply 1 l/min) in a fermentor with a working volume of 1200 ml, using the 2-fold concentrated mineral medium with 2% (v/v) methanol described earlier (de Boer et al., 1990b). Mycobacterium gasti 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/l of yeast extract and 1 ml/l of stock trace elements solution (Vishniac & Santer, 1957) were added. Corynebacterium sp. XG
(Bastide et al., 1989) and *Bacillus methanolicus* 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* 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 18,000 g ($k_v 6.98$ 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/g of cells), then disintegrated by passage (3-5 times) through a French pressure cell, 4°C, $1.5 \times 10^5$ kN/m$^2$. The homogenate was centrifuged at 4°C, 25,000 g ($k_v 6.98$ cm), 30 min. Crude extract was used for enzyme assays and enzyme purification.

**Enzyme assays.**

Methanol:NDMA oxidoreductase activity of MFF 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 35.400 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 of ethanol was used instead of methanol. Formaldehyde dismutase activity was assayed in a cuvette, 5 ml working volume, at 30°C, pH 6.3, by following formic acid production using a pH-stat as described (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 the substrate per 1 min.

Enzyme purification.

Purification of the MPP's from both A. methanolicum and M. gasteri 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 pH 7.5. A gradient of KCl 0-1 M was applied at a flow rate of 0.5 ml/min within 1 h. Active fractions were collected, dialyzed 1 h against 1 M potassium phosphate, pH 7.5, then applied on a Phenyl-superose HR 5/5 column, equilibrated with the same buffer as used for dialysis. Finally, the enzyme was eluted in a gradient of potassium phosphate 1-0.05 M, pH 7.5, 30 min, at a flow rate of 0.5 ml/min. 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 $V_{\text{max}}$, $K_m$ 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 ($M_r$ 670,000) gamma globulin ($M_r$ 158,000), ovalbumin ($M_r$ 44,000) and myoglobin ($M_r$ 17,000).
Table 1. Methanol:NDMA and ethanol:NDMA oxidoreductase activities in crude extracts of Amycolatopsis methanolica and Mycobacterium gastri cells grown in batch flasks with different substrates (1% w/v final concentrations).

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>Specific activities (mU mg⁻¹) of extracts tested with methanol ethanol methanol ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>A. methanolica 3 5 9 12</td>
</tr>
<tr>
<td>Ethanol</td>
<td>M. gastri 0 0 0 0</td>
</tr>
<tr>
<td>Propanol</td>
<td>A. methanolica 1 6 16 37</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>M. gastri NG NG 12 12</td>
</tr>
<tr>
<td>Butanol</td>
<td>A. methanolica 2 1 0 0</td>
</tr>
<tr>
<td>Ethyleneglycol</td>
<td>M. gastri NG NG 0 0</td>
</tr>
<tr>
<td>Glycerol</td>
<td>A. methanolica 0 0 28 23</td>
</tr>
<tr>
<td>Glucose</td>
<td>M. gastri 0 0 0 0</td>
</tr>
</tbody>
</table>

NG, no growth.

Assay of methylformate. MFF enzyme samples were incubated with 40 mM of methanol and 40 mM of formaldehyde at 21°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 cutoff of 10 kDa; 1 μl of the filtrate was applied onto a HP-1 column (cross-linked methyl-silicone; 30 m x 0.53 mm; thickness layer 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. 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: $\alpha_2$-macroglobulin ($M_r$ 180,000), $\beta$-galactosidase ($M_r$ 116,000), fructose-6-phosphate kinase ($M_r$ 84,000), pyruvate kinase ($M_r$ 58,000), fumarase ($M_r$ 48,500), lactic acid dehydrogenase ($M_r$ 36,500), triosephosphate isomerase ($M_r$ 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 using BSA (Bio-Rad, USA) 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 alcohols 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 Cl. The alcohol:NDMA oxidoreductase-specific activities detected in Gram-positive bacteria 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 only detected 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). The results thus suggest that *A. methanolica* and *M. gastri* employ NAD-dependent alcohol dehydrogenase for ethanol oxidation, but not for methanol oxidation. We therefore decided to study the methanol:NDMA oxidoreductases in these organisms in more detail.

**PPLC 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.*, 1992). In cells of *M. gastri*, grown on methanol only one enzyme was found catalyzing both NDMA-linked ethanol- and methanol-oxidizing reactions. However, when the strain was grown on propanol, PPLC analysis indicated the presence of an additional protein active with ethanol, but not with methanol. This protein

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg ml(^{-1}))</th>
<th>Specific activity (mU mg(^{-1}))</th>
<th>Total activity (mU)</th>
<th>Yield (%)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Crude extract</td>
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<td>1</td>
</tr>
<tr>
<td>2. Q-Sepharose</td>
<td>0.3</td>
<td>61</td>
<td>158</td>
<td>35</td>
<td>9.1</td>
</tr>
<tr>
<td>3. Phenyl-Superose</td>
<td>0.2</td>
<td>73</td>
<td>68</td>
<td>15</td>
<td>10.9</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Crude extract</td>
<td>3.5</td>
<td>28</td>
<td>1943</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>2. Q-Sepharose</td>
<td>1.5</td>
<td>60</td>
<td>1479</td>
<td>76</td>
<td>2.1</td>
</tr>
<tr>
<td>3. Phenyl-Superose</td>
<td>2.8</td>
<td>96</td>
<td>1352</td>
<td>68</td>
<td>3.4</td>
</tr>
</tbody>
</table>
eluted at the same position as the ethanol:NDMA oxidizing enzyme from *A. methanolicum*. 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. methanolicum* and *M. gastri* (Table 2). This is in fact the first case that an enzyme of *A. methanolicum* with methanol-oxidizing activity was purified to homogeneity, 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 activity was observed with pyridine dinucleotides and with common artificial electron acceptors such as DCPIP and tetrazolium salts. The *in vivo* electron acceptor for these enzymes remains to be identified.

**Relative molecular mass and quaternary structure of MFF**

The relative molecular masses of the purified native MFF enzymes from *A. methanolicum* and *M. gastri*, as studied by gel-filtration chromatography, were estimated as *M*<sub>r</sub> 268,000 and *M*<sub>r</sub> 255,000, respectively. SDS gel electrophoresis revealed the presence of a single subunit species in both enzyme preparations, with estimated subunit relative molecular masses of *M*<sub>r</sub> 50,000 and *M*<sub>r</sub> 49,000 for the enzymes from *A. methanolicum* and *M. gastri*, respectively. Both the native enzymes thus might contain 4-5 identical subunits. However, electron microscopic studies revealed that both enzymes have decameric structures (Bystrykh *et al.*, 1993), similar to the NAD<sup>+</sup>-dependent methanol dehydrogenase in *B. methanolicus* 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 MFF enzymes are able to catalyze 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
Table 3. pH optima for activity and stability of MFF's from *A. methanolina* and *M. gastri*. pH areas with 80% of maximal activity are shown.

<table>
<thead>
<tr>
<th>Type of activity</th>
<th>Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>A. methanolina</em></td>
</tr>
<tr>
<td>pH activity</td>
<td></td>
</tr>
<tr>
<td>dismutase</td>
<td>6.2-6.5</td>
</tr>
<tr>
<td>dehydrogenase</td>
<td>6.0-6.7</td>
</tr>
<tr>
<td>reductase</td>
<td>4.5-5.0</td>
</tr>
<tr>
<td>pH stability</td>
<td>6.5-8.0</td>
</tr>
</tbody>
</table>

Dismutase, dehydrogenase and reductase activities refer to formaldehyde dismutase, methanol:NDMA oxidoreductase and NADH-dependent formaldehyde reductase activities, respectively.

coupled to oxidation of NADH. The ability of MFF to catalyze 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 MFF from both *A. methanolina* 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 hydrolyzed 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 are comparable, namely pH 6.3. The formaldehyde reductase reaction displayed a lower pH optimum, namely pH 4.5-5.0 (Table 3).

MFF enzyme activity increased with temperature up to 55°C.
Figure 1. The effect of MFF protein (from _M. gastri_) concentrations on methanol:NDMA oxidoreductase (A) and formaldehyde reductase (B) activities. Squares show specific activities, circles correspond to total activities.

Table 4. Substrate specificities of the MFF's from _Amycolatopsis methanolica_ and _Mycobacterium gastri_.

<table>
<thead>
<tr>
<th>Substrate</th>
<th><em>Amycolatopsis methanolica</em></th>
<th><em>Mycobacterium gastri</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( V_{\text{max}} ) \text{mU mg}^{-1}</td>
<td>( K_m ) mM</td>
</tr>
<tr>
<td>NDMA (+ methanol)</td>
<td>65</td>
<td>0.018</td>
</tr>
<tr>
<td>Methanol</td>
<td>57</td>
<td>2.65</td>
</tr>
<tr>
<td>Ethanol</td>
<td>90</td>
<td>0.057</td>
</tr>
<tr>
<td>Propanol</td>
<td>77</td>
<td>0.002</td>
</tr>
<tr>
<td>Butanol</td>
<td>76</td>
<td>0.002</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>73</td>
<td>0.110</td>
</tr>
<tr>
<td>Isobutanol</td>
<td>73</td>
<td>0.001</td>
</tr>
<tr>
<td>2-methoxyethanol</td>
<td>63</td>
<td>0.010</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>51</td>
<td>0.007</td>
</tr>
<tr>
<td>Ethyleneglycol</td>
<td>66</td>
<td>0.047</td>
</tr>
<tr>
<td>Glycerol</td>
<td>72</td>
<td>0.970</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td>40</td>
<td>17.0</td>
</tr>
</tbody>
</table>

The limit of the \( K_m \) measurement was 0.001 mM. \( \infty \), no inhibition.
Above this temperature, the enzyme inactivated within a few minutes. Both enzymes are completely stable during incubation for 1 h at 30°C and pH 6.3.

As shown for the methanol:NDMA oxidoreductase and formaldehyde reductase reactions of purified MFF from *M. gastri*, plots of these MFF enzyme activities versus protein concentration are not linear (Fig. 1). Fixed protein concentrations (about 20 μg of protein per ml assay mixture) therefore were 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 methanol:NDMA oxidoreductase activity of MFF from *A. methanolina*. Pyrazole (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 pyrazole did not inhibit.

Figure 2. Transformed s(v) versus s plots of alcohol:NDMA oxidoreductase activities of MFF, (A) from *M. gastri* with non-inhibitory substrates: methanol (▲), glycerol (●) and ethylene-glycol (■); (B) from *A. methanolina* with inhibitory substrates: ethanol (▲), isopropanol (■) and formaldehyde (●).
Kinetics of NDMA-linked oxidation of alcohols

The MFF enzymes from both A. methanolicus 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 found to be very broad (Table 4). Methanol, glycerol and ethyleneglycol did not inhibit the enzymes (Fig. 2A), whereas most of the other substrates did (Fig. 2B). The kinetics of NDMA-linked oxidation of methanol was found to obey ping-pong mechanism (Fig. 3). Comparison of the apparent kinetic parameters for primary alcohols with the true parameters revealed insignificant differences, suggesting that the apparent kinetic parameters in Table 4 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 C₄ (Table 5). 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.
Table 5. Aldehyde reductase and formaldehyde dismutase reactions catalyzed by the MFF's from *A. methanolica* and *M. gastri*

<table>
<thead>
<tr>
<th>Substrate</th>
<th><em>A. methanolica</em></th>
<th><em>M. gastri</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( V_{\text{max}}' )</td>
<td>( K_m' )</td>
</tr>
<tr>
<td></td>
<td>mU mg(^{-1})</td>
<td>mM</td>
</tr>
<tr>
<td>NADH (with CH(_2)O)</td>
<td>100</td>
<td>0.6</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>14</td>
<td>0.01</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>15</td>
<td>0.7</td>
</tr>
<tr>
<td>Propionaldehyde</td>
<td>72</td>
<td>246</td>
</tr>
<tr>
<td>Butyraldehyde</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Formaldehyde dismutase reaction

| Formaldehyde               | 85000           | 19.5        | 132000            | 12.4        |

Figure 4. Transformed s(v) versus s plots of aldehyde reductase activities of MFF from *M. gastri* with acetaldehyde (•), propionaldehyde (○) and butyraldehyde (■). Curves represent kinetic parameters shown in Table 5.

Finally, the enzyme gradually deviated from Michaelis-Menten function with increasing carbon chain length of the substrate (Fig. 4).
Formaldehyde dismutase reaction.

The formaldehyde dismutase reaction catalyzed by MFF from *A. methanolicum* and *M. gastri* showed Michaelis-Menten kinetics with respect to formaldehyde. No activity was found with acetaldehyde, propionaldehyde or butyraldehyde. The $K_m$ 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 $V_{\text{max}}$ 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 $V_{\text{max}}$ and $K_m$ values for formaldehyde in these reactions must be a reflection of different limiting steps in the reaction cycles.

Discussion

The results presented show that several Gram-positive bacteria able to oxidize methanol (*A. methanolicum*, *M. gastri*) contain a highly active formaldehyde dismutase. The enzymes studied also display alcohol:NDMA oxidoreductase activity with a very broad substrate specificity towards primary alcohols and aldehydes, including $C_1$ substrates. Both these MFF enzymes possess a firmly bound pyridine dinucleotide cofactor which is catalytically active (Bystrykh et al., 1993). The kinetics of this type of enzyme is generally of the ping-pong type (Kovar et al., 1984; Kato et al., 1986; Frey, 1987), as was shown to be the case for MNO (Fig. 3).

From the various reactions catalyzed by MFF the highest activity by far was observed with the formaldehyde dismutase assay (compare Tables 4 and 5). This may reflect the fact that neither NDMA nor NADH are physiological substrates for the MFF 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 transhydrogenase 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 MFF enzymes and formaldehyde dismutase from P. putida share the ability to catalyze 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-methylo trophic 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. methanolicus and M. gastri are methylo trophic bacteria and still sensitive to formaldehyde. A. methanolicus 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. methanolicus we observed formaldehyde accumulation in the culture and a decrease in MFF activity (data not shown). It thus appears doubtful that MFF fulfils the same in vivo function as formaldehyde dismutase from P. putida. A further difference is that the P. putida formaldehyde dismutase does not display methanol:NDMA oxidoreductase activity, unlike the MFF enzymes from A. methanolicus and M. gastri. The prosthetic groups of these enzymes are also different, with NADPH in MFF (Bystrykh et al., 1993) and NADH in formaldehyde dismutase (Kato et al., 1986). Finally, amino acid sequencing data and computer image analysis revealed rather strong similarities between the MFF enzymes and methanol dehydrogenase from B. methanolicus C1 (Vonck et al., 1991; Bystrykh et al., 1993). All three these 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 MFF enzymes have a similar function in the Gram-positive bacteria Amycolatopsis methanolicus and Mycobacterium gastri. Further work is certainly required to
elucidate the in vivo role of the MFF enzymes. The enzyme in *A.
methanolicum* may be an essential component of a multienzyme
methanol dehydrogenase complex, resulting in the n-MDH or TD-MDH
activities reported earlier (Duine *et al.*, 1984; van Ophem *et

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Chapter 5

Nicotinoprotein (NAD(P)-containing) alcohol/aldehyde oxidoreductases. Purification and characterization of a novel type from *Amycolatopsis methanolica*.

**SUMMARY**

Extracts of Gram-positive bacteria like *Rhodococcus rhodochrous*, *Rhodococcus erythropolis* and *Amycolatopsis methanolica*, but not those of several Gram-negative ones, showed dehydrogenase activity for ethanol as well as for methanol when p-nitroso-N,N-dimethylaniline (NDMA) was used as electron acceptor. Chromatography of extracts of the first two organisms revealed one activity for both substrates, that of *A. methanolica* two activities, one of which is able to oxidize methanol and has been purified [Bystrykh, L.V., Govorukhina, N.I., van Ophem, P.W., Hektor, H.J., Dijkhuizen, L., and Duine, J.A., unpublished results]. The other one, indicated as NDMA-dependent alcohol dehydrogenase (NDMA-ADH), was purified to homogeneity. It is a trimeric enzyme consisting of subunits with a relative molecular mass of 39 kDa and one firmly bound NAD as cofactor. Although NDMA-ADH shows structural similarity with the long-chain, zinc-containing, NAD(P)-dependent alcohol dehydrogenases (EC 1.1.1.1) with respect to the N-terminal sequence up to residue 41 (56% identical positions with horse liver alcohol dehydrogenase), the enzymes are catalytically different since NDMA-ADH is unable to use NAD(P)(H) as a coenzyme and NAD(P)-dependent alcohol dehydrogenases are inactive with NDMA (in the absence of NAD). Comparison of the NDMA-ADH properties with those of the methanol-oxidizing enzyme of *A. methanolica*, *Mycobacterium gastri*, and *Bacillus methanolicus* Cl, and formaldehyde dismutase (EC 1.2.99.4) of *Pseudomonas putida* F61 revealed large differences in structural as well as catalytic properties, in spite of the fact that all are "nicotinoproteins" (enzymes which have bound NAD(P) as a cofactor). It is concluded, therefore, that NDMA-ADH is a novel type of nicotinoprotein alcohol dehydrogenase.
INTRODUCTION

In the search for a methanol-oxidizing activity in *Amycolatopsis methanolica*, an assay was applied able to detect alcohol dehydrogenases having NAD(P) as cofactor, using p-nitroso-N,N-dimethylaniline (NDMA) as electron acceptor [1]. However, upon applying anion-exchange chromatography on extracts exhibiting such a property, two separated activities were found in the eluted fractions, one showing activity with alcohols including methanol, the other except methanol. The enzyme responsible for the first activity (the so-called methanol-oxidizing, formaldehyde-reducing, and formaldehyde dismutating enzyme (MFF)) has been purified and characterized [1, 2]. A similar enzyme appeared to occur in another Gram-positive methanol utilizer, *Mycobacterium gastri* MB19 [1].

The present paper describes the purification and characterization of the enzyme responsible for the other activity, indicated here as NDMA-dependent alcohol dehydrogenase (NDMA-ADH). To reveal the physiological significance of NDMA-ADH, induction experiments with *A. methanolica* and subsequent chromatography of the extracts was carried out. Since NDMA-ADH appeared to contain bound NAD as cofactor, amino acid sequencing of the N-terminal part was performed in order to determine its relatedness with other alcohol/aldehyde oxidoreductases containing NAD(P) as bound cofactor (the so-called "nicotinoproteins") and the common NAD(P)-dependent alcohol dehydrogenases. In addition, a search was made for the presence of NDMA-dependent alcohol dehydrogenases in a number of Gram-positive and -negative bacteria.

Abbreviations: C12Ind: 2,6-dichlorophenol-indophenol; FA1DM: formaldehyde dismutase; FD-FA1DH: factor-dependent formaldehyde dehydrogenase; HL-ADH: horse liver alcohol dehydrogenase; K/P1: potassium phosphate buffer; MeDH: methanol dehydrogenase; MFF: methanol-oxidizing, formaldehyde-reducing, and formaldehyde dismutating enzyme; NDMA: p-nitroso-N,N-dimethylaniline; NDMA-ADH: NDMA-dependent alcohol dehydrogenase; PQQ: pyrroloquinoline quinone.
Cultivation of the organisms.

Amycolatopsis methanolica (NCIB 11946; LMD 80.32) was grown on a mineral salt medium [3], both batch-wise and fed-batch-wise with methanol as a carbon and energy source, as described [4]. The organism was also grown (batch-wise) on the following substrates: 1 % (v/v) ethanol; 0.2 % (v/v) 1-butanol; 0.1 % 1-hexanol; 0.5 % (w/v) sodium acetate; 0.5 % (w/v) glucose. The other organisms were grown (batch-wise) according to the published procedures: Rhodococcus rhodochrous (LMD 89.129) and Rhodococcus erythropolis (DSM 1069) on 0.5 % (v/v) ethanol and the latter organism also on 2 mM 3,4-dimethoxybenzoic acid [5]; Mycobacterium gastri MB19 on 1 % (v/v) methanol [6]; Hyphomicrobiurn X on 0.4 % (v/v) methanol [7]; Comamonas testosteroni on 1 % (v/v) ethanol [8]; Pseudomonas aeruginosa (ATCC 17933) on 1 % (v/v) ethanol [9]; Pseudomonas BB1 on 0.5 % (v/v) ethanol [10]; Acetobacter pasteurianus (LMD 88.49) on 0.5 % (v/v) ethanol [11]; Xanthobacter autotrophicus (NCIB 11171) on 1 % (w/v) ethyleneglycol [12].

Enzyme assays.

p-Nitroso-N,N-dimethylaniline-dependent alcohol dehydrogenase (NDMA-ADH) activity was assayed by measuring the rate of decrease in absorbance at 440 nm of NDMA (35 μM final concentration) in 10 mM potassium phosphate buffer (K/Pi), pH 7.0. The reaction was started by adding ethanol (1 mM final concentration). Other substrates were used at concentrations as indicated in the text. Activities were calculated by using a molar absorption coefficient for NDMA at 440 nm of 35,400 M⁻¹cm⁻¹ [13]. To assay sugars as possible (physiological) electron acceptors instead of the (artificial) NDMA, a coupled assay was performed in which the putative acetaldehyde formed from ethanol was assayed with aldehyde dehydrogenase (formate ester dehydrogenase) [14] in the following way: a solution containing 100 μM Wurster’s Blue, 1 or 10 mM of the tested sugar and 20 μg aldehyde dehydrogenase [14] in 50 mM K/Pi, pH 7.0, was mixed with NDMA-ADH (20 μg). The mixture was incubated for 2 min and after recording the endogenous rate of dye reduction at 600 nm, ethanol was added to a final concentration of 1 mM. The following enzymes were assayed as described: the methanol-oxidizing
activity of the methanol-oxidizing, formaldehyde-reducing, and formaldehyde
dismutating enzyme (MFF) [1] (50 mM methanol was used as a substrate);
formaldehyde reductase [1]; formaldehyde dismutase (FAldM) [15] (10 mM KOH
was used as a titrant); factor-dependent formaldehyde dehydrogenase (FD-
FAldH) (at room temperature) [16]; formate ester dehydrogenase [14]. The
assay for NADH dehydrogenase was carried out with NADH-specific NADH
dehydrogenase from A. methanolicum. The assay was carried out at room
temperature, using 0.1 M KPB, pH 7.0, with 40 μM 2,6-dichlorophenol-
indophenol (Cl₂Ind) as electron acceptor. After recording the endogenous
rate of dye reduction at 600 nm, NADH was added to a final concentration of
0.25 mM. All assays were performed at 37 °C, except where indicated
otherwise.

Chromatography of NDMA-dependent alcohol dehydrogenases.

Cells were disrupted as described [17], yielding the cell free extract.
These extracts were applied to a Mono-Q 5/5 column (Pharmacia) equilibrated
with 20 mM K/P₄₅, pH 7.2. Elution occurred with a gradient from 0.1 to 0.7 M
KCl in 20 mM K/P₄₅, pH 7.2, in 15 min at a flow rate of 1 ml/min. The 1 ml
fractions were screened for NDMA-ADH plus MFF (with ethanol), MFF (with
methanol) and FAldM activity.

Purification of NDMA-ADH.

Harvested cells (62 g wet weight) of methanol-grown A. methanolicum were
washed twice with 20 mM K/P₄₅, pH 7.2, and disrupted with a French pressure
cell as described [17]. Fractionated precipitation with (NH₄)₂SO₄ was
applied to the extract. NDMA-ADH activity precipitated between 25 and 55 %
saturation and was collected by centrifugation (20 min at 48,000 x g). The
pellet was dissolved in a minimal volume of 10 mM K/P₄₅, pH 7.0, and applied
to a Phenyl-Sepharose Hi-Load column (12 x 2.2 cm; Pharmacia), equilibrated
with 1.5 M (NH₄)₂SO₄ in 10 mM K/P₄₅, pH 7.0. After washing the column with
the same buffer, elution occurred with a linear gradient from 1.5 M to 0 M
(NH₄)₂SO₄ in 10 mM K/P₄₅, pH 7.0, in 3 hours at a flow rate of 3 ml/min.
NDMA-ADH activity eluted in the final part of the gradient. After pooling
and concentrating the active fractions by pressure filtration, using a membrane with a cutoff of 10 kDa, the enzyme solution was applied to a Mono-Q 10/10 column, equilibrated with 20 mM K/Pi, pH 7.2. After washing the column with the same buffer, elution occurred with a gradient from 0.15 M to 0.45 M KCl in 20 mM K/Pi, pH 7.2, in 30 min at a flow rate of 2 ml/min. NDMA-ADH eluted at 0.30 M KCl. After pooling and concentrating, as described above, buffer exchange with 10 mM K/Pi, pH 7.0, occurred on a PD-10 column (Pharmacia). The enzyme was further purified using adsorption chromatography on a hydroxylapatite HT (BioRad) column (11 x 1 cm). After equilibrating the column with 10 mM K/Pi, pH 7.0, the preparation was applied, the column washed with the same buffer and tyra enzyme eluted with a gradient from 10 to 100 mM K/Pi, pH 7.0, in 30 min at a flow rate of 1 ml/min. NDMA-ADH activity eluted at 70 mM K/Pi. After pooling and concentrating, further purification was obtained by adsorption chromatography on a Superose-12 gel filtration column (Pharmacia) (normally used as a gel filtration column) equilibrated with 1.0 M K/Pi, pH 7.0. The pooled and concentrated active fractions were applied to the column and elution occurred with a gradient from 1 to 0.1 M K/Pi, pH 7.0, in 1 h at a flow rate of 0.5 ml/min. NDMA-ADH eluted at 0.9 M K/Pi.

Analytical methods.

Protein determinations were performed according to Bradford [18] with bovine serum albumin as a standard. Methylformate was determined using gas chromatography on a HP-1 column (Crosslinked methyl-silicone; 30 m x 0.53 mm; thickness layer: 2.65 μm; Hewlett-Packard) as described [1]. Detection of pyrroloquinoline quinone (PQQ) was performed with a biological assay [19]. Identification and quantification of the nicotinamide cofactor was carried out by denaturing the enzyme and purifying the compound by chromatography, as follows. Buffer exchange of an NDMA-ADH preparation (11 nmol) occurred on a PD-10 column equilibrated with 0.1 M Tris/HCl, pH 8.5, containing 6 M urea. After concentrating the eluted enzyme by pressure filtration, the cofactor was detached by heating the sample for 2 min at 100
Table 1: Induction of NDMA-dependent dehydrogenase activity for ethanol and methanol in *A. methanolicum*.

Extracts of *A. methanolicum*, grown on different carbon sources, were tested with the NDMA-ADH assay using 1 mM ethanol or 50 mM methanol as substrates. Specific activities are given in nmol NDMA converted/min/mg protein.

<table>
<thead>
<tr>
<th>Substrate in assay</th>
<th>Cells grown on 1 mM ethanol</th>
<th>Cells grown on 50 mM methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol (fed-batch)</td>
<td>18</td>
<td>5</td>
</tr>
<tr>
<td>Methanol</td>
<td>17</td>
<td>5</td>
</tr>
<tr>
<td>Ethanol</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>1-Butanol</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>1-Hexanol</td>
<td>17</td>
<td>2</td>
</tr>
<tr>
<td>Acetate</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>Glucose</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

°C. The sample was centrifuged for 3 min at 16,000 x g and the supernatant was applied to a Mono-Q 5/5 column equilibrated with 10 mM Tris/HCl, pH 8.5, containing 6 M urea. Elution occurred with a gradient from 0 to 1 M KCl in the same buffer in 15 min at a flow rate of 0.5 ml/min. Monitoring of the elutate was carried out at 254 nm. The elution time of the peak observed was compared with that of authentic samples (10 nmols of NAD, NADH, NADP, and NADPH), treated in the same way. The observed peak was collected and tested with a dehydrogenase assay specific for NADH (see paragraph "Enzyme assays"). Electron microscopy of the enzyme was performed as described in [20]. N-terminal amino acid sequence determination was carried out on a 477A pulsed liquid sequenator (Applied Biosystems, Foster City, USA) starting from 1.2 nanomoles of protein.
Polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis was performed on commercially available gradient gels (8 - 25 %) using the Pharmacia Phastsystem equipment according to the instructions given by the manufacturer. The procedure followed to determine the molecular mass of the native enzyme was according to Andrews [21]. To denature the enzyme, a solution of it was heated for 5 min at 100 °C in the presence of 5 % (w/v) sodium dodecylsulfate and 25 % (v/v) 2-mercaptoethanol. Protein staining occurred with Phastgel Blue and activity staining for native enzyme was performed using a mixture as described for the NDMA-ADH assay, inspecting the gels for the disappearance of the yellow colour. Standards from the High Molecular Weight kit (Pharmacia) were used for the comparison with native protein and the Low Molecular Weight kit (Pharmacia) for that with the denatured protein.

![Graph](image)

**Figure 1**: Chromatographic separation of NDMA-ADH and MFF of extracts from *A. methanolic* grown on methanol.

Fractions were screened for NDMA-dependent activity with ethanol (o) as well as with methanol (+). Peak I corresponds with NDMA-ADH, peak II with MFF.

RESULTS.

Detection, induction and assay of NDMA-ADH.

Cell free extracts of methanol-, other primary alcohol-, and acetate-grown, but not glucose-grown, *A. methanolic* showed NDMA-dependent dehydrogenase activity for ethanol and methanol (Table 1). Chromatography of the extracts on a Mono-Q column using a KCl gradient yielded two separated activities with ethanol, one eluting at 0.3 M KCl and indicated as Activity I, the other eluting at 0.7 M KCl and indicated as activity II (Figure 1).
Table 2: NDMA-ADH and MFF content of *A. methanolica* extracts.

Extracts of cells grown on different substrates were chromatographed on Mono-C-Q and the amount of NDMA-dependent activity with ethanol as substrate were calculated by adding up the activities of the fractions in which the enzymes eluted (fractions in peak I for NDMA-ADH, those in peak II for MFF). Recoveries were mostly within 90%. Activities are given in nmol NDMA reduced/min.

<table>
<thead>
<tr>
<th>Extract prepared from cells grown on:</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NDMA-ADH</td>
</tr>
<tr>
<td>Methanol</td>
<td>226</td>
</tr>
<tr>
<td>Ethanol</td>
<td>6</td>
</tr>
<tr>
<td>1-Butanol</td>
<td>79</td>
</tr>
<tr>
<td>1-Hexanol</td>
<td>200</td>
</tr>
<tr>
<td>Acetate</td>
<td>112</td>
</tr>
</tbody>
</table>

Activity II originates from the already described MFF [1], in view of its chromatographic and catalytic behaviour (active with methanol and showing formaldehyde dismutase activity). Activity I appeared to be clearly distinct as it did not convert methanol and did not dismutate formaldehyde. It originates from the here described NDMA-dependent alcohol dehydrogenase (NDMA-ADH).

As appears from Table 1, the ratio of the activity for ethanol and methanol varies with the growth substrate used. Chromatography of the extracts and quantification of the activities revealed that this can be ascribed to variation in the MFF/NDMA-ADH ratio, the content of MFF remaining nearly constant, that of NDMA-ADH varying significantly (Table 2).

On optimizing the assay for NDMA-ADH, maximal activities were found at 45 °C and at pH 7.0, as measured in K/Pi-buffers. No activity was observed when using the following electron acceptors: 1 mM concentrations of NAD, NADP or of the NAD-analogs 3-acetylpyridine adenine dinucleotide or thionicotinamide adenine dinucleotide (neither at pH 7.0, 8.0 nor 9.0, the latter buffered with 0.1 M sodiumpyrophosphate); phenazine methosulfate (0.1 mM); Cl₂Ind (40 μM); Wurster’s Blue (100 μM); (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-
Table 3: Purification of NDMA-ADH.

1 U = 1 μmol NDMA converted/min.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Recovery (%)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free extract</td>
<td>2370</td>
<td>40.3</td>
<td>0.019</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Precipitation</td>
<td>1500</td>
<td>22.5</td>
<td>0.017</td>
<td>56</td>
<td>0.89</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>396</td>
<td>15.0</td>
<td>0.038</td>
<td>37</td>
<td>2.0</td>
</tr>
<tr>
<td>Mono-Q</td>
<td>9.5</td>
<td>6.5</td>
<td>0.68</td>
<td>16</td>
<td>36</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>2.8</td>
<td>3.8</td>
<td>1.4</td>
<td>9.4</td>
<td>74</td>
</tr>
<tr>
<td>Superose adsorption</td>
<td>0.78</td>
<td>2.4</td>
<td>3.1</td>
<td>6.0</td>
<td>163</td>
</tr>
</tbody>
</table>

Figure 2: Absorption spectrum of NDMA-ADH.

The absorption spectrum of the purified enzyme (0.19 mg/ml) was measured in 0.1 M K/P₁, pH 7.0.

tetrazolium bromide (2 mM); cytochrome c (50 μM). Increasing the phosphate concentration in the assay to 1 M or the amount of enzyme added (both conditions increase the specific activity of tetrazolium dye-dependent alcohol dehydrogenase activity present in the Gram-positive bacteria investigated here [22]) did not increase the specific activity of NDMA-ADH. Also the incorporation of 0.1 M NH₄Cl in the assay (activating PQQ-containing, dye-linked alcohol dehydrogenases [23]), did not augment NDMA-ADH activity.
Purification and characterization of NDMA-ADH.

Applying the purification steps as described in the Methods section, NDMA-ADH was purified approx. 160-fold at a rather low yield (Table 3). The loss in activity can be partially explained from the removal of MFF (contributing to NDMA-ADH activity) in the steps applied before the hydroxyapatite step. Gelfiltration of NDMA-ADH on Superose-12 using 0.1 M K/Pi, pH 7.0, as buffer (this concentration of buffer preventing adsorption to the column material) indicated a relative molecular mass of approx. 120 kDa. Polyacrylamide gel electrophoresis of the native enzyme (0.4 μg; the detection limit using the Coomassie Brilliant Blue staining method is 20 - 30 ng protein per protein band) showed one major protein band at a M_r of 110.000 and a minor one with a M_r of 300.000. However, when activity staining was performed only the 110 kDa protein showed activity, confirming the molecular mass found using gel filtration. Since gel filtration did not lead to removal, the appearance of the 300 kDa band is probably due to denatured, associated NDMA-ADH caused by electrophoresis. Electrophoresis in the presence of sodium dodecylsulfate revealed one band with a M_r of 39.000. Electron microscopy of the enzyme did not show the presence of decamers, as has been observed with MFF [2] and methanol dehydrogenase (MeDH) from Bacillus methanolicus Cl [20].

The absorption spectrum of the purified enzyme showed a peak at 280 nm and a shoulder in the 320 - 340 nm region (Figure 2), thereby resembling the absorption spectra of MFF [2] and of MeDH [N. Arfman, unpublished results], but not that of (rabbit: muscle) glyceraldehyde-3-phosphate dehydrogenase (which also contains tightly bound NAD) which has an absorption maximum at 360 nm [24]). Samples of enzyme denaturated by heating were inactive in the PQQ assay and also flavins were absent, as judged from the absorption spectrum. However, the detached chromophore appeared to be NADH, as it eluted from the Mono-Q column at the same KCl concentration as authentic NADH, the absorption spectrum showed maxima at 264 and 340 nm and the collected peak appeared to be active with the NADH-specific NADH dehydrogenase. From the surface area of the eluted peak in the chromatogram and the calibration curve with NADH reference samples, for a typical preparation it was calculated that 11 nmol of NDMA-ADH yielded 12 nmol of NADH. Since apo-enzyme seemed to be absent (no increase in activity on addition of nucleotides (see below) to the assay), this indicates that NDMA-ADH contains one mol of NADH per enzyme molecule.

Determination of the N-terminal amino acids (54) revealed the sequence as indicated in Scheme 1. Alignment with MFF [2] showed 18 %, with MeDH from Bacillus methanolicus Cl 7 % [20], with FD-PgLDH 23 % [16], and with horse liver alcohol dehydrogenase (HL-ADH) 56 % identical positions [25] (Scheme 1).
Scheme 1: N-terminal amino acid sequences of alcohol dehydrogenases.

Data for FD-FALDH, NDMA-ADH and MFF, all from A. methanolicus, are from [16], from this work and from [2], respectively, those for methanol dehydrogenase (MeDH) from Bacillus methanolicus CI are from [20] and those for horse liver alcohol dehydrogenase (HL-ADH) are from [25].

Substrate specificity.

NDMA-ADH is not only able to use ethanol as a substrate, but other primary alcohols and benzylalcohol as well (Table 4). With secondary alcohols and formaldehyde, but not with 2-methyl-2-propanol, low activities were observed (Table 4). Acetaldehyde gave still lower activity. Since product identification was not carried out in these cases, it is not known whether they are genuine substrates. Substrate inhibition occurred for all substrates indicated in Table 4, the extent expressed by the $K'_m$ values. From the ratio $V'_{max}/K'_m$ it is clear that NDMA-ADH prefers 1-propanol, 1-butanol and benzylalcohol as substrates (the $K'_m$ value for benzylalcohol must be below 5 $\mu$M since at this concentration the activity was still maximal).

For 1 mM concentrations of ethanol, it appeared that the $K'_m$ for NDMA was 83 $\mu$M while the $V'_{max}$ was 19.2 $\mu$mol/min/mg protein. No substrate inhibition was observed in the concentration range of NDMA tested (15 - 55 $\mu$M).

Since NDMA contains an aldehyde-like moiety, it might be reasoned that a sugar could be the physiological electron acceptor for NDMA-ADH. However, of all the common sugars tested, none was able to induce acetaldehyde formation from ethanol (the coupled assay used was able to detect acetaldehyde in the $\mu$M range).

It has already been found that MFF shows NADH-dependent formaldehyde
Table 4: Substrate specificity of NDMA-ADH.

Data could be reasonably fitted by an extended version of the Michaelis-Menten equation in which substrate inhibition is assumed. Apparent kinetic parameters were calculated with this equation. The range of substrate concentrations used is given in brackets. 1 U = 1 μmol NDMA converted/min.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$</th>
<th>$K_i$</th>
<th>$V_{max}$</th>
<th>$V_{max}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>tested</td>
<td>(mM)</td>
<td>(mM)</td>
<td>(U/mg protein)</td>
<td>(U/mg protein)</td>
</tr>
<tr>
<td>Ethanol (50 μM - 0.1 M)</td>
<td>0.082</td>
<td>33</td>
<td>5.90</td>
<td>72</td>
</tr>
<tr>
<td>1-Propanol (5 μM - 10 mM)</td>
<td>0.0039</td>
<td>4.0</td>
<td>6.09</td>
<td>1560</td>
</tr>
<tr>
<td>1-Butanol (5 μM - 1 mM)</td>
<td>0.0025</td>
<td>0.65</td>
<td>6.09</td>
<td>2440</td>
</tr>
<tr>
<td>2-Propanol (1 - 50 mM)</td>
<td>13.4</td>
<td>14.5</td>
<td>5.94</td>
<td>0.44</td>
</tr>
<tr>
<td>2-Butanol (1 - 50 mM)</td>
<td>10.1</td>
<td>4.4</td>
<td>5.70</td>
<td>0.56</td>
</tr>
<tr>
<td>Benzylalcohol (5 μM - 2 mM)</td>
<td>&lt;0.005</td>
<td>0.35</td>
<td>5.7</td>
<td>&gt;1140</td>
</tr>
<tr>
<td>Formaldehyde (5 - 200 μM)</td>
<td>6.0</td>
<td>29</td>
<td>4.96</td>
<td>0.83</td>
</tr>
</tbody>
</table>

Figure 3: Lineweaver - Burk plot of NDMA-ADH and pyrazole as inhibitor.

NDMA-ADH activity was measured with the following pyrazole concentrations: 0 μM (a); 5 μM (b); 10 μM (c); 20 μM (d).
reductase activity, formaldehyde dismutase activity, and probably related with the latter property, methylformate production when incubated with formaldehyde plus methanol [1]. When these assays were applied to the fractions obtained from the Mono-Q column (Figure 1), those containing MFF showed a positive response for all these activities, those containing NDMA-ADH did not. To probe NDMA-ADH further, pure enzyme was investigated for dismutase activity with acetaldehyde, propionaldehyde and benzaldehyde and for NAD(P)H-dependent reductase activity with the same substrates (0.1, 0.2 and 1.0 mM concentrations were tested at pH 5.0, 6.0 and 7.0). However, all the results were negative.

**Inhibitors.**

Studies with pyrazole, a competitive inhibitor of ethanol for NAD-

Table 5: Inhibitors of NDMA-ADH.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (mM)</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>NiCl₂</td>
<td>1</td>
<td>85</td>
</tr>
<tr>
<td>ZnSO₄</td>
<td>1</td>
<td>60</td>
</tr>
<tr>
<td>KCN</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>NaN₃</td>
<td>2</td>
<td>75</td>
</tr>
<tr>
<td>NaN₃</td>
<td>10</td>
<td>55</td>
</tr>
<tr>
<td>AMP, ADP, or ATP</td>
<td>1</td>
<td>90</td>
</tr>
<tr>
<td>AMP, ADP, or ATP</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>1</td>
<td>60</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>2</td>
<td>40</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>10</td>
<td>5</td>
</tr>
</tbody>
</table>
dependent alcohol dehydrogenase [26], showed the same behaviour with NDMA-ADH (Figure 2) and having a $K_i$ of 1.2 $\mu$M. Although acetaldehyde is a (very poor) substrate, it also acts as an inhibitor (Table 5). Inhibition occurred in a time-dependent manner as observed in the following experiments: when ethanol and acetaldehyde (both 2 mM final concentrations) were added simultaneously to the assay mixture 15% inhibition was observed; when acetaldehyde was added (2 min incubation) and subsequently ethanol, 67% inhibition was found. Other possible inhibitors tested were: 1 mM concentrations of NAD, NADP, NADH or NADPH; 1 mM and 10 mM concentrations of AMP, ADP or ATP; 1 mM concentrations of Ca$^{2+}$, Co$^{2+}$, Cu$^{2+}$, Fe$^{2+}$, Fe$^{3+}$, Mg$^{2+}$, Mn$^{2+}$, Mo$^{6+}$, Ni$^{2+}$, Zn$^{2+}$ or 0.1 mM Hg$^{2+}$ salts; 1 mM EDTA; 1 mM $N_3$; 2 mM KCN.

From Table 5 it is clear that metal ions reacting with SH-groups (Cu$^{2+}$, Hg$^{2+}$, Ni$^{2+}$, and Zn$^{2+}$) and the nucleotides AMP, ADP, and ATP are inhibitors. NAD(H) and NADP(H) did neither inhibit nor stimulate NDMA-ADH activity.

**NDMA-ADH and/or MFF in other bacteria.**

Extracts of *Rhodococcus rhodochrous* grown on ethanol and *Rhodococcus erythropolis* grown on ethanol or on 3,4-dimethoxybenzoic acid, all showed NDMA-dependent activity with methanol and ethanol as well as FAldM activity.

**Table 6: NDMA-dependent methanol and ethanol dehydrogenase and formaldehyde dismutase activities in extracts of *Rhodococcus* species.**

<table>
<thead>
<tr>
<th>Organism (growth substrate)</th>
<th>ethanol$^a$</th>
<th>methanol$^a$</th>
<th>FAldM$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>R. rhodochrous (EtOH)</td>
<td>2</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td>R. erythropolis (EtOH)</td>
<td>3</td>
<td>2</td>
<td>3.3</td>
</tr>
<tr>
<td>R. erythropolis (MBA)</td>
<td>4</td>
<td>2</td>
<td>0.3</td>
</tr>
</tbody>
</table>

---

$^a$nmol NDMA converted/min/mg protein; $^b$nmol formic acid produced/min/mg protein.
(Table 6), but insignificant activity with ethanol and NAD(P). Since extracts of methanol-grown *Mycobacterium gastri* MB19 and *A. methanolica* showed similar behaviour, although *M. gastri* only contains MFF [1], it was interesting to investigate this aspect for the *Rhodococci*. When the extracts were applied to the Mono-Q column, in all cases only one peak containing all three activities was detected, the peak eluting, however, at a concentration of 0.3 M KCl. No NDMA-dependent activities were found in extracts of the Gram-negative bacteria tested.

**DISCUSSION.**

Extracts of alcohol-grown *A. methanolica* contain NDMA-dependent dehydrogenase activity for methanol and ethanol (Table 1). Chromatography of these extracts on a Mono-Q column (Figure 1) revealed that all contain the already known MFF [1, 2] and the here described NDMA-ADH. Since the level of MFF remained nearly constant but that of NDMA-ADH varied according to the growth substrate used, one of the better inducers, methanol, was chosen to produce cells from which the enzyme could be purified.

NDMA-ADH of *A. methanolica* was purified to homogeneity. The enzyme oxidizes alcohols only in the presence of NDMA, not in that of NAD(P) or artificial dyes commonly used in assays for dye-linked dehydrogenases. It is inactive when assayed with aldehydes and NAD(P)H. This indicates that the internally bound NADH neither reacts nor is displaced by externally added NAD(P). Most probably NDMA regenerates the enzyme by oxidizing the internally bound NADH, just as it does with one of the intermediates in the catalytic cycle of common NAD(P)-dependent alcohol dehydrogenases (EC 1.1.1.1) where it oxidizes NADH when it is still bound to the enzyme before it is released [13]. In view of the fundamental differences in catalytic behaviour, it seemed interesting to compare the structural properties of NDMA-ADH and the NAD(P)-dependent alcohol dehydrogenases.

The N-terminal amino acid sequence of NDMA-ADH has significant similarity (Scheme 1) with that of (class I) alcohol dehydrogenase (56 % identical positions out of the first 41 amino acids with HL-ADH). However, in contrast to the dimeric/tetrameric alcohol dehydrogenases (EC 1.1.1.1) it has a trimeric structure and contains 1 firmly bound NADH per enzyme molecule. Recently, a trimeric representative has been found in this group (class III alcohol dehydrogenase or factor-dependent formaldehyde dehydrogenase) and it was also isolated from *A. methanolica* [16]. However, the N-terminal amino acid sequence of this enzyme has scarcely any similarity with that of NDMA-ADH (although it has with HL-ADH [16]), shows different substrate
Table 7: Comparison of nicotinoprotein alcohol/aldehyde oxidoreductases.

Data for NDMA-ADH from *A. methanolicus* are from this work, those for MFF from *A. methanolicus* from [1], those for FAiDM from *Pc. putida* F61 from [28], those for methanol dehydrogenase (MeDH) from *Bacillus methanolicus* Cl from [20, N. Arfman, unpublished results]. a low activity which increases drastically upon addition of helper protein [20]; b n.d. = not determined.

<table>
<thead>
<tr>
<th>Properties</th>
<th>NDMA-ADH</th>
<th>MFF</th>
<th>FAiDM</th>
<th>MeDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular mass (enzyme)</td>
<td>120 kDa</td>
<td>500 kDa</td>
<td>285 kDa</td>
<td>430 kDa</td>
</tr>
<tr>
<td>Molecular mass (subunit)</td>
<td>39 kDa</td>
<td>49 kDa</td>
<td>44 kDa</td>
<td>43 kDa</td>
</tr>
<tr>
<td>Active with methanol + NDMA</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Active with ethanol + NDMA</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>Dismutase activity</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>Active with methanol + NAD</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>yes&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Active with ethanol + NAD</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>yes&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Active with aldehydes + NADH</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
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<tr>
<td>Bound cofactor</td>
<td>NAD(H)</td>
<td>NADP(H)</td>
<td>NAD(H)</td>
<td>NAD(H)</td>
</tr>
<tr>
<td>Inhibitors:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrazole</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>n.d.&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ADP</td>
<td>yes</td>
<td>n.d.</td>
<td>no</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

specificity and does not contain bound NAD(H) [16]. Thus, the trimeric composition of these enzymes seems just fortuitous as their catalytic and structural properties are quite different. Similarly, although NDMA-ADH has structural resemblance and similarity in substrate (Table 4) and inhibitor (pyrazole, Table 5) specificity with respect to the NAD-dependent alcohol dehydrogenases, the decisive argument to distinguish it from this group is the bound NADH, acting as a cofactor instead of a coenzyme.

Recently [27], a number of alcohol/aldehyde oxidoreductases have been discovered which have also bound NAD(P) as cofactor, the enzymes named "nicotinoproteins" in analogy with other groups of cofactor-containing enzymes like flavoproteins and quinoproteins. Since NDMA-ADH is a
nicotinoprotein by definition, it is interesting to compare its properties with the others. As shown in Table 7 (and in Scheme 1 for two other nicotinoproteins of which N-terminal amino acid sequences are known), there exists large diversity in structural and catalytic properties. This suggests that the active sites of these nicotinoproteins are significantly different, as is well illustrated by the fact that only a few can use NDMA as electron acceptor and the exceptional behaviour of MeDH, reacting with external NAD as electron acceptor in the presence of a helper protein (evidence has been provided that this derives from transfer of reduction equivalents, not from exchange of bound NADH by added NAD [N. Arfman, unpublished results]). In the case of NDMA-ADH, the inability to use NAD as electron acceptor could result from: 1) the high redox-potential of the bound NAD/NADH couple; 2) the instability of the enzyme in the region where the assay should theoretically have its pH optimum (high for alcohol oxidation, low for aldehyde reduction); 3) inaccessibility of the active site for the rather large NAD(P)H. Possibility (1) is unlikely since the enzyme is also inactive with NADH and aldehydes, and the NAD-analogues, thio-NAD and acetylpyridine adenine dinucleotide, having a much higher redox-potential than NAD, were also inactive as electron acceptor. Possibility (2) is unlikely too since one would expect to observe some activity in the pH ranges tested (7 - 9 for ethanol and NAD(P), 5 - 7 for acetaldehyde and NAD(P)H). Therefore, possibility (3) is most likely, the active site allowing entrance of the nucleotides AMP, ADP and ATP and pyrazole as inhibitors while NAD(P)(H) are not since they are neither inhibitors nor electron acceptors (donors). The fact that an artificial dye like Wurster's Blue, comparable in size with NDMA, is unable to oxidize the bound NADH could be due to the lack of a positioning effect on this compound. Thus, it has been suggested that the aldehyde-mimicking nitroso group of NDMA attaches to the Zn\(^{2+}\) ion in normal NAD-dependent alcohol dehydrogenases [29]. Other effects may also play a role since in this connection it is somewhat unexpected that the enzyme does not show the dismutase activity, as observed with MFF and FALDM, and is unable to use aldose/ketose sugars as electron acceptors, as observed with the nicotinoprotein glucose/fructose oxidoreductase [30]. However, this inability may also result from an inadequate redox-potential of the bound NAD/NADH couple or inability to bind these small compounds in a proper way. Unusual binding of NAD(H) can be ruled out since this is not reflected by the absorption spectrum of the enzyme, being similar to that of MFO and MeDH and dissimilar from that of glyceraldehyde-3-phosphate dehydrogenase in which NAD is bound in an unusual way [31]. Inhibition studies (Table 3) strongly suggest the occurrence of an essential SH-group in the active site. Further studies are required to reveal any role in NAD-binding.
Since it is clear that NDMA-ADH does not directly communicate with the NAD/NADH pool or the common sugars in the cytosol, it is tempting to speculate that the enzyme is in fact an alcohol dehydrogenase which transfers its reducing equivalents to a component of the respiratory chain. In this connection it should be mentioned that evidence has been provided for the existence of multienzyme complexes, consisting of an alcohol- and an NADH dehydrogenase, and most probably playing a role in methanol conversion by this organism [16, 17]. Whether MFF and/or NDMA-ADH form part of these complexes remains to be elucidated. Also the role of the enzyme is unclear. Although the induction (Table 1) by higher aliphatic alcohols is in line with its substrate specificity (Table 4), the induction by methanol and acetate is not obvious. In addition, it should be reminded that MFF has an overlapping (except for methanol) and FD-FAIADH a partly overlapping substrate specificity with NDMA-ADH. Although this suggests that NDMA-ADH is superfluous in A. methanolicus, it could also be concluded that its genuine role is still not understood.

To study the distribution of nicotinoprotein alcohol/aldehyde oxidoreductases, extracts of Gram-positive and Gram-negative bacteria were screened with assays sited to detect MFF, FAIAD and NDMA-ADH. As shown in Table 6, R. erythropolis and R. rhodochrous show activity reminiscent of MFF, but chromatography revealed only one enzyme with an elution behaviour similar to that of NDMA-ADH. Thus, although no conclusion can be drawn on the type present, it suggests that nicotinoprotein alcohol/aldehyde oxidoreductases may play an important role in Gram-positive bacteria, in line with the fact that the organisms and growth conditions investigated did not lead to the induction of the normal NAD(P)-dependent alcohol dehydrogenases. Although the results with the Gram-negative bacteria tested were negative, it should be reminded that FAIAD was found in formaldehyde-resistant Pseudomonas putida F61 [28]. Therefore, the distribution of these enzymes might be broad, implying that alcohol oxidation is not only catalysed by flavoprotein, quinoprotein, and NAD(P)-dependent alcohol oxidoreductases, but also to a significant extent by a diversity of nicotinoprotein alcohoh-dehydrogenases, one of which is NDMA-ADH.

ACKNOWLEDGEMENTS

The investigations were supported by the Foundation for Chemical Research (SON), which is subsidized by the Netherlands Scientific Organisation (NWO), and by the National incentive program on fundamental research in Life Sciences initiated by the Belgian Science Policy Programming Department. The authors want to thank Janet Vonck, University of Groningen, for performing the electron microscopy.
REFERENCES


Chapter 6

Different Types of Formaldehyde-Oxidizing Dehydrogenases in Nocardia Species 239: Purification and Characterization of an NAD-Dependent Aldehyde Dehydrogenase

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Three different dehydrogenases able to oxidize formaldehyde were found in the Gram-positive myco- troph, Nocardia sp. 239: an NAD-dependent aldehyde dehydrogenase (NA-ADH), an NAD- and factor-dependent formaldehyde dehydrogenase (FD-FDH), and a dye-linked aldehyde dehydrogenase (DL-ADH). The ratio of the activities observed for the two NAD-linked enzymes varied with growth conditions: batch-wise grown cells had nearly the same activities for both enzymes; in fed batch-wise grown cells (methanol limitation) only FD-FDH was detected. The latter is clearly involved in formaldehyde oxidation, since the enzyme and the factor were found only in methanol-grown cells and the enzyme is specific for formaldehyde. In contrast, the two aldehyde dehydrogenases may have significance for aldehyde dissimulation in general, since both activities could also be demonstrated in ethanol- and butanol-grown cells (but not in glucose-grown cells) and higher aldehydes are even better substrates than formaldehyde. NA-ADH was purified to homogeneity. The enzyme seems to be a homotramer since it showed a relative molecular mass of 200,000 and the denaturated form of 55,000. Other characteristics are as follows: the enzyme showed substrate inhibition for the aldehydes tested; optimal activity was found at pH 9.2; the reverse reaction was not observed; the enzyme was specific for NAD; GSH, K\textsuperscript{+}, or NH\textsubscript{4}\textsuperscript{+} addition did not stimulate formaldehyde oxidation; the order of NAD and substrate addition to the enzyme was not important; several compounds able to block SH groups were inhibitory. Comparison with NAD-linked aldehyde dehydrogenases from Gram-negative bacteria showed that the Nocardia enzyme is distinct from the enzyme of Pseudomonas putida (EC 1.2.1.46) and of Hyphomicrobiun X. © 1990 Academic Press, Inc.

\footnote{1 To whom correspondence should be addressed.}

Formaldehyde is an intermediate in the biotransformation of methane, methanol, methylamine, and methoxylated compounds. Depending on the type of organism or growth condition, conversion of formaldehyde occurs via assimilation and/or dissimilation pathways. Different dehydrogenases, which could be principally involved in formaldehyde dissimulation, have been detected in Gram-negative bacteria: NAD-linked (formaldehyde dehydrogenases, one type (EC 1.2.1.46) found in a betaine-grown Pseudomonas putida strain (1–3) and the other in methanol-grown Hyphomicrobiun X (4); NAD-linked, GSH-dependent formaldehyde dehydrogenase (EC 1.2.1.1) from different bacteria (see Ref. (5) for a compilation, although it should be realized that these activities have been measured mostly with cell-free extracts and the dependency on GSH has not always been unambiguously established): NAD-linked, factor-dependent formaldehyde dehydrogenase described for Methylococcus capsulatus strain Bath (6); dye-linked aldehyde dehydrogenase, detected in Hyphomicrobiun sp. (7, 8) and Pseudomonas methanica (EC 1.2.99.3) (9).

Only a few Gram-positive methanol-utilizing bacteria are known and their enzymology of C\textsubscript{2} compounds dissimilation has been scarcely studied. Only one formaldehyde dehydrogenase has been characterized so far, namely, NAD-linked, factor-dependent formaldehyde dehydrogenase from the nonmethylo troph Rhodococcus erythropolis (10). For both this enzyme and that from M. capsulatus (6), the identity of the factor is unknown, although from the few properties published it appears already that they are different from each other. From preliminary work on the methanol-utilizing Nocardia sp. 239, a type of methanol dehydrogenase that is quite different from the well-known methanol dehydrogenase (EC 1.1.99.8) present in Gram-negative bacteria was suggested (11). It has been postulated that the novel enzyme occurs in a multienzyme complex, together with
ALDEHYDE DEHYDROGENASE FROM Nocardiopsis SPECIES 239

![Graphs showing formaldehyde-oxidizing activities in DEAE-Sepharose fractions. The DEAE-Sepharose elution patterns are shown for cell-free extracts (900 mg protein) prepared from nethanol-grown cells, cultured batch-wise (a) or fed batch-wise (b). Activities were measured with the NA-ADH assay (●), the FD-FDH assay (○), and the DL-ADH assay (△). The peaks marked I, II, and III correlate with NA-ADH, FD-FDH, and DL-ADH, respectively.]

NAD-linked formaldehyde dehydrogenase. Since we have embarked on the characterization of this complex, one of the aims was to identify the formaldehyde dehydrogenase activity in it. This appeared to be somewhat complicated since two distinct NAD-dependent dehydrogenases as well as a dye-linked one were discovered in cell-free extracts, as reported here. The purification and characterization of one of the NAD-dependent enzymes are also described.

MATERIALS AND METHODS

Materials. Formaldehyde solutions were prepared by heating paraformaldehyde in water for 4 h at 100°C. Paraformaldehyde, acetone, acetaldehyde, propionaldehyde, benzaldehyde, octanal, NAD, NADH, NADP, GSH, 2,6-dichlorphenol-indophenol (DCPIP), and phenazine methosulfate were from Merck (Darmstadt, FRG). Butyraldehyde was from Hopkin and Williams Ltd. (London, UK). DNease and baker’s yeast formate dehydrogenase (EC 1.2.1.2) were from Boehringer (Mannheim, FRG). Nitroblue tetrazolium, dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), and p-chloromercuribenzoate (PCMB) were from Sigma (St. Louis, MO). DEAE-Sepharose CL-6B (fast flow) and PD-10 columns were from Pharmacia (Uppsala, Sweden). All other chemicals were of an analytical grade.

Cultivation of the organism. Nocardiopsis sp. 239 (LMD 80.32) was grown aerobically in a 20-liter fermentor at 37°C on a mineral salt medium (12), supplemented with 1% (v/v) methanol, 1% (v/v) ethanol, or 1% (w/v) glucose. Batch-wise growth was continued until the optical density at 540 nm was approx. 3. For fed-batch-wise (methanol limited) growth, medium with 1% (v/v) methanol was pumped to a fermentor (containing 16 liters of mineral salt medium) at a rate of 21 ml/h in a time span of 100 h. Fermentors were inoculated with 1.5 liters of a preculture, grown on mineral salt medium with 0.5% (v/v) methanol for 40 h at 37°C.

Preparation of cell-free extract. Frozen cells were mixed with an equal volume of 20 mM potassium phosphate buffer, pH 7.2 (KPB). Cells were disrupted by passing the mixture twice through a precooled French pressure cell at 110 MPa. To lower the viscosity, DNease was added. The suspension was centrifuged for 30 min at 48,000g and 4°C, giving the cell-free extract.

Enzyme assays. NAD-linked aldehyde dehydrogenase (NA-ADH) activity was measured at room temperature by determining the rate of NADH formation at 340 nm in 0.12 M sodium pyrophosphate buffer, pH 9.0, containing 2.5 mM NAD. Enzyme was added and the mixture incubated for 30 s. The reaction was started by adding formaldehyde (1 mM final concentration). Factor-dependent formaldehyde dehydrogenase (FD-FDH) activity was measured in the same way, except that 6 mM formaldehyde was used and, in addition, DTT (final concentration 1 mM) as well as 100 μl factor solution were added. To avoid complication of formaldehyde, in some experiments DTT was omitted. In that case, all the solutions were degassed and subsequently flushed with N2. Covettes were filled in an anaerobic cabinet under an N2/H2 (97.5%/2.5%) atmosphere and stoppered with Soba seals. After transfer to the spectrophotometer, the reaction was started by adding an aerobically formaldehyde solution via a syringe provided with a hypodermic needle.

Dye-linked aldehyde dehydrogenase (DL-ADH) activity was determined by measuring the reduction of DCPIP at 600 nm in 0.12 M sodium pyrophosphate, pH 9.0, containing 40 μM DCPIP. After addition of the enzyme, the reaction was started by adding formaldehyde to a concentration of 20 μM. A check on formate production was made with formate dehydrogenase.

To detect the reverse reaction of NA-ADH, the conversion of NADH (250 μM) in the presence of sodium formate (1 mM) was followed. Experiments were performed in 0.1 M potassium phosphate buffer (pH 7.0 and 8.0) and in 0.12 M sodium pyrophosphate buffer, pH 9.0.

Activities were calculated by using molar absorption coefficients for NADH at 340 nm of 6220 M⁻¹ cm⁻¹ and for DCPIP at 600 nm of 22 × 10³ M⁻¹ cm⁻¹ (13).

Preparation of factor and factor-free cell-free extract. Factor was prepared by applying 2 ml of cell-free extract (obtained from methanol-grown cells, cultured fed batch-wise) to a PD-10 gel filtration column equilibrated with KPB. Elution occurred with KPB. The first 3.5
TABLE I
Formaldehyde-Oxidizing Activities in Cell-Free Extracts of Cells Grown under Different Conditions

<table>
<thead>
<tr>
<th>Growth on</th>
<th>NA-ADH assay</th>
<th>DL-ADH assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol (batch-wise)</td>
<td>167</td>
<td>25</td>
</tr>
<tr>
<td>Methanol (fed batch-wise)</td>
<td>35</td>
<td>67</td>
</tr>
<tr>
<td>Ethanol (batch-wise)</td>
<td>26</td>
<td>40</td>
</tr>
<tr>
<td>Glucose (batch-wise)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Note: Activities were measured at 37°C with the assays indicated under Materials and Methods. Formaldehyde oxidation rates were calculated from the rates of NADH production and DCPIP reduction and the activities are given in nmol formaldehyde/min/mg protein.

ml of eluate contained factor-free cell-free extract, while the following 5 ml contained the factor. Factor solution was stored at -20°C.

Purification of NA-ADH. All purification steps were carried out at 4°C, except where indicated otherwise. Cell-free extract (900 mg protein) was applied to a column of DEAE-Sephacel CL-6B (3.2 × 12.6 cm), equilibrated with XBP and the column was washed with three volumes of the same buffer. Elution occurred with 300 ml of a gradient from 0 to 1 M KCl in KPB at a flow rate of 1 ml/min. Fractions with NA-ADH activity were pooled and concentrated under N₂ pressure (3 bar) over a membrane (a cutoff of 10,000) to approx 6 ml and the concentrate was desalted on a PD-10 gel filtration column equilibrated with 10 mM sodium pyrophosphate/HCl buffer, pH 8.0 (NaPPB). The pooled fractions were further concentrated to 3.0 ml, as described above, and applied in 500-μI portions to a Mono-Q HR 10/10 column, used in combination with the Pharmacia FPLC system at room temperature. The column was eluted with 90 ml of a KCl gradient from 0 to 0.2 M in NaPPB at a flow rate of 3 ml/min. After pooling and concentrating, as described above, gel filtration was performed on a Superose-12 column, equilibrated with 0.1 M potassium phosphate buffer, pH 7.0, in the FPLC system with a flow rate of 0.5 ml/min.

Analytical methods. Protein determinations were performed according to Bradford with bovine serum albumin as a standard (14). Formaldehyde was determined according to Avigad (15) and formic acid according to Höpner and Knappe (16). Methanol and ethanol consumption by the growing bacteria was followed by gas chromatography on a Porapak Q column. The cofactor pyrroloquinoline quinone (PQQ) was determined with a biological assay (17).

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was performed on commercially available gradient gels (8-25%) using the Pharmacia Phast System equipment according to the instructions given by the manufacturer. The enzyme was denatured by heating the solution for 5 min at 100°C in the presence of 5% sodium dodecyl sulfate (SDS) and 25% β-mercaptoethanol. Protein staining occurred with Phast gel blue. For molecular weight determinations the standards used were the High Molecular Weight Kit (native gel electrophoresis) and the Low Molecular Weight Kit (SDS gel electrophoresis), both from Pharmacia. Enzyme activity staining was performed with the procedure described for NAD-dependent enzymes (18) using formaldehyde (1 mM) as substrate.

RESULTS

Different Types of Formaldehyde-Oxidizing Enzymes

In cell-free extracts of Nocardiopsis cells, grown on methanol, formaldehyde-oxidizing activity could be demonstrated by NADH formation as well as by DCPIP reduction. Upon purification, large losses of NAD-dependent activity for formaldehyde were sometimes observed, but could be restored by addition of a low-molecular-weight heat-stable factor prepared from cell-free extract. When cell-free extract of batch-wise grown cells was applied to

TABLE II
Substrate Specificity

<table>
<thead>
<tr>
<th>Enzyme/Aldehyde tested</th>
<th>Kₐ (μM)</th>
<th>Kᵦ (mM)</th>
<th>Vₘₐₓ (μmol aldehyde/min/mg protein)</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA-ADH/</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formaldehyde (0.1–20 mM)</td>
<td>650</td>
<td>6</td>
<td>9.5</td>
<td>1</td>
</tr>
<tr>
<td>Acetaldehyde (0.1–10 mM)</td>
<td>82</td>
<td>5</td>
<td>4.9</td>
<td>4</td>
</tr>
<tr>
<td>Propionaldehyde (0.01–10 mM)</td>
<td>6</td>
<td>2</td>
<td>0.6</td>
<td>6</td>
</tr>
<tr>
<td>Benzaldehyde (0.1–4 mM)</td>
<td>330</td>
<td>2</td>
<td>9.3</td>
<td>2</td>
</tr>
<tr>
<td>FD-FDH/</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formaldehyde (0.2–5 mM)</td>
<td>790</td>
<td>2</td>
<td>0.3</td>
<td>1</td>
</tr>
<tr>
<td>DL-ADH/</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formaldehyde (8–200 mM)</td>
<td>7300</td>
<td>ni*</td>
<td>1.7</td>
<td>1</td>
</tr>
<tr>
<td>Acetaldehyde (0.1–5 mM)</td>
<td>360</td>
<td>ni</td>
<td>4.3</td>
<td>52</td>
</tr>
<tr>
<td>Propionaldehyde (0.1–5 mM)</td>
<td>94</td>
<td>ni</td>
<td>4.7</td>
<td>217</td>
</tr>
</tbody>
</table>

Note: Aldehydes were tested with the following enzyme preparations: NA-ADH, purified enzyme; FD-FDH and DL-ADH, fractions from the DEAE-Sephacel step. The range of substrate concentrations used is given in parentheses. Kᵦ values were calculated assuming Michaelis-Menten kinetics and using the following equation: v = Vₘₐₓ*[S]/(Kₐ + [S] + ([S]/Kᵦ)), where [S] is the substrate concentration. Relative activities represent the Vₘₐₓ/Kₐ ratio of the substrate concerned divided by that of formaldehyde. Aldehyde oxidation rates were calculated from the rates of NADH production or DCPIP reduction.

*ni, no substrate inhibition detected.
a DEAE-Sepharose column (conditions are given under "Purification of NA-ADH"), activity eluted in three separate peaks (Fig. 1a): peak I detectable with the NA-ADH as well as the FD-FDH assay, indicating that no factor is required for activity; peak II, practically only detectable with the FD-FDH assay, indicating that a factor is needed for activity; peak III, only detectable with the DL-ADH assay (for clarity reasons, the symbols are omitted in certain parts of the figure). Therefore, three different enzymes able to oxidize formaldehyde are present in this organism under the specified growth condition: an NAD-linked, GSH- and factor-independent aldehyde dehydrogenase (NA-ADH) (eluting at a concentration of approx. 0.18 M KCl); an NAD-linked and factor-dependent formaldehyde dehydrogenase (FD-FDH) (eluting at a concentration of 0.29 M KCl); an NAD(P)-independent, dye-linked aldehyde dehydrogenase (DL-ADH) (eluting at a concentration of 0.36 M KCl).

The Effects of Growth Conditions and Carbon Source

The regime applied during growth determines whether methanol is the growth limiting factor or not. It was found that after batch-wise grown cells were harvested, 0.1-0.3% methanol was left in the culture medium, while this value was usually below 0.1% for fed batch-wise growth. Data obtained for NAD-linked and dye-linked formaldehyde dehydrogenase activities of cell-free extracts from these cells (Table I) suggested that the growth limiting factor exerts an effect on the type of enzyme synthesized. This could be substantiated by the results of DEAE-Sepharose fractionation: when the same amount of cell-free extract of both type of cells was applied to a DEAE-Sepharose column, for batch-wise grown cells half (60%) of the NAD-dependent activity corresponds with NA-ADH (peak I, Fig. 1a), while NA-ADH is nearly absent (1-2%) in fed batch-wise grown cells (peak I, Fig. 1b).

Nocardi a sp. 239 is able to grow on ethanol, and NAD-dependent formaldehyde-oxidizing activity has been demonstrated in cell-free extracts (12). To examine whether this is due to the presence of NA-ADH and/or FD-FDH, factor and factor-free cell-free extracts were prepared from cell-free extracts of ethanol-grown cells (batch-wise). It appeared that factor and FD-FDH activities were absent, indicating that all the NAD-dependent activity corresponds with NA-ADH. In addition to the NA-ADH activity, DL-ADH activity could be demonstrated in cell-free extracts of cells grown on ethanol (Table I).

Cell-free extracts from glucose-grown cells showed neither NAD- nor dye-linked aldehyde dehydrogenase activities (Table I).

Substrate Specificities

NA-ADH, FD-FDH, and DL-ADH were tested with a number of aldehydes (Table II). From the relative activities (the ratio of the apparent kinetic parameters \( V_{\text{max}}/K_{\text{m}} \)), it appears that higher aldehydes are better substrates than formaldehyde for NA-ADH and DL-ADH. Butyraldehyde and octanal were also active with the two ADHs and are probably even better substrates (the high affinities precluded measurement of the \( K_{\text{m}} \) values). FD-FDH appeared to be specific for formaldehyde since the other aldehydes showed no activity. Sub-

### TABLE III

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (U*)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free extract</td>
<td>900</td>
<td>125</td>
<td>0.14</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>DEAE</td>
<td>146</td>
<td>112</td>
<td>0.77</td>
<td>90</td>
<td>5.5</td>
</tr>
<tr>
<td>Mono Q</td>
<td>29</td>
<td>104</td>
<td>3.6</td>
<td>84</td>
<td>25.5</td>
</tr>
<tr>
<td>Superose</td>
<td>15</td>
<td>82</td>
<td>5.4</td>
<td>65</td>
<td>39.1</td>
</tr>
</tbody>
</table>

\*1 U = 1 \( \mu \)mol NADH produced/min/mg protein.

### TABLE IV

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (mM)</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>0.1</td>
<td>2</td>
</tr>
<tr>
<td>PCMB</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>PMSF</td>
<td>0.1</td>
<td>50</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>Methanol</td>
<td>250</td>
<td>65</td>
</tr>
<tr>
<td>Ethanol</td>
<td>170</td>
<td>78</td>
</tr>
<tr>
<td>1-Propanol</td>
<td>130</td>
<td>31</td>
</tr>
<tr>
<td>1-Butanol</td>
<td>110</td>
<td>15</td>
</tr>
</tbody>
</table>

Note. Purified NA-ADH was added to the reaction mixture and incubation was performed in the usual way. Inhibitor was then added and the incubation continued for another 1 min, after which the reaction was started by adding formaldehyde.

### TABLE II

Inhibitors of NA-ADH
TABLE V
Comparison of NAD-Dependent Aldehyde Dehydrogenases

<table>
<thead>
<tr>
<th>Properties</th>
<th>Enzyme</th>
<th>Noc</th>
<th>Ps</th>
<th>HX</th>
</tr>
</thead>
<tbody>
<tr>
<td>( M, \text{ (native)} )</td>
<td>200,000</td>
<td>150,000</td>
<td>nd*</td>
<td></td>
</tr>
<tr>
<td>( M, \text{ (subunit)} )</td>
<td>55,000</td>
<td>75,000</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>pH optimum</td>
<td>9.2</td>
<td>7.8</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Relative activities with</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>4 ( \times 10^{-3} )</td>
<td>nd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propionaldehyde</td>
<td>6 ( \times 10^{-6} )</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhibitors&lt;sup&gt;6&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( H_2O_2 )</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>( Ni^{2+} )</td>
<td>-</td>
<td>+</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>( Hg^{2+} )</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>PCMB</td>
<td>+</td>
<td>+</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>PMSF</td>
<td>+</td>
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<td>nd</td>
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<tr>
<td>primary alcohols</td>
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<td>+</td>
<td>nd</td>
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<td>Stimulators&lt;sup&gt;6&lt;/sup&gt;</td>
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<tr>
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<td>-</td>
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<td>Importance of NAD/substrate addition</td>
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<td>order</td>
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<td>No</td>
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</tr>
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</table>

Note: Data for the enzyme from Nocardia sp. 239 (Noc) are from this work, those for the Pseudomonas putida C-83 enzyme (Ps) from Ando and co-workers (1–3), and those for the Hyphomicrobiurn X enzyme (HX) from Pools and Duine (4). Relative activities were calculated by dividing the \( V_{max}/K_m \) value of the substrate concerned by that of formaldehyde.

* nd, not determined.
+ *, effective; −, non-effective.

strate inhibition (with the substrates indicated) was observed for NA-ADH and FD-FDH, but not for DL-ADH.

**NA-ADH**

**Purification.** NA-ADH was purified from cell-free extracts of cells grown batch-wise on methanol with a yield of 65% (Table III). The final preparation was homogeneous, as judged by polyacrylamide gel electrophoresis, giving a single band with protein as well as with enzyme activity staining. The native enzyme preparation showed a band with a relative molecular mass of 200,000 and SDS–polyacrylamide gel electrophoresis gave one band at 55,000, suggesting that NA-ADH is a homotetrameric enzyme.

**Optimal conditions and stability.** The order of addition of the components to the assay was not critical. The pH optimum for NA-ADH activity is between 9.0 and 9.5, as measured in NaPP buffer. The optimal temperature is 37°C. Good stability was observed at pH values between 7.0 and 8.0 in KPB. Storage at −40°C for several months and freezing–thawing cycles did not affect the activity.

**Properties.** Alcohols (primary alcohols up to \( n \)-pentanol, and 2-propanol) were not substrates in the concentration range tested (up to 1 M). The enzyme was active with NAD, but not with NADP. The \( K_m \) for NAD with 1 mM formaldehyde as substrate was 0.62 mM.

Formaldehyde oxidation to formate occurs with a concomitant stoichiometric reduction of NAD since 258 nmol formaldehyde was oxidized to 254 nmol formate and production of 235 nmol NADH. No reverse reaction was observed under the indicated conditions (using 0.06 mg enzyme, 14 nmol NADH had disappeared from the reaction mixture in 1 h at pH 7.0).

Since the final preparation showed the typical protein absorption spectrum (\( \lambda_{max} = 278 \) nm) with no absorbance above 300 nm, chromophores are absent. In accordance with this, PQQ was not detected in denaturated protein samples.

**Inhibitors.** The following compounds were tested for inhibition or stimulation in the assay with formaldehyde (1 mM) as a substrate: salts of Ba<sup>2+</sup>, Ca<sup>2+</sup>, Co<sup>2+</sup>, Cr<sup>3+</sup>, Cu<sup>2+</sup>, Fe<sup>2+</sup>, Fe<sup>3+</sup>, Mg<sup>2+</sup>, Mn<sup>2+</sup>, Mo<sup>6+</sup>, Ni<sup>2+</sup>, Zn<sup>2+</sup>, iodoacetic acid, primary alcohols from methanol up to butanol, and 2-propanol (all 1 mM); primary alcohols from methanol up to butanol at higher concentrations (Table IV); HgCl<sub>2</sub> (0.1 mM); 0.1 M K<sup>+</sup> or NH<sub>4</sub><sup>+</sup> salt concentrations; 10 mM concentrations of KCN, GSH, DTT, and EDTA; 2 mM H<sub>2</sub>O<sub>2</sub>; 0.1 mM concentrations of PMSF and PCMB. Compounds showing any effect are presented in Table IV. To compare the Nocardia enzyme with that of P. putida C-83, 1 mM Ni<sup>2+</sup> salt was tested (complete inhibition was reported for the latter enzyme (1)). Under the conditions described by Ando et al. (1), no inhibition was observed for the Nocardia enzyme. The inhibitory effect of H<sub>2</sub>O<sub>2</sub> could not be overcome by adding NAD to the reaction mixture before the H<sub>2</sub>O<sub>2</sub> (as was found for NAD-dependent aldehyde dehydrogenase of Hyphomicrobiurn X (4)).

None of the compounds tested stimulated the NA-ADH activity.

**DISCUSSION**

Cell-free extracts of methanol-grown Nocardia sp. 239 showed formaldehyde oxidation with NAD as well as with DCPIP as electron acceptor. The reason is that several formaldehyde-oxidizing enzymes are produced by this organism. DEAE-Sepharose chromatography revealed three well-separated distinct activities which belong to NA-ADH, FD-FDH, and DL-ADH.

DL-ADH was not further purified. The enzyme was also present in ethanol-grown cells but not in glucose-grown cells. In view of this induction pattern and its broad substrate specificity for aldehydes, it could be argued that the enzyme has a role in aldehyde oxidation in general. Dye-linked aldehyde dehydrogenases have also been found in Hyphomicrobiurn species: a constitutive one in Hyphomicrobiurn X (7) and another one induced...
in *Hyphomicrobium* ZV 580 (8) when grown on methylamine. In the case of *Hyphomicrobium* X, it was concluded that a role in formaldehyde oxidation was highly improbable (7). Because of the lack of data, the extent of similarity cannot be established.

FD-FDH was not further purified. However, from the properties determined it is already clear that the enzyme is quite different from the other two; it has a strict specificity for formaldehyde; NAD (not NADP) is used as a coenzyme; a low-molecular-weight factor is required for activity. This factor is not GSH or coenzyme A (to be published elsewhere). As could be expected, FD-FDH activity is absent in ethanol-grown cells. On the other hand, it is uncertain whether all formaldehyde oxidation is catalyzed by this enzyme, since methanol-grown cells also contain the aldehyde dehydrogenases. FD-FDH from *Nocardia* might be similar to the factor-requiring formaldehyde dehydrogenase reported for *R. erythropolis* (10). This idea is strengthened by the fact that the factor preparations are interchangeable (unpublished results; further work on FD-FDH is in progress).

NA-ADH was purified to homogeneity. It has a broad specificity for aldehydes and is induced during growth on methanol and ethanol, but not on glucose. For those reasons, it could have a general role in cellular aldehyde oxidation, but not a crucial one in formaldehyde oxidation since it was nearly absent in fed batch-wise grown cells on methanol. Comparison with other NAD-dependent, non-GSH, non-factor-requiring aldehyde dehydrogenases from Gram-positive bacteria is impossible since to our knowledge such an enzyme has not been characterized so far from these organisms. Thus, to classify the enzyme, it was attempted to make a comparison with formaldehyde-oxidizing, NAD-linked, factor-independent dehydrogenases from Gram-negative bacteria. As is apparent from the structural and catalytic data given in Table V, the enzyme is quite different from the nearly formaldehyde-specific enzyme from *P. putida* C-83 (1). Enzymes with a substrate specificity similar to that of the *Nocardia* NA-ADH have been found in many Gram-negative bacteria. In the case of mammalian organisms, several isozymes of different enzymes, indicated as EC 1.2.1.3 and EC 1.2.1.5, are in agreement with the definition. Unfortunately, such a subdivision does not exist for bacteria. However, the differences in sensitivity to inhibitors and stimulators between the enzyme from *Nocardia* and that from *Hyphomicrobium* X (Table V) (the latter is a type EC 1.2.1.5 enzyme (4)) suggest that such a situation might also exist in bacteria. It is clear that information on the primary structures of the enzymes is required before such a distinction can be made.

*Nocardia* sp. 239 contains a genuine formaldehyde dehydrogenase (FD-FDH) and two dehyde dehydrogenases (NA-ADH, DL-ADH) which could have a helper role in formaldehyde oxidation. To corroborate the latter, chemostat experiments will be required to relate precise growth conditions to enzyme levels and to aldehyde respiration capacities of cells. FD-FDH is a novel enzyme, first detected in *R. erythropolis* (10) and most probably a similar one that has now also been found in *Nocardia* sp. 239. The dependency of the activity on a factor implies that the presence of this enzyme may have been overlooked in cases where only the cell-free extract of the organisms was investigated. Insight into its distribution and properties could provide an answer to the question of whether it is an alternative or a complement for NAD-linked, GSH-dependent formaldehyde dehydrogenase (EC 1.2.1.1).

**ACKNOWLEDGMENTS**

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**REFERENCES**

Chapter 7

NAD-linked, factor-dependent formaldehyde dehydrogenase or trimeric, zinc-containing, long-chain alcohol dehydrogenase from *Amycolatopsis methanolica*

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NAD-linked, factor-dependent formaldehyde dehydrogenase (FD-FAIDH) of the Gram-positive methylotrophic bacterium, *Amycolatopsis methanolica*, was purified to homogeneity. It is a trimeric enzyme with identical subunits (molecular mass 40 kDa) containing 6 atoms Zn/enzyme molecule. The factor is a heat-stable, low-molecular-mass compound, which showed retention on an Aminex HPX-87H column. Inactivation of the factor occurred during manipulation, but activity could be restored by incubation with dithiothreitol. The identity of the factor is still unknown. It could not be replaced by thiol compounds or cofactors known to be involved in metabolism of C1 compounds. Of the aldehydes tested, only formaldehyde was a substrate. However, the enzyme showed also activity with higher aliphatic alcohols and the presence of the factor was not required for this reaction. Methanol was not a substrate, but high concentrations of it could replace the factor in the conversion of formaldehyde. Presumably, a hemiacetal of formaldehyde is the genuine substrate, which, in the case of methanol, acts as a factor leading to methylformate as the product. This view is supported by the fact that formate could only be detected in the reaction mixture after acidification. Inhibition studies revealed that the enzyme contains a reactive thiol group, being protected by the binding of NAD against attack by heavy-metal ions and aldehydes. Studies on the effect of the order of addition of coenzyme and substrate suggested that optimal catalysis required NAD as the first binding component. Substrate specificity and the induction pattern clearly indicate a role of the enzyme in formaldehyde oxidation. However, since FD-FAIDH was also found in *A. methanolica* grown on n-butanol, but not on ethanol, it may have a role in the oxidation of higher aliphatic alcohols as well. FD-FAIDH and the factor from *A. methanolica* are very similar to a combination already described for *Rhodococcus erythropolis* [Eggeling, L. & Sahm, H. (1985) Eur. J. Biochem. 150, 129—134].

NAD-linked, glutathione-dependent formaldehyde dehydrogenase (GD-FAIDH) resembles FD-FAIDH in many respects. Since glutathione has so far not been detected in Gram-positive bacteria, FD-FAIDH could be the counterpart of this enzyme in Gram-positive bacteria. Alignment of the N-terminal sequence (31 residues) of FD-FAIDH with that of GD-FAIDH from rat liver indeed showed similarity (30% identical positions). However, comparable similarity was found with class I alcohol dehydrogenase from this organism and with cytosolic alcohol dehydrogenase from *Saccharomyces cerevisiae*, isozyme 1. Therefore, it is concluded that the trimeric FD-FAIDH is related to the dimeric, tetrameric, zinc-containing, long-chain alcohol dehydrogenases.

Oxidation of methanol to formaldehyde by Gram-positive bacteria occurs by enzymes quite different from those found in Gram-negative bacteria and yeasts [1–3]. Therefore, it seemed interesting to investigate whether Gram-positive organisms also use unique enzymes for the oxidation of formaldehyde to formate.

Methanol-grown *Amycolatopsis methanolica* contains three different enzymes which are able to oxidize formaldehyde [4]: an NAD-dependent general aldehyde dehydrogenase, which has been purified and characterized [4]; dye-linked aldehyde dehydrogenase, detectable with 2,6-dichlorophenol indophenol as electron acceptor; an NAD-linked, factor-dependent formaldehyde dehydrogenase (FD-FAIDH). This re-
port describes the purification and characterization of FD-FA1DH from this organism. Since similarity was possible with FD-FA1DH from *Rhodococcus erythropolis* [5] and NAD-linked glutathione-(GSH)-dependent formyldehydrogenase (GD-FA1DH) found in eukaryotes [6] and Gram-negative bacteria [6, 7], an examination was made of possible relatedness with these enzymes.

**METHODS**

**Cultivation of the organisms**

*A. methanolica* [8] (NCIB 11946, LMD 80.32; previously known as *Streptomyces* sp. 239 [9] and *Nocardia* sp. 239 [10]) was grown batch wise and fed batch wise at 37°C on a mineral salt medium [10] supplemented with methanol [3]. The organism was also grown (batch wise) on each of the following substrates: 1% (by vol.) ethanol; 0.2% (by vol.) 1-butanol; 0.1% (by vol.) 1-hexanol; 0.5% (mass,vol.) sodium acetate; 0.5% (mass/vol.) glucose. *Rhodococcus erythropolis* (DSM 1069) was grown batch wise on 1% (by vol.) ethanol or on 2 mM 3,4-dimethoxybenzoic acid [5]. *Myxobacterium gutst* MB19 (LMD 87.103; kindly provided by Dr N. Kato) was grown batch wise on 1% methanol (by vol.) as described by Kato et al. [11].

**Enzyme assays**

FD-FA1DH activity was measured at room temperature by determining the rate of NADH formation at 340 nm. This occurred by mixing 0.92 ml 0.12 M sodium pyrophosphate (adjusted to pH 9.0 with a concentrated HCl solution), containing 2.5 mM NAD and 1.0 mM dithiothreitol with 50 μl factor solution (prepared using the 'heating procedure', as described below) and with 20 μl enzyme solution or factor-depleted cell-free extract. The mixture was incubated for 30 s and the reaction was started by adding formaldehyde (6.0 mM final concentration). On using methanol as a factor, 20 μl was added to the mixture while dithiothreitol and factor solution were added from the assay and the concentration of formaldehyde was raised to 25 mM. FD-FA1DH activity was also determined using alcohols as substrate. In this case, activity was measured in 0.1 M sodium carbonate, pH 10.2, containing 2.5 mM NAD. To 0.95 ml of this mixture, 20 μl enzyme was added. The reaction was started by the addition of ethanol (0.43 M final concentration). NADH-linked, factor-independent aldehyde dehydrogenase activity was measured using the NA-ADH assay [4] with 1 mM formaldehyde or 0.5 mM acetaldheyde as a substrate. The FD-FA1DH activity of cell-free extracts from *R. erythropolis* was measured as described [5]. NAD-linked, GSH-dependent GD-FA1DH from *Candida boidini* (Sigma, St Louis, MO, USA) was assayed as described by Schütte et al. [12].

Activities were calculated using a molar absorption coefficient for NADH at 340 nm of 6220 M⁻¹ cm⁻¹ [13].

**Preparation of factor solution and factor-depleted cell-free extract and partial purification of the factor**

Factor solution was prepared by applying 2.5 ml cell-free extract (protein concentration approximately 20 mg/ml), prepared from methanol-grown cells (fed batch), to a Pharmacia PD-10 (Sephadex G-25) column equilibrated with 20 mM potassium phosphate (buffer A), pH 7.2. Elution with the same buffer provided a first fraction (3.5 ml) containing factor-depleted cell-free extract and a second fraction (5 ml) containing the factor (designated as factor obtained by the 'desalting procedure'). Alternatively, factor solution was prepared by denaturing the proteins in the cell-free extract. In this case, the extract was kept in a boiling water bath for 5 min and denaturated protein was removed by centrifugation for 15 min at 40000 × g (designated as factor obtained by the 'heating procedure').

Partial purification of factor was performed as follows. The pH of the factor solution (prepared by the 'heating procedure') was adjusted to just below pH 2.0 with 1 M H₃SO₄ and the mixture was centrifuged for 3 min at 16000 × g. A 200-μl portion of the supernatant was injected onto an Aminex ion-exclusion column HPX-87H (Bio-Rad), equilibrated with 0.01 M H₂SO₄. Elution occurred with the same solution at a flow rate of 0.5 ml/min. Eluting compounds were detected with a Waters Differential Refractometer. The pH of the collected fractions was adjusted to neutrality and 100 μl samples were tested in the FD-FA1DH assay with formaldehyde as a substrate.

**Purification of FD-FA1DH**

70 g thawed cell paste of *A. methanolica* (grown fed batch wise on methanol) was washed twice with buffer A, pH 7.2, and cell-free extract was prepared as described by Duine et al. [1]. The extract (46 mg protein/ml) was fractionated with ammonium sulfate. The enzyme precipitated over 30–50% ammonium sulfate saturation. The precipitate was dissolved in 25 ml 10 mM potassium phosphate (buffer B), pH 7.0 and applied to a phenyl–Sepharose HP column (12 cm × 2.6 cm) equilibrated with 1.5 M (NH₄)₂SO₄ in buffer B, pH 7.0. After washing the column with the same buffer, elution occurred with a gradient of 1.0–0 M ammonium sulfate in buffer B, pH 7.0, over 3 h at a flow rate of 3 ml/min. FD-FA1DH eluted at a concentration of 0.2 M ammonium sulfate. After pooling and concentrating the active fractions (using an Amicon membrane filter with a cutoff of 10 KDa), ammonium sulfate was removed on a PD-10 column equilibrated with buffer A, pH 7.2. The enzyme solution was applied to a Mono-Q 10/10 column, equilibrated with buffer A, pH 7.2. After washing with the same buffer, elution occurred with a gradient of 0.20–0.50 M KCl in buffer A, pH 7.2 over 30 min at a flow rate of 2 ml/min. The enzyme eluted at 0.30 M KCl. After pooling and concentrating (as described above), the enzyme was further purified on a 5'-AMP—Sepharose 4B (Pharmacia) column (4.8 cm × 2.2 cm), equilibrated with 0.1 M potassium phosphate (buffer C), pH 7.0. Elution was carried out with a gradient of 0–4 M NaCl in buffer C, pH 7.0 over 4 h at a flow rate of 0.5 ml/min. After pooling and concentrating the active fractions, gel filtration was carried out on a Superose-12 column in buffer C, pH 7.0. For sequencing experiments, an additional step consisting of gel permeation appeared to be necessary. This was carried out on a Zorbax VGF-250 HPLC column (9.5 mm × 250 mm; DuPont, USA) in 0.2 M sodium phosphate, pH 7.0 at a flow rate of 1 ml/min. Ammonium-sulfate fractionation and affinity chromatography on 5'-AMP—Sepharose were performed at 4°C, the other steps at room temperature.

**Analytical methods**

Protein determinations were performed according to Bradford with bovine serum albumin as a standard [14]. Metal ions were determined by atomic absorption spectrophoto-
Molecular-mass determinations were carried out on a Superose-12 column calibrated with Blue Dextran (3000 kDa), ferritin (440 kDa), γ-globulin (150 kDa), yeast alcohol dehydrogenase (140 kDa), bovine serum albumin (67 kDa), β-lactoglobulin (40 kDa), cytochrome c (13 kDa) and K,[Fe(CN)](329). The specific absorption coefficient of the enzyme at 280 nm was calculated from an experiment in which the pure enzyme was injected on a Superose-12 gel-filtration column, in buffer C, pH 7.0 measuring the A_{280}/A_{405} ratio by photodiode array detector, and using the equation: A_{280}/A_{405} = 34.14. A_{280}/A_{405} = 0.02 [16]. Formate production was assayed with NAD-dependent yeast formate dehydrogenase according to Höpner and Knappe [17]. Acetaldehyde was determined according to Avigad [18].

Polycrylamide gel electrophoresis and isoelectric focusing

PAGE and IEF were performed on commercially available gels (8–25% gradient gel for native PAGE, 12.5% homogeneous gels for SDS/PAGE, and IEF 5–8 gels for IEF) using the Pharmacia Phastsystem equipment according to the instructions provided by the manufacturer. For SDS/PAGE, the enzyme was denaturated by heating the solution for 5 min at 100 °C in the presence of 5% SDS and 25% 2-mercaptoethanol. Protein staining occurred with Phastgel Blue. For molecular-mass determinations, the standards used were the high molecular mass kit (native PAGE) and the low molecular mass kit (SDS/PAGE). For the isoelectric point determination, the pl calibration kit was used as a standard. Enzyme-activity staining was performed with the procedure described for NAD-dependent enzymes [19], using a freshly prepared factor solution (prepared by the 'desalting procedure') and 6 mM formaldehyde as substrate, while diithiothreitol was omitted from the reaction mixture.

To sequence the amino acids of the purified protein, electrophoresis was carried out in a 7-cm SE250 Mighty Small II vertical slab unit ( Hoefer Sc., USA). Subsequently, electroblotting was performed overnight at pH 8.4 (50 mM Tris-H₂BO₃) in a Mini-Trans-Blot module (Bio-Rad, USA), on a poly(vinylidene difluoride) membrane [20].

N-terminal amino acid sequence analysis

The N-terminal amino acid sequence of the electrophorated FD-FA1DH was determined by automated Edman degradation in a 477A pulsed liquid sequenator with on-line detection of the phenylthiohydantoin—amino-acids (Applied Biosystems, USA), using the Blott-cartridge.

RESULTS

Assay conditions

In the search for the appropriate assay conditions for FD-FA1DH, part of the data provided by Eggeling and Sahm [5, 21] for the enzyme from R. erythropolis was very useful. Using 0.1 M pyrophosphate, an optimal pH of 9.0 was found. Taking this activity as 100%, other buffers at this pH gave the following values: 25 mM 2-(cyclohexylamino)ethanesulfonic acid/NaOH, 80%; 50 mM Tris·HCl, 80%; 0.1 M NH₄Cl/NH₃, 80%; 0.1 M glycine/NaOH, 40%; 0.1 M Na₂B₄O₇/HCl, 0%. A slight increase in activity (10%) was achieved by adding 0.5 M sodium or potassium salts in the case of the 2-(cyclohexylamino)ethanesulfonic acid buffer. Addition of NH₄Cl did not result in activation.

Fig. 1. Titration of enzyme with factor. Factor was prepared by applying the 'heating procedure' to cell-free extract (26 mg protein/ml) for A. methanolica cells grown fed batch wise on methanol. Pure FD-FA1DH (6 μg) was assayed with varying amounts of factor solution, keeping the volume of the mixture at 1.0 ml (supplementation with buffer A at pH 7.2).

Purified enzyme assayed in the absence of factor showed an activity which was 4% that observed in the presence of 50 μl factor (prepared by the 'heating procedure' from cells grown fed batch wise on methanol). The relationship between the amount of factor in the assay and the activity observed is shown in Fig. 1. Aging or manipulation of the factor preparations led to a decrease in activity, but this could be restored by prior incubation with diithiothreitol, as routinely performed in the assay procedure.

The order of addition of coenzyme and substrate appeared to be important; when substrate (either formaldehyde or ethanol) was added to the enzyme before NAD, much lower activities were observed than for the reversed order, as carried out in the standard assay. The decrease in activity caused by formaldehyde was a time-dependent process.

Activity observed with alcohols did not require the presence of factor (or of diithiothreitol). With formaldehyde as a substrate, high concentrations of methanol in the assay medium (0.63 M) could replace the factor in the absence of diithiothreitol.

Purification and characterization of FD-FA1DH

As indicated in Table 1, the activity of FD-FA1DH with formaldehyde co-purified with that for ethanol. The final preparation was investigated by PAGE under non-denaturing conditions. Enzyme, as well as protein staining, revealed only one band with a molecular mass of 117 kDa. The rather constant activity ratios for the two substrates (Table 1) and the homogeneity indicate that activity for formaldehyde and ethanol both reside in the same enzyme molecule. Gel filtration on Superose-12 revealed a molecular mass for the enzyme of 120 kDa. PAGE under denaturing conditions (in the presence of SDS) gave a band staining for protein with a molecular mass of 40 kDa. In view of the high similarity with the enzyme from R. erythropolis and the strong evidence provided for a trimeric structure in the latter case [5], it is concluded that FD-FA1DH from A. methanolica is also a trimeric enzyme. IEF showed that the enzyme has an isoelectric point of 6.4.

The absorption spectrum of the enzyme showed no absorbance above 300 nm. The specific absorption coefficient, A₄₅₀ nm, appeared to be 0.84. Metal analysis for Fe, Mg, Cu and Zn showed that only Zn was present in an amount of 6.1
Table 1. Purification scheme. FD-FA1DH was assayed with formaldehyde and factor (50 µl) prepared according to the 'heating procedure' and with ethanol. Activities are given in U/mg protein (1 U is defined as 1 nmoI NADH produced/min). The ratio of specific activities represents specific activity with formaldehyde/speciﬁc activity with ethanol.

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<th>Purification factor</th>
<th>Specific activity using ethanol as a substrate</th>
<th>Ratio of specific activities</th>
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<tr>
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Fig. 2. N-terminal amino acid sequences. Numbering is according to the sequence derived for FD-FA1DH. Data for S. cerevisiae alcohol dehydrogenase I (Sc ADH 1) are from [22] and for GD-FA1DH and class-1 alcohol dehydrogenase (ADH) are from [23]. Identification of amino acids given in brackets is provisional. The residues marked with an asterisk are strictly conserved in ADH.

Attempts to identify the factor

Several chromatographic materials were used in attempts to adsorb (and thus purify) the factor. These included DEAE-Sepharose, Q-Sepharose, Mono-Q, carboxymethyl-Sepharose, Mono-S, hydroxyapatite, phenyl-Sepharose, activated thiol-Sepharose, concanavalin-A-Sepharose, dihydroxyboryl-SP 500, Seppak cartridges, silica gel 60, fructose, controlled pore glass, aluminium oxide (acid, neutral and basic). All results were negative, but retention was observed on an Aminex HPX-87H column under acid conditions. Factor activity was present in fractions (Fig. 3, retention time 10.5 min, peak 1) eluting later than the front peak (retention time 7.3 min). Since such a column is normally used for liquid chromatography of sugars, such compounds were tested for activity. However, replacement of the factor was not possible with ribose, glucose, fructose, galactose, sucrose, lactose, inositol, mannitol or sorbitol.

Inactivation of the factor due to aging and restoration of activity by incubating with thiol compounds like dithiothreitol, suggested that the factor might also be a thiol compound. However, GSH was inactive. Reversibly, the factor could not replace GSH in the assay for GD-FA1DH from C. boidinii. Other thiol compounds like cysteine, 2-mercaptoethanol or sodium sulfide were also inactive, but as with GSH, they could replace diithiothreitol in reactivating the factor. Other compounds acting as scavengers of formaldehyde (tetrahydrofuranolactone [24], coenzyme A [25]) were inactive, and neither were cysteamine and D,L-lipoic acid.

The fact that methanol can replace the factor gives some clue to the role of the factor. As suggested already by Eggeling and Sahm [5], the hemiacetal of methanol and formaldehyde (CH₃-O-CH₂OH) could be the genuine substrate in this case. If so, methylnorformate would be the product, not formate, and in the case of the factor, the corresponding formate ester. To investigate this, the standard assay for formaldehyde with either the factor or methanol, was carried out, except that 0.25 mM NAD was used and the reaction was continued to completion (as judged from NADH formation). Subsequently, membrane filtration was applied to the mixture (using a membrane with a cutoff for proteins with a molecular mass larger than 30 kDa). A negative result was obtained when using the method of Höpner and Knappe [17] for formate detection in the filtrate. However, if the filtrate was first acidified (and the proteins were removed by centrifugation), the stoichiometric amount (280 nmoI) of formate was found (and 240 nmoI NADH). To obtain further evidence for the
Table 2. Inhibitors for FD-FA1DH. Purified FD-FA1DH was mixed with or without factor, after which the inhibitor was added. The mixture was incubated for 1 min and the reaction was started by adding formaldehyde (6 mM final concentration) or ethanol (0.43 M final concentration).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Activity with</th>
<th>Activity with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td>formaldehyde</td>
<td>ethanol</td>
</tr>
<tr>
<td>None</td>
<td>100%</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>CuCl₂</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NiCl₂</td>
<td>1</td>
<td>100%</td>
<td>65</td>
</tr>
<tr>
<td>ZnCl₂</td>
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<td>100%</td>
<td>65</td>
</tr>
<tr>
<td>KCN</td>
<td>2</td>
<td>45</td>
<td>30</td>
</tr>
<tr>
<td>1,10-phenanthroline</td>
<td>5</td>
<td>20</td>
<td>35</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>1</td>
<td>85</td>
<td>95</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>10</td>
<td>50</td>
<td>95</td>
</tr>
</tbody>
</table>

putative reaction mechanism, methylformate (final concentration 50 mM) was tested in the reverse reaction together with 0.25 mM NADH in buffers ranging over pH 5.0 – 7.6. However, no activity was observed.

Substrate specificity and inhibitors

Of the aldehydes tested (from formaldehyde to octanal and benzaldehyde), only formaldehyde was active. The Kₘ and the Vₘₚ were 9.6 mM and 30.9 μmol NADH produced · min⁻¹ · (mg protein)⁻¹, respectively, as tested with 1 mM diithiothreitol and 50 μl factor prepared by the ‘heating procedure’. Substrate inhibition occurred and the Kᵢ with formaldehyde was 8.7 mM (it should be noted that results for the determination of the kinetic parameters are probably heavily biased by the presence of diithiothreitol, as this thiol compound forms an adduct with formaldehyde). Acetaldehyde appeared to be an inhibitor for the enzyme when formaldehyde was the substrate (Table 2). However, when the addition of substrate (formaldehyde or ethanol) preceded the addition of acetaldehyde, no inhibition was observed, suggesting that acetaldehyde is able to bind to the enzyme, although it is not a substrate.

Of the alcohols tested (primary alcohols from methanol to decanol; secondary alcohols 2-propanol and 2-butanol), primary alcohols (except methanol) appeared to be substrates. As shown in Table 3, the higher aliphatic alcohols are the best substrates. To avoid enzyme inactivation, a non-saturating concentration of 0.43 M ethanol was routinely used throughout the assay. A significant higher pH optimum (pH 10.2) was found for the alcohols than for formaldehyde (the same phenomenon was found for the R. erythropolis enzyme); the pH optimum with ethanol was 9.5, as measured in 0.1 M pyrophosphate, while with formaldehyde this was pH 8.0 [5] and for GD-FA1DH [27]). The product is the corresponding aldehyde, as was checked for using ethanol as a substrate (180 nml acetaldehyde was formed with a concomitant production of 170 nml NADH). Substrate inhibition was not observed. The reverse reaction occurred, as judged from the activity observed in buffer C at pH 7.0, containing 0.25 mM NADH and 1 mM octanal as a substrate (80% of the activity observed in the forward reaction in the standard assay with ethanol).

Table 3. Apparent kinetic parameters for alcohols. Alcohols were tested with purified FD-FA1DH. The range of substrate concentrations used was given in parenthesis. Lineweaver-Burk plots were used as a check for Michaelis-Menten kinetics. The kinetic parameters were calculated with Marquard analysis [38]. 1 U = 1 μmol NADH produced/min.

<table>
<thead>
<tr>
<th>Alcohols</th>
<th>Apparent kinetic parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Km (mM)</td>
</tr>
<tr>
<td>Ethanol (0.02 – 1.0 M)</td>
<td>343</td>
</tr>
<tr>
<td>1-Propanol (20 – 400 mM)</td>
<td>324</td>
</tr>
<tr>
<td>1-Butanol (5 – 100 mM)</td>
<td>84.0</td>
</tr>
<tr>
<td>1-Pentanol (1.5 – 30 mM)</td>
<td>26.5</td>
</tr>
<tr>
<td>1-Hexanol (0.2 – 4 mM)</td>
<td>3.4</td>
</tr>
<tr>
<td>1-Octanol (0.1 – 2 mM)</td>
<td>1.2</td>
</tr>
<tr>
<td>12-Hydroxydodecanoic acid (0.1 – 2 mM)</td>
<td>1.7</td>
</tr>
</tbody>
</table>

NADP could not replace NAD in the assay with either formaldehyde or ethanol as a substrate.

Several compounds were tested as inhibitors with formaldehyde as well as with ethanol as a substrate (1 min incubation before substrate addition), such as metal ions (1 mM concentrations of Ca²⁺, Co²⁺, Cu²⁺, Fe²⁺, Fe³⁺, Mg²⁺, Mn²⁺, Mo⁶⁺, Ni²⁺, Zn²⁺ or 0.1 mM Hg²⁺ salts); metal chelators (1 mM EDTA or Ni²⁺; 2 mM CN⁻; 5 mM 1,10-phenanthroline); pyrazole, ADP and ATP (all 10 mM). As shown in Table 2, only metal ions reacting with thiol groups (Cu, Hg, Ni and Zn), as well as chelators like KCN and 1,10-phenanthroline, were inhibitors. However, the inhibitory effect of Zn and Ni was only observed with ethanol as a substrate, not with formaldehyde. Also, when methanol replaced the factor, thereby excluding an interfering effect of diithiothreitol, no inhibition of formaldehyde oxidation could be demonstrated with the two metal ions. Inactivation by thiol-group inhibitors could be prevented by adding first NAD in the case where formaldehyde was a substrate, but not when ethanol was used as a substrate.

Induction and distribution of FD-FA1DH

Cells grown on methanol (both batch wise as well as fed batch wise) contained FD-FA1DH and factor, but those grown on ethanol, acetate or glucose did not. Cell-free extracts from cells grown on ethanol or acetate showed NAD-dependent formaldehyde-dehydrogenase activity which was not dependent on the presence of factor. Therefore this activity can be ascribed to the presence of a NAD-dependent general aldehyde dehydrogenase already reported for this organism [4]. This was confirmed by demonstrating activity with acetaldehyde, a compound which is not a substrate for FD-FA1DH. However, FD-FA1DH activity [10 nmol NADH produced · min⁻¹ · (mg protein)⁻¹] and factor appeared to be present in the cell-free extract of cells grown on n-butanol and n-hexanol, suggesting that production of FD-FA1DH and factor is also induced during growth on higher aliphatic alcohols.

As reported already by Eggeling and Sahm, cells of R. erythropolis grown on 3,4-dimethoxybenzoic acid contain FD-FA1DH, those grown on ethanol do not [5, 21]. This was confirmed in the present study and it was also found that only
## Table 4. Comparison of FD-FAIDH and factors.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Activity with factor-depleted enzyme from</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A. methanolica</td>
</tr>
<tr>
<td></td>
<td>mmol NADH - min⁻¹ (mg protein)⁻¹</td>
</tr>
<tr>
<td>None</td>
<td>25</td>
</tr>
<tr>
<td>From A. methanolica</td>
<td>78</td>
</tr>
<tr>
<td>From R. erythropolis</td>
<td>59</td>
</tr>
</tbody>
</table>

Cells grown on the first substrate contained factor. This factor was also active in the assay of FD-FAIDH from A. methanolica. Reversely, factor obtained from methanol-grown cells of A. methanolica was active in the assay for FD-FAIDH from R. erythropolis (Table 4). The Gram-positive bacterium, *Mycobacterium gastri*, is able to grow on methanol and, just like *A. methanolica*, possesses the ribulose monophosphate pathway for intracellular formaldehyde fixation [10, 11]. However, neither the presence of NAD-dependent FAIDH activity, nor FD-FAIDH (not even when factor solution, prepared from cell-free extracts of *A. methanolica* or *R. erythropolis*, was added to the assay) could be detected.

## DISCUSSION

Comparison of FD-FAIDH from *A. methanolica* with that of *R. erythropolis* (Table 4) [5] revealed the following similarities: the enzyme is a trimer with subunits of 40 kDa; it contains 6 atoms Zn/enzyme molecule [although not mentioned in [5], Zn has also been detected in the *R. erythropolis* enzyme (L. Eggeling and H. Sahm, unpublished results)]; it oxidizes formaldehyde as well as higher aliphatic alcohols. Only small differences were detected with respect to pH optimum of the activity and the effect of dichothioretil. Also, the factors appear to be similar, if not identical, since mutual exchange was possible (Table 4). Thus FD-FAIDH is not restricted to one organism, and, in view of the possibility that factor dependency could have been overlooked in assays with cell-free extracts, it might have a wide distribution. However, it has no universal role in formaldehyde oxidation by Gram-positive bacteria since FD-FAIDH is absent in *M. gastri*. In the latter organism as well as in the thermotolerant *Bacillus* sp., 3-hexulose-6-phosphate synthase is responsible for the dissimilation of formaldehyde [11, 28]. In this connection, it is interesting to mention that cell-free extracts of *M. gastri* have substantial levels of formate dehydrogenase [29], while *A. methanolica* does not but contains a formate-ester dehydrogenase [30].

High concentrations of methanol mimicked the effect of the factor, most probably by forming the hemiacetal adduct of methanol and formaldehyde. This was substantiated by the fact that formate is not the product, liberation of formate requiring acidification (or heat treatment) [5]. It is likely that formate esters are intermediates in me hanol dissimilation by *A. methanolica*, since a formate ester dehydrogenase (not a formate ester hydrolase) occurs in this organism [30].

Formally, a hemiacetal can be regarded as an alcohol so that FD-FAIDH could act as an alcohol dehydrogenase, in accordance with its activity for higher aliphatic alcohols. Such a dual substrate specificity can be explained by assuming that the hydrophobic tail of the higher aliphatic alcohols is mimicked by a similar moiety in the factor so that both contact the same residues in the active site. However, this is contradicted by the observation that the factor did not adsorb to reverse-phase column materials, but on the contrary behaved as a hydrophilic compound analogous to sugars in the chromatography on the Aminex column. Thus, the latter suggests that factor-formaldehyde adduct and the alcohols could occupy different sites on the enzyme. Support for this view can be derived from the fact that the assays for formaldehyde and ethanol have different pH optima and the extent of inhibition for these two substrates is also different, as shown in the case of Ni²⁺ and Zn²⁺ and acetaldheyde (although differences in the assay systems as well as differences in affinity of the substrates could also explain this, as has been demonstrated for GD-FAIDH [31]). Therefore, although the preliminary results suggest that the dual substrate specificity originates from different active sites, a definite conclusion must await indepth kinetic investigations.

The observation that the order of substrate and coenzyme addition is not irrelevant, points to the existence of a sequentially ordered reaction mechanism in which the coenzyme is the first reactant. An thiol group in the enzyme seems to play a crucial role in this, since incubation with aldehydes (the 'substrate' formaldehyde as well as the 'non-substrate' acetaldheyde) resulted in a time-dependent inactivation. In accordance with this, NAD addition before the incubation with inhibitor prevented inactivation by the aldehydes as well as by the thiol group inhibitors Cu²⁺ and Hg²⁺.

Based on the properties of the factor, namely its hydrophilicity, its ability to be reactivated by dithiorthioform and its participation in enzyme formaldehyde oxidation, a number of sugars, thiol compounds and common cofactors involved in C1 biochemistry were tested for their ability to replace the factor. However, since the results were all negative, no clue was provided with respect to the identity of the factor. Summarizing what is presently known about the factor, it is a heat stable compound, behaving as a weak acid on isoelectric focusing [5] and it is reducible with dithiorthioform and other thiol compounds. The observed retention on the Aminex HPX-87H column (Fig. 3) could provide a tool to purify and characterize the factor in the future.

Although methanol mimics the effect of factor in the *in vitro* assay, the high concentrations required are probably not met in *vivo*. In contrast, ¹³C-NMR spectroscopy of Gram-positive *Staphylococcus aureus* cells (but not cells of the Gram-negative *Pseudomonas putida*) incubated with methanol and ¹³C formaldehyde and ¹H formaldehyde showed the presence of methylformate [32]. Therefore, it cannot be excluded that the formation of hemiacetal also occurs in vivo, the adduct being oxidized by FD-FAIDH and thus explaining the occurrence of methylformate. However, another likely candidate for methylformate formation seems to be a formaldehyde dimutase, which is present in cell-free extracts of *A. methanolica* and able to convert formaldehyde into methylformate in the presence of methanol (P. W. van Ophem and J. A. Duine, unpublished results).

NAD-linked, factor-independent formaldehyde dehydrogenase from the Gram-negative *P. putida* C-83 [33 – 35], shows some similarities with FD-FAIDH. The enzyme is able to convert formaldehyde and (at a lower rate) some lower al-
phatic aldehydes at a pH optimum of 8.9 and higher aliphatic alcohols at a pH optimum of 10.8; it contains 2 atoms of Zn/subunit. However, it does not require the presence of factor and its structure is quite different (the molecular mass is 150 kDa and it consists of two subunits).

As far as is presently known [36, 37], Gram-positive bacteria do not possess GSH, a compound which is essential for the activity of GD-FAIDH. GD-FAIDH has several properties in common with GD-FAIDH; the inability to oxidize formaldehyde as such; the requirement of a co-substrate for formaldehyde oxidation; the enzyme being composed of subunits of 40 kDa and containing 2 atoms Zn/subunit; an isoelectric point of about 6.4; the protective effect of NAD for the thiol group and substrate inhibitors. Therefore, GD-FAIDH might be a Gram-positive counterpart of GD-FAIDH, the latter being present in Gram-negative bacteria, yeasts and other eukaryotes [6, 7]. In addition, GD-FAIDH and GD-FAIDH are able to oxidize higher aliphatic alcohols (Table 3) [27, 31, 38, 39] and reduce the corresponding aldehydes: pyrazole is a very weak inhibitor for these enzymes [40], this work). Differences exist with respect to co-substrate specificity, since lipoic acid was able to replace GSH in the assay for GD-FAIDH [31], but not factor in the GD-FAIDH assay, and GSH and factor are not mutually exchangeable. Also, with respect to substrate specificity, differences exist; 2 M ethanol does not saturate GD-FAIDH [40].

Alignment of the N-terminal sequence of FD-FAIDH (31 amino acids) with that of GD-FAIDH [23] (which is identical to class-III alcohol dehydrogenase [27, 38], as demonstrated for the enzymes from rat liver and human liver [39]) revealed a significant similarity (approx. 30% identical residues, Fig. 2). However, approximately the same similarity was observed for class-I alcohol dehydrogenase from rat liver [23] and cytosolic alcohol dehydrogenase from S. cerevisiae isozyme 1 (Fig. 2), even though lower similarities were found for the bacterial alcohol dehydrogenases from Zymomonas mobilis, Escherichia coli [41], and Bacillus stearothermophilus [42], while almost no homology exists with mammalian aldehyde dehydrogenases [43], and references therein) or with aldehyde dehydrogenase from Pseudomonas aeruginosa [44]. Thus, this indicates that no special similarity exists for GD-FAIDH but a general similarity exists for eukaryotic alcohol dehydrogenases, as further confirmed by the fact that the residues at positions 26 and 30 are proline and glutamic acid, respectively, amino acids which are strictly conserved at these positions in all eukaryotic alcohol-dehydrogenases sequenced so far [22, 23]. Although it is clear that FD-FAIDH belongs to the group of dimeric/tetrmeric zinc-containing long-chain alcohol dehydrogenases, more structural information is required to enable determination of its precise position in this group of enzymes.

The investigations were supported by the Foundation for Chemical Research (SON), which is subsidized by the Netherlands Scientific Organisation (NWO), and by the National incentive program on fundamental research in Life Sciences initiated by the Belgian Science Policy Programming Department. The authors thank Paul Poels for his attempts to purify the factors, J. K. Koot and J. Padmos for the atomic absorption spectrometry and Marcel Kesselring and Karin Zuijderduin for their technical assistance.

REFERENCES
Chapter 8

Dye-linked dehydrogenase activities for formate and formate esters in *Amycolatopsis methanolica*

Characterization of a molybdoprotein enzyme active with formate esters and aldehydes

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Department of Microbiology and Enzymology, Delft University of Technology, The Netherlands

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Cell-free extracts of methanol-grown *Amycolatopsis methanolica* contain dye-linked dehydrogenase activities for formate and methyl formate. Fractionation of the extracts revealed that the (unstable) activity for formate resides in membrane particles, while that for methyl formate belongs to a soluble enzyme that was purified and characterized. The enzyme, indicated as formate-ester dehydrogenase, appeared to be a molybdoprotein (4 Fe, 3 or 4 S, 1 Mo and 1 FAD were found for each enzyme molecule), with a molecular mass of 186 kDa and consisting of two subunits of equal size. Product identification suggests that the formate moiety in the ester becomes hydroxylated to a carbonate group after which the unstable alkyl carbonate decomposes into CO₂ and the alcohol moiety. Based on structural and catalytic characteristics, the enzyme appears to be very similar to an enzyme isolated from *Comamonas testosteroni* [Poels, P. A., Groen, B. W. & Duine, J. A. (1987) Eur. J. Biochem. 166, 575–579] which was at that time considered to be an aldehyde dehydrogenase. Formate-ester dehydrogenase activity appeared to be present in several other bacteria. Possible roles for the *A. methanolica* enzyme in CO₂ dissimilation (oxidation of methyl formate to methanol and CO₂ or a factor-formate adduct to factor plus CO₂) or in general aldehyde oxidation, are discussed.

Much is known about the pathways and enzymes involved in the dissimilation of C₁ compounds [1, 2]. However, this applies to Gram-negative bacteria and yeasts, not to Gram-positive bacteria. For the latter group, unique dehydrogenases have been found to catalyse the oxidation of methanol [3–5] and formaldehyde [6–8] in some of these organisms. Therefore, it seemed interesting to study the formate-oxidizing enzyme of a Gram-positive bacterium, presumably utilizing a direct linear pathway for formaldehyde dissimilation.

The Gram-positive methanol-utilizing *Amycolatopsis methanolica* contains a methanol dehydrogenase [3] which is quite different from the classical methanol dehydrogenase found so far in all Gram-negative methanol-utilizing bacteria. The organism also has several dehydrogenases able to oxidize formaldehyde to formate, one of which is specific for formaldehyde and requires the presence of an unknown factor for activity [7, 8]. The only activity reported for formaldehyde oxidation concerns a dye-linked dehydrogenase [9], however, this finding could not be reproduced by others [10–12]. One of the explanations for this could be that formate oxidation is a minor route in the dissimilation, explaining the low respiration rates of whole cells with formate [10]. As a consequence of this assumption, an alternative, major route should exist.

As shown in [8], factor-dependent formaldehyde dehydrogenase, an enzyme comparable to glutathione-dependent formaldehyde dehydrogenase (FAIDH), produces factor-formate. High concentrations of methanol can replace the factor, leading to methyl formate production. Although the high concentrations of methanol required seem to preclude *in vivo* methyl formate production via this enzyme, there are indications that methyl formate could be an intermediate in the dissimilation of formaldehyde by bacteria. This ester has been detected by *in vivo* ¹³C-NMR spectroscopy of *Staphylococcus aureus* cells in the presence of ¹³C-labelled formaldehyde [13]. In their report, the authors suggested that the ester could be formed via formaldehyde dismutase, an enzyme first detected in a formaldehyde-resistant *Pseudomonas putida* strain [14]. Recently, we discovered such an activity in *A. methanolica* and *in vitro* methyl formate production occurred when formaldehyde dismutation was carried out in the presence of methanol (P. W. van Opheim and J. A. Duine, unpublished results). Thus, formate-ester production is a likely event in this organism. If it is assumed that the esters are not converted by a hydroxylase (see below) (a special hydroxylase has been found for S-formylglutathione, the product of glutathione-dependent FAIDH [15, 16], enzymes could exist which oxidize these compounds. For those reasons, a search was made for formate and formate-ester oxidoreductases. After the discovery of for-

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*Abbreviations: Cl.Ind. 2,6-dichloroindophenol; AIDH, aldehyde dehydrogenase; FDH, formate dehydrogenase; FD, factor-dependent; FAIDH, formaldehyde dehydrogenase; FEDH, formate-ester dehydrogenase; LDH, lactate dehydrogenase; MMT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium boronide.*

*Enzymes: NAD-linked, glutathione-dependent formaldehyde dehydrogenase (EC 1.2.1.1); NAD-linked, factor-dependent formaldehyde dehydrogenase (EC 1.2.1.1); NAD-linked, factor-dependent formaldehyde dehydrogenase (EC 1.2.1.1); NAD-linked, factor-dependent formaldehyde dehydrogenase (EC 1.2.1.1); NAD-linked, factor-dependent formaldehyde dehydrogenase (EC 1.2.1.1); NAD-linked, factor-dependent formaldehyde dehydrogenase (EC 1.2.1.1).*
Table 1. Purification of FEDH. Activity was measured with Wurster’s Blue (100 μM) using ethyl formate (10 mM for FEDH) or acetaldehyde (1 mM for AIDH) as a substrate. 1 U = 1 μmol Wurster’s Blue reduced/min. The ratio of AIDH activity/FEDH activity is also given.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein</th>
<th>FEDH activity</th>
<th>Recovery</th>
<th>Purification factor</th>
<th>AIDH specific activity</th>
<th>Ratio of specific activities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>U</td>
<td>U/mg</td>
<td>%</td>
<td>-fold</td>
<td>U/mg</td>
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<td>100</td>
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<td>1.23</td>
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<td>36</td>
<td>3.33</td>
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</tr>
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<td>22</td>
<td>7.18</td>
<td>18</td>
<td>69</td>
<td>20.1</td>
</tr>
</tbody>
</table>

mate dehydrogenase (FDH) and formate-ester dehydrogenase (FEDH) activities, an attempt has been made to purify and characterize these enzymes.

MATERIALS AND METHODS

Materials

Methyl formate, ethyl formate, chloral hydrate, trimethyl orthoformate and N-methylformamide were from Janssen Chimica, Tilburg, The Netherlands; all aldehydes, formamide, methyl butyrate and xanthine were from Merck, Amsterdam, The Netherlands; ethyl acetate and N,N-dimethylformamide were from Baker, Deventer, The Netherlands; N-formylglycine was from Fluka, Brussels, Belgium; lactate dehydrogenase (LDH, from rabbit muscle) and yeast FDH were from Boehringer Mannheim, FRG; all chromatography columns, the Phast system electrophoresis equipment and the kits with standards for electrophoresis and isoelectric focusing were from Pharmacia, Uppsala, Sweden; membrane filters were from Amicon, Beverly, MA, USA.

Cultivation of the organisms

_A. methanolica_ was grown in batch culture at 37°C on a mineral-salt medium [10] supplemented with 1% (by vol.) methanol, 1% ethanol, 0.2% 1-butanol, 0.1% 1-hexanol, 0.5% (mass/vol.) glucose or 0.5% (mass/vol.) sodium acetate. The organism was also grown in a chemostat under methanol limitation (dilution rate = 0.0075 h⁻¹), as described [17]. _Rhodococcus erythropolis_ was grown in 1% (by vol.) ethanol or on 2 mM 3,4-dimethoxybenzoic acid as described by Eggeling and Sahm [6]. _Mycobacterium gastri_ MB19 was grown on 1% (by vol.) ethanol or 1% (by vol.) methanol according to Kato et al. [18]. The latter two organisms were grown in batch culture.

Enzyme assays

FEDH activity was assayed at 37°C in 0.1 M potassium phosphate (buffer A), pH 7.8, with 100 μM Wurster’s Blue (synthesized according to Michaelis and Granick) [19] as electron acceptor. After recording the endogenous rate at 600 nm, a freshly prepared ethyl formate solution (10 mM final concentration) was added and enzyme activity was determined by subtracting the endogenous rate from the observed rate. 2,6-Dichloroindophenol (Cl₂Ind)-linked FDH activity was assayed at 30°C in buffer A, pH 6.3, with 50 μM Cl₂Ind, measured at 600 nm, or 2 mM 3,4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), measured at 550 nm, as electron acceptor. After recording the endogenous rate, sodium formate solution (0.8 mM final concentration) was added and the enzyme activity was determined by subtracting the endogenous rate from the observed rate. NAD-linked factor-dependent (FD) FAIDH and Cl₂Ind-linked aldehyde dehydrogenase (AIDH) activities were measured as described elsewhere [7].

Factor-formate-hydrolase activity was measured by determining formate production as follows: FD-FAIDH was incubated with factor and formaldehyde under the common assay conditions [7] for 10 min. Protein was then removed by pressure filtration using a membrane filter with a 10-kDa cutoff. To convert interfering NADH into non-interfering NAD, 500 μl filtrate was mixed with 500 μl buffer A, pH 7.0, containing 2 mM pyruvate. 20 μl LDH was added and the mixture was incubated [20] for 1 min. LDH was removed by pressure filtration (as above) and 200 μl filtrate was incubated with 100 μl cell-free extract [prepared from methanol-grown cells, in 50 mM potassium phosphate (buffer B), pH 7.0] for 1 h. This incubation occurred with and without the addition of 2 mM dithiothreitol (it has been reported [14] that dithiothreitol reactivates S-formylglutathione hydrolase). After pressure filtration, the formate content of the filtrate was determined with yeast FDH as described by Höpner and Knappe [21], except that 20 mM Me₅P, pH 7.0, was used as assay buffer. All incubations were performed at room temperature.

Calculations were using a molar absorption coefficient of 9000 M⁻¹ · cm⁻¹ for Wurster’s Blue at 600 nm [22] and 8100 M⁻¹ · cm⁻¹ for MTT-formazan at 550 nm [23]. The

Fig. 1. Absorption spectrum of purified formate-ester dehydrogenase. The absorption spectrum of the enzyme (0.35 mg protein/ml) was measured in 0.1 M potassium phosphate, pH 7.0.
value for Cl₂Ind was calculated from the equation relating the molar absorption coefficient to pH [24].

Membrane particles

Cell-free-extract-containing membrane particles with Cl₂Ind-FDH activity was prepared from cells grown in the chemostat under methanol limitation, as described [3]. The extract was first centrifuged for 5 min at 27000 x g. Subsequently, the supernatant was centrifuged for 30 min at 40000 x g, yielding a sediment which was suspended in 0.1 M Mes/NaOH, pH 6.1, designated as the crude membrane fraction.

Purification of FEDH

Frozen cell paste (48 g) of A. methanolica, grown in batch culture on methanol, was suspended in an equal volume of 10 mM potassium phosphate (buffer C), pH 7.0, and converted into cell-free extract as described [3]. The cell-free extract was fractionated with (NH₄)₂SO₄, and proteins precipitating in the range 25–55% saturation were collected by centrifugation. The precipitate was dissolvend in 20 ml buffer C, pH 7.0. The solution was applied to a phenyl-Sepharose column (HiLoad XK 26/10), equilibrated with buffer C, pH 7.9, containing 1.5 M (NH₄)₂SO₄. The column was washed with 3 vol. of the same buffer. Elution occurred in 3 h with a 1.5–0-M (NH₄)₂SO₄ gradient in buffer C, pH 7.0, at a flow rate of 3 ml/min. Active fractions were pooled and concentrated by pressure filtration over a membrane (with a 10-kDa cutoff) to approximately 15 ml. After dialysis for 16 h against 1.20 mM potassium phosphate (buffer D), pH 7.2, the solution was concentrated to 5 ml as indicated above. Subsequently, anion-exchange chromatography was carried out on a MonoQ 10/10 column. The column was equilibrated with buffer D, pH 7.2 and the enzyme solution was injected each time as a 500-μl aliquot. After washing the column with 3 vol. buffer D, pH 7.2, elution occurred in 30 min with a gradient of 0.30 M–0.60 M KCl in buffer D, pH 7.2, at a flow rate of 2 ml/min. Active fractions were pooled and concentrated to 1.5 ml. The solution was injected each time as a 200-μl aliquot on a gel-filtration column (Superose 12) equilibrated with buffer A, pH 7.0. The column was operated at a flow rate of 0.5 ml/min. Active fractions were pooled and stored at –20°C. All operations, except cell disruption (which was performed using a French Pressure cell at 4°C), were carried out at room temperature.

Molybdoprotein AIDH from Comamonas testosteroni (partly purified as described) [25] was a gift of B. W. Groen.

Analytical method

Protein determinations were performed according to Bradford [26] with bovine serum albumin as a standard. Molecular mass determinations were carried out on Superose-12 column calibrated with Blue Dextran (3000 kDa), ferritin (440 kDa), 7-globulin (150 kDa), yeast alcohol dehydrogenase (140 kDa), bovine serum albumin (67 kDa), β-lactoglobulin (40 kDa), cytochrome c (13 kDa) and K₃[Fe(CN)]₆ (320 Da). The specific absorbance of the enzyme at 280 nm was determined as described [27], using the equation $A_{280}^{\text{ref}} = (3.414 	imes \frac{A_{208}}{A_{280}}) - 0.02$. Fe content as well as Mo content were determined by atomic-absorption spectrophotometry [25]. The Fe content was also determined with a chemical assay [28]. S was determined with the procedure given by Canela and Nin [29] as well as that by Meyer [30]. FAD was determined as described by Poels et al. [25]. Detection of pyrroloquinoline quinone was performed with a bioassy described by Groen et al. [31]. Acetaldehyde was determined according to Avigad [32]. Acetic acid and ethanol were determined enzymically with commercial kits (Boehringer Mannheim) according to the instructions given by the manufacturer.

Electrophoresis and isoelectric focusing

PAGE was performed on commercially available gradient gels (8–25%) and IEF on pH 5–8 gels. To denature the
enzyme, it was heated in solution for 3 min at 100°C in the presence of 5% (mass/vol.) SDS and 25% (by vol.) 2-mercaptoethanol. Protein staining was with Phastgel Blue. Staining for FEDH activity was performed by incubating the gels in buffer A, pH 7.8, containing 10 mM ethyl formate and an appropriate amount of Wurster's Blue.

RESULTS

FEDH activity

Freshly prepared cell-free extracts of *A. methanolicum* grown under methanol limitation (prepared with buffer D, pH 7.2) showed very low activities (2–3 nmol·min⁻¹·mg protein⁻¹) for formate in the assay with Cl₂Ind or MTT as electron acceptor. The activity appeared to be unstable (50% loss in 5 min). An improvement was achieved by preparing the extract in 0.1 M Mes/NaOH, pH 6.3 (50% loss in 20 min). Higher specific activities (26 nmol·min⁻¹·mg protein⁻¹) were found in the membrane particles and these contained more than half of the total activity. The lability of the enzyme precluded its purification.

FEDH

Assay conditions

In the search for methyl-formate-oxidizing activity, the nucleotide coenzymes NAD and NADP (1 mM), O₂ (0.2 mM as measured with a Clark electrode cell) and the following dyes were tested as electron acceptor: $\text{Cl}_2[\text{Fe(CN)}_6]^-$ (1 mM), Wurster’s Blue (100 μM), phenazine methosulfate (0.1 mM), Cl₂Ind (40 μM) and phenazine methosulfate (0.1 mM)/Cl₂Ind (40 μM). Only with Wurster’s Blue and with the combination phenazine methosulfate/Cl₂Ind, were significant and low activities observed, respectively. Using Wurster’s Blue and 0.1 M potassium phosphate buffers, the pH optimum was 7.8. No substantial changes in activity were found on increasing the concentration of potassium phosphate or on using 50 mM Tris/Cl, 25 mM Mops/NaOH or 25 mM Hepes/NaOH (all pH 7.8) as buffer. Both ethyl formate and ethyl formate were substrates, but since the latter is a less volatile compound, it is the preferable substrate for the assay.

Purification and structural properties

As shown in Table 1, FEDH was isolated in high yield. The final preparation appeared to be homogeneous, as protein staining after electrophoresis showed a single band for native (PAGE) as well as denatured enzyme (SDS/PAGE). Upon IEF a single band was also obtained and this corresponded to an isoelectric point of 6.3. Both PAGE and gel filtration on Superose 12 indicated a molecular mass of 186 kDa. Since SDS/PAGE revealed a molecular mass of 89 kDa for the denatured protein, the enzyme seems to consist of two sub-units of equal size.

Analysis of a heat-denatured enzyme preparation for the presence of the cofactor pyrroloquinoline quinone gave a negative result. However, the absorption spectrum of the native enzyme (Fig. 1) is reminiscent of a molybdenoprotein [33]. Therefore, an attempt was made to demonstrate the presence, and determine the content, of the common cofactors of molybdenoproteins. Based on a molecular mass of 186 kDa and the determined specific absorbance ($A_{380}^{1%}$) of 1.03, the spectrophotometric analysis methods revealed 4.3 atoms Fe and 1.2 molecules FAD for each enzyme molecule. Atomic absorption spectroscopy indicated the presence of 4.1 atoms Fe and 1.0 atom Mo for each enzyme molecule. Using the method of Caneva and Nin [29], 2.9 atoms S were found for each enzyme molecule, while the method of Meyer [30] gave values of 4.3 and 2.7 S atoms for two different FEDH preparations. Not only the cofactor composition and the shape of the absorption spectrum (Fig. 1) are typical of a molybdenoprotein, but also the cofactor content, except for S (molybdenoproteins contain 4 Fe-S clusters [33]). However, a low S content could be due to storage effects [34], in line with the varying content found in different preparations, as mentioned above. Finally, the absorbance ratios ($A_{380}/A_{550}$ of 5.3 and $A_{450}/A_{550}$ of 2.7) are also similar to those found in molybdenoproteins [33]. In view of these properties and the strong resemblance with AIDH from *C. testosteroni* (see below), it is highly likely that FEDH also contains a pterin cofactor.

| Table 5. Induction of FEDH. FEDH activities of cell free extracts of *A. methanolicum*, grown on various substrates, were measured with ethylformate (10 mM). 1 U = 1 mmol Wurster’s Blue converted/min. |

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>FEDH activity U/g protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% methanol</td>
<td>100</td>
</tr>
<tr>
<td>1% ethanol</td>
<td>17</td>
</tr>
<tr>
<td>0.2% 1-butanol</td>
<td>45</td>
</tr>
<tr>
<td>0.1% 1-hexanol</td>
<td>290</td>
</tr>
<tr>
<td>0.5% glucose</td>
<td>0</td>
</tr>
<tr>
<td>0.5% sodium acetate</td>
<td>15</td>
</tr>
</tbody>
</table>

| Table 6. Comparison of FEDH from *A. methanolicum* and dye-linked aldehyde dehydrogenase from *C. testosteroni*. Data for FEDH from *A. methanolicum* is shown. |

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Molecular mass</th>
<th>pH</th>
<th>pH optimum</th>
<th>Number of S/Fe/Mo atoms enzyme molecule</th>
<th>Number of FAD/ molybdeno-pterin</th>
<th>$K_m$</th>
<th>Activity with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>isomeric</td>
</tr>
<tr>
<td>FEDH</td>
<td>186</td>
<td>89</td>
<td>6.3</td>
<td>7.8</td>
<td>3–4:4:1:1:0</td>
<td>3.6</td>
<td>&lt; 2.5</td>
</tr>
<tr>
<td></td>
<td>186</td>
<td>92</td>
<td>n.d.</td>
<td>8.2</td>
<td>3.7:4.0:1:0</td>
<td>1.1:1.2</td>
<td>0.10*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.9*</td>
</tr>
<tr>
<td>Dye-linked AIDH</td>
<td>186</td>
<td>89</td>
<td>6.3</td>
<td>7.8</td>
<td>3–4:4:1:1:0</td>
<td>3.6</td>
<td>&lt; 2.5</td>
</tr>
<tr>
<td></td>
<td>186</td>
<td>92</td>
<td>n.d.</td>
<td>8.2</td>
<td>3.7:4.0:1:0</td>
<td>1.1:1.2</td>
<td>0.10*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.9*</td>
</tr>
</tbody>
</table>

+ : active; - : not active.


Substrate specificity

Only formate esters (the esters of methanol and ethanol were tested) were active (Table 2), but not formate itself, ethyl acetate, or methyl butyrate (at 10 mM concentrations). Since the formate moiety in the esters seemed essential, also formamide and its N-methylated derivatives were tested. Only N-methylformamide showed activity, but not 20 mM N-formylglycine, 100 mM formamide or 100 mM N,N-dimethylformamide (Table 2). Since FEDH appears to be very similar (see below) to dye-linked aldehyde dehydrogenase from *C. testosteroni* [25]; a number of aldehydes were also investigated. As shown in Table 2, aliphatic aldehydes (from formaldehyde to octanal) and the aromatic aldehyde, benzaldehyde, were substrates, acetaldehyde being the best aliphatic aldehyde substrate (even better than ethyl formate; see Table 3). Activity was also observed with 100 mM trimethyl orthoformate and with 50 mM chloral hydrate. Neither alcohols (10 mM concentrations of methanol or ethanol), nor a ketone like acetone (10 mM) nor xanthine (2 mM) were substrates.

Product identification

To identify the product and stoichiometry of aldehyde oxidation, 125 nmol acetaldehyde was incubated with enzyme (4 µg) and 243 nmol Wurster’s Blue for 4 min under the common assay conditions. It appeared that 235 nmol Wurster’s Blue was reduced with concomitant formation of 117 nmol acetic acid, indicating that aldehydes are oxidized to their corresponding acids.

From a mechanistic point of view, molybdenoprotein aldehyde and xanthine oxidoreductase act as a hydroxylase [25]. Therefore, it could be expected that FEDH converts formate esters into the corresponding carbonate compounds. Thus, using ethyl formate as a substrate, the expected product is ethyl carbonate. Since the latter compound is unstable [35], decomposing to CO₂ and ethanol, a procedure was developed to determine the putative ethanol formed, avoiding hydrolysis of the substrate. For that purpose, 250 nmol ethyl formate was incubated with enzyme (20 µg) and 224 nmol Wurster’s Blue for 10 min under the common assay conditions. Subsequently, product and enzyme were separated by pressure membrane filtration, using a filter with a 30-kDa cutoff. Reduction of 215 nmol Wurster’s Blue led to the appearance of 119 nmol ethanol in the filtrate. Ethanol production by hydrolysis of the ester could be excluded since when the experiment was performed in the absence of Wurster’s Blue, only about 10 nmol ethanol was detected in the filtrate. Insignificant substrate hydrolysis was further confirmed by the absence of formate in the filtrate of the complete experiment. Contamination of ethyl formate with acetaldehyde could also be excluded since no acetaldehyde was detected in the filtrate. Product identification was not performed for methyl formate, chloral hydrate, or trimethyl orthoformate. Therefore, it is uncertain whether these compounds are real substrates. Summarizing, substrates can be defined by the formula: Y-X-C(H)O, in which X is either O [Y is CH₃(CH₂)₃], but not H; H (formaldehyde) or CH₂ [Y is H or CH₃(CH₂)₃].

Inhibitors

In order to probe the molybdenoprotein nature of FEDH, several common inhibitors [33] for this type of enzymes were tested. As shown in Table 4, most of these compounds appeared to be effective, although significant differences exist with xanthine dehydrogenase [33] (not so surprising when it is realized that xanthine is not a substrate). The following compounds did not inhibit the activity: 1 mM concentrations of Ca²⁺, Co²⁺, Mg²⁺, Mn²⁺, Ni²⁺, Mo⁶⁺ and Zn²⁺ salts, 1 mM EDTA, 2 mM KCl or 10 mM NaN₃.

Induction and distribution

Cell-free extracts of *A. methanolica* grown on methanol or ethanol contain dye-linked AIDH active with Cl₁Ind [7]. Since Cl₁Ind can be replaced by Wurster’s Blue (however, the activity is ten-times lower, data not shown), it could be argued that FEDH is identical to the previously detected Cl₁Ind-AIDH. However, this possibility can be excluded since FEDH is unable to use Cl₁Ind as an electron acceptor. Anion-exchange chromatography of a cell-free extract (from cells grown on methanol) on a Mono-Q column with a KCl gradient revealed two activities when the fractions were assayed with acetaldehyde and Wurster’s Blue. The first activity eluted at 0.4 M KCl and appeared to be FEDH (as it was active with ethyl formate), the second activity eluted at 0.5 M KCl and appeared to be the previously detected Cl₁Ind-AIDH [7] since ethyl formate was not a substrate and the enzyme was active with Cl₁Ind and acetaldehyde. Thus *A. methanolica* produces two different enzymes able to catalyse dye-linked aldehyde oxidation, one of these is FEDH, which is able to oxidize aldehydes as well as formate esters.

Induction of FEDH occurred when *A. methanolica* was grown on methanol, ethanol, 1-butanol, 1-hexanol or acetate, but not on glucose (Table 5). Highest activity was found for cells grown on methanol (either in batch culture or in fed-batch culture [7]) or on 1-hexanol.

Methanol-grown as well as ethanol-grown cells of *M. gastri* LB19 contained FEDH activity, although rather low

methanolica are from this study while those for dye-linked AIDH from *C. testosteroni* are from [25] or form this work (*), n.d., not determined.

<table>
<thead>
<tr>
<th>benzoaldehyde</th>
<th>methyl formate</th>
<th>ethyl formate</th>
<th>formate formamide</th>
<th>N,N-methylformamide</th>
<th>N,N-dimethylformamide</th>
<th>chloral hydrate</th>
<th>trimethyl orthoformate</th>
<th>Inhibition with methanol</th>
<th>A₂₈₀/A₄₅₀</th>
<th>A₂₈₀/A₃₅₀</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>5.3</td>
<td>2.7</td>
<td>90</td>
<td>15</td>
</tr>
<tr>
<td>+</td>
<td>*</td>
<td>*</td>
<td>+</td>
<td>*</td>
<td>*</td>
<td>+</td>
<td>+</td>
<td>4.9</td>
<td>3.1</td>
<td>+</td>
<td>0</td>
</tr>
</tbody>
</table>
Although several aldehydes and formylated compounds were active with the enzyme (Table 2), product analysis, carried out only for acetaldehyde and ethyl formate, showed that they are genuine substrates oxidized in a stoichiometrical way with respect to the dye.

The behaviour during purification as well as the substrate and electron-acceptor specificities showed that FEDH is quite different from the previously reported [7] Clβ2α-AIDH of A. methanolicus. Dye-linked AIDH activities have been reported for several Gram-negative bacteria [36–40], most of the enzymes considered to be quinoproteins [39,40] e.g. AIOH from Acetobacter aceti, currently rubricated as EC 1.2.99.3 [40a], although this EC number was originally given to a haemoprotein AIOH from Methyllosinus trichosporum [38,40b]. From comparison of structural, as well as enzymic properties, it appears that FEDH is nearly identical to dye-linked AIDH found in ethanol-grown C. testosteroni (Table 6), originally described as a dye-linked molybdoprotein AIDH [25]. Both enzymes are able to oxidize formylated compounds and this property was also exhibited by cell-free extracts from M. garsi and R. erythropolis. It is clear, therefore, that several quite different types of dye-linked AIDH exist in bacteria and that the molybdoprotein type, also having FEDH activity, is distributed among Gram-positive, Gram-negative, methylo-trophic and non-methylotrophic organisms.

Whole cells of A. methanolicus have a low capacity to respire formate when compared to methanol or formaldehyde [10]. Although this could be due to the fact that formic acid is a weak acid, being able to decrease the respiration rate by affecting the proton-motive force [41], another explanation could be that this organism has only low levels of FEDH, in line with the activities observed in cell-free extracts. In accordance with this view, it should be noted that FD-FADH has an important role in the conversion of formaldehyde [7,8], while its product, the factor-formyl adduct, is not hydrolysed. Thus, FEDH is a good candidate for converting factor-formyl adduct to CO2 and factor by oxidation.

Another indication for a role of FEDH in formate-ester oxidation comes from the observation of methyl formate production when certain Gram-positive bacteria are treated with formaldehyde [13], most probably formed from the hemiacetal adduct of formaldehyde and methanol. Although the hemiacetal seems to be an inadequate substrate for FD-FADH [8], it is a much better substrate for a recently detected formyldehyde dismutase, leading to significant methyl formate production in vitro (P. van Ophem and J. A. Duine, unpublished results). Thus, FEDH could have a role in methyl formate oxidation as well as in factor-formate oxidation.

The induction with higher aliphatic alcohols, the high activity with aldehydes and the similarity with AIDH from C. testosteroni could indicate that FEDH has a dual role. It is interesting to note that the preceding enzyme in the pathway of methanol designated here, namely FD-FADH [8], not only oxidizes factor-formyl adduct, but also higher aliphatic alcohols. Thus, the duality of both enzymes, as found in vitro, could have a physiological meaning.

Future research will be directed towards eliminating the enzyme from A. methanolicus, by mutation, in order to corroborate the roles suggested here. As formate-ester formation has not been considered so far in dissimilation routes, it would be worthwhile to look for FEDH activity in other organisms in which formyl esters might be formed in the metabolism of formaldehyde. In this context, it should be realized that although it is generally presumed that the formylglutathione adduct is converted by a specific hydrolase into formate and

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**Factor-formate-hydrolase activity**

In order to obtain the factor-formate needed to detect the putative factor-formate hydrolase, FD-FADH was incubated with factor and formaldehyde as indicated in Materials and Methods, thereby yielding approximately 12 μmol NADH in the filtrate after pressure filtration. Assuming a stoichiometric conversion, 1 mol NAD should react with 1 mol substrate (hydroxymethylated factor), yielding 1 mol factor-formate. However, after incubating the reaction mixture with cell-free extract (100 μl added to 200 μl filtrate), no formate could be detected in the final filtrate, neither for the reaction carried out with nor without diithiothreitol addition. Surprisingly, also when formate (100 nmol) was added to the mixture, the result of the enzyme assay for formate was negative. Since formate as such is active in the standard assay, this suggested that the yeast FDH was inhibited by a compound present in the reaction mixture. Inhibition was not due to NADH since the LDH and pyruvate added, completely converted NADH into NAD within 30 s, as judged from the absorbance at 340 nm. To detect the putative inhibitor, formaldehyde, diithiothreitol, factor, pyruvate, lactate, methyl formate and a mixture of formaldehyde, diithiothreitol, factor and NAD in 0.1 M sodium pyrophosphate/HCl, pH 9.0, (without incubation according to [8]) were tested. However, none of these inhibited the yeast FDH. These preliminary experiments strongly suggest that factor-formate is the inhibitor, suggesting that either no hydrolase exists for this compound in this organism or that inadequate assay conditions were applied.

**DISCUSSION**

Cell-free extracts of A. methanolicus showed a dye-linked FDH activity, as already reported by Kato et al. [9]. Highest specific activity was found in membrane particles, suggesting that the activity derives from a membrane-bound enzyme. The reasons why others [10–12] could not find this activity are most probably due to the low specific activity, or the low levels of enzyme produced and its instability.

In contrast to the case for formate, significant, stable, dye-linked dehydrogenase activity for formate esters appeared to be present in cell-free extracts. The enzyme exhibiting this activity was purified to homogeneity. Its absorption spectrum and cofactor composition and content show that FEDH is a typical molybdoprotein. This statement is also valid in a mechanistic sense, since FEDH acts as a hydroxylase, as judged from the conversion of ethyl formate into ethanol and CO2, with ethyl carbonate as a likely (labeled) intermediate.
glutathione, this has only been proven in a restricted number of cases [15, 42, 43] and oxidation of the adduct has been claimed to occur by a FDH [44]. Finally, as aldehydes and formate esters can be regarded chemically as compounds terminally substituted with a carbonyl group, testing the capability of the already known molybdenoprotein oxidoreductases for formate ester oxidation would be interesting in order to probe the generality of this reaction.

The investigations were supported by the Foundation for Chemical Research, which is subsidized by the Netherlands Scientific Organisation. The authors want to thank B. W. Green, for performing the experiments with the C. testosteroni enzyme, J. Padmos, Delft University of Technology, for performing the atomic absorption spectrometry and Prof. Dr L. Dijkhuizen, University of Groningen, for critical reading of the manuscript.

REFERENCES

Chapter 9

General discussion.

Introduction.

Methanol dissimilation by *Amycolatopsis methanolica* is not straightforward, in that sense that a multitude of steps and enzymes for a particular step have been found, as shown in the preceding chapters (Fig. 1). This chapter discusses the likeliness of these steps and the involvement of the enzymes in this, and/or in other dissimilation pathways. For that purpose, an overview is presented on the induction of the enzymes/activities found at growth on a number of substrates (Table 1). Since several methanol-oxidizing activities have been detected in extracts of *A. methanolica* (of which one has been purified and characterized), their relatedness is discussed by comparing the properties (Table 2). Finally, the possibility that enzymes similar to those found in *A. methanolica* are involved in similar conversions in other (Gram-positive) bacteria will also be considered.

The methanol oxidation step.

Three distinguishable methanol-oxidizing activities/enzymes (Table 2) have been detected in extracts of *A. methanolica* (Fig. 1: step 1): 1. novel methanol dehydrogenase (n-MDH) [3, Chapter 2]; 2. tetrazolium-dependent alcohol dehydrogenase (TD-ADH) [4, Chapter 3]; 3. methanol-oxidizing, formaldehyde-reducing, and formaldehyde-dismutating enzyme (MFF) [Chapter 4].

MFF from *A. methanolica* as well as that from *Mycobacterium gastri* and methanol dehydrogenase from *Bacillus Cl* [5] show structural similarities: all are decameric proteins; similarity exists in amino acid sequences of certain peptides (50 - 60 % identity); Zn$^{2+}$ is present in (nearly stoichiometrical) amounts; the enzymes have bound NAD(P) as cofactor, distinguishing them from the common NAD(P)-dependent alcohol dehydrogenases using NAD(P) as a coenzyme (for that reason, the name "nicotinoproteins" is proposed here for the enzymes using NAD(P) as a cofactor). However, mechanistically, significant differences exist: MFF shows formaldehyde dismutase (FADDM) activity, the methanol dehydrogenase does not; exchange of
Enzymes (the most likely route involved in bold):
1. n-MDH; TD-ADH; MFF.
2. FD-FALDH; Y-X-H = factor.
3. aldehyde dehydrogenases (NAD-linked, dye-linked and FEDH).
4. MFF.
5. MFF; (FD-FALDH).
6. dye-linked FDH.
7. FEDH.
8. non-enzymatically.

reduction equivalents between bound NADH and external NAD occurs for methanol dehydrogenase, not for MFF; a helper protein to stimulate the latter process occurs in *Bacillus* C1, not in *A. methanolicus* or in *M. gastri*. To explain this, it must be concluded that the active sites of the enzymes are different. If it is assumed that the catalytic properties, as found in vitro, reflect the in vivo situation, also the role of the enzymes will be different in the respective organisms. From the preliminary characterizations, it seems that methanol dehydrogenase is a cytosolic
Table 1: Induction of activity of enzymes possibly involved in the methanol dissimilation pathway of *A. methanolicum*.

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*A. methanolicum* was grown with the substrates indicated (concentrations are given in brackets). The organism was also grown fed-batch-wise on methanol (FB). The following activities were measured of the cell free extracts: TD-ADH (tetrazolium dependent alcohol dehydrogenase); NA-ALDH (NAD-dependent aldehyde dehydrogenase using 0.5 mM acetaldehyde as a substrate); FD-FALDH (NAD- and factor-dependent formaldehyde dehydrogenase); FEDH (formate ester dehydrogenase); NDMA-ADH (NDMA-dependent alcohol dehydrogenase); MFF (methanol-oxidizing, formaldehyde reducing, and formaldehyde-dismutating enzyme). PQQ determinations were carried out for the free as well as the bound form.

---

**Activities**

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>PQQ</th>
<th>TD-ADH</th>
<th>NA-ALDH</th>
<th>FD-FALDH</th>
<th>FEDH</th>
<th>NDMA-ADH</th>
<th>MFF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol (FB)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Methanol (1 %)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ethanol (1 %)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-/+</td>
<td>-/+</td>
<td>+</td>
</tr>
<tr>
<td>1-butanol (0.2 %)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-/+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1-hexanol (0.1 %)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-/+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acetate (0.5 %)</td>
<td>-</td>
<td>+</td>
<td>-/+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glucose (0.5 %)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
</tbody>
</table>

+ = present; -/+ = low activity present; - = no activity present.

---

enzyme, having indirect contact with the respiratory chain via the NAD/NADH pool in the cytosol while MFF forms part of a complex in the respiratory chain (*vide infra*). Taking also the absence of MFF in *Corynebacterium XG* into account, it appears therefore that methanol oxidation in Gram-positive methanol-utilizers is not such a uniform process as that in their Gram-negative counterparts.

MFF is the only methanol-oxidizing enzyme of *A. methanolicum* characterized so far. However, in view of the other activities for methanol oxidation in this organism, the question must be posed whether it is the sole enzyme catalyzing this oxidation, and if so, how it can be transformed into forms.
Table 2: Comparison of methanol-converting activities present in *A.* methanolica.

<table>
<thead>
<tr>
<th>Properties</th>
<th>n-MDH</th>
<th>TD-ADH</th>
<th>MFF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reproducibly detectable</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Electron acceptor</td>
<td>DCP/IP</td>
<td>MTT/INT</td>
<td>NDMA</td>
</tr>
<tr>
<td>Cofactor</td>
<td>PQQ/NAD</td>
<td>?</td>
<td>NADP</td>
</tr>
<tr>
<td>Stimulation by salts</td>
<td>NH$_4^+$</td>
<td>high PO$_4^{3-}$/SO$_4^{2-}$</td>
<td>none</td>
</tr>
<tr>
<td>Found in multi-enzyme complex</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>Able to convert other alcohols</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Induced during growth on other primary alcohols</td>
<td>?</td>
<td>yes</td>
<td>yes</td>
</tr>
</tbody>
</table>

manifesting itself as n-MDH or TD-ADH activity.

When comparing methanol-grown cells with ethanol-grown cells of *A.* methanolica, similar kinetic parameters are found for ethanol oxidation but different ones for methanol oxidation (Chapter 3). This could be interpreted that a general enzyme exists for oxidizing alcohols, including methanol, and an extra one for methanol, being induced at growth on methanol. In that view, the general enzyme could be MFF and/or TD-ADH. Since MFF and TD-ADH are found in cells grown on a variety of substrates (except on glucose) and have overlapping substrate specificities, TD-ADH may consist of MFF and (a number of) redox components, one of which reacts with the special tetrazolium-dyes required for the assay. If this view is correct, the implication is that not every Gram-positive methanol-utilizer produces these components since *Bacillus* Cl has an MFF-like enzyme (methanol dehydrogenase) but does not show TD-ADH activity. On the other hand, the presence of TD-ADH activity does not imply a functional usage of the methanol-oxidizing capability of the enzyme by the organism. Thus, although *Rhodococcus erythropolis* has TD-ADH activity and is able to dissipilate formaldehyde, this organism does not grow on methanol. This could be related to the absence of a formaldehyde assimilating pathway or to a non-functionality of TD-ADH (and MFF) in growth on methanol. In the latter view, the methanol-oxidizing ability of these enzymes should be regarded as fortuitous and
interpreted as the enzyme having another function. Although MFF shows FAÏDM activity, a role of this seems unlikely in _A. methanolicus_ (see below). Although the broad substrate specificity for primary alcohols could be functional for growth on these compounds, this is also unlikely since the organism can use an NAD-dependent alcohol dehydrogenase and an NDMA-linked alcohol dehydrogenase (Chapter 5) for that purpose. Therefore, it is tentatively concluded that TD-ADH (and MFF) have a role in methanol dissimilation, although the exclusivity of this is debatable (vide infra).

The extra enzyme could be n-MDH, being different from MFF and TD-ADH (specific for methanol) and rather unique for Gram-positive methanol-utilizers since its activity has so far only been demonstrated in _A. methanolicus_ [3] and in the Thermoactinomycete strain 381 [6]. Since PQQ is lacking in the organisms in which n-MDH has not been found, and PQQ was detected in partially purified n-MDH, it is tempting to speculate that n-MDH contains a quinoprotein. The unreproducible behaviour of this enzyme with respect to detection might be related to its complex structure, one of the components being labile or easily detached during preparation of cell free extract.

NDMA-ADH does not use methanol as a substrate but primary alcohols, the affinity for the enzyme increasing with their chain length. The enzyme showed neither FAÏDM activity nor methylformate production. It contains tightly bound NAD(H), indicating that bound NAD(P) is not a prerequisite per se for methanol oxidation and FAÏDM activity. Apparently, the nature of the active site of a nicotinoprotein enzyme is the crucial factor in this, just as in the case of quinoprotein alcohol dehydrogenases. Alignment of the N-terminal sequence of NDMA-ADH showed absence of homology with MFO from _A. methanolicus_, but significant homology with horse liver alcohol dehydrogenase. If further structural analysis can confirm the tentative results, the surprising conclusion is that _A. methanolicus_ contains two structurally and mechanistically quite different nicotinoprotein alcohol dehydrogenases, implying that binding of NAD(P) is not restricted to a special type of protein or alcohol dehydrogenase.

Induction experiments (Table 1) showed that NDMA-ADH is induced during growth on methanol, acetate, 1-butanol and 1-hexanol, but not on ethanol. Thus, with respect to the physiological role of NDMA-ADH a broad function in alcohol oxidation could be imagined, but real insight is still lacking.

The formaldehyde oxidation step.

_A. methanolicus_ produces two NAD-dependent dehydrogenases able to oxidize formaldehyde: a general aldehyde dehydrogenase, and a factor-dependent
formaldehyde dehydrogenase (FD-FA1DH) [7, Chapter 6]. The aldehyde dehydrogenase (Fig. 1; step 3) is induced not only during batch-wise growth on methanol, but on other alcohols as well. Furthermore, the kinetic parameters indicate that higher aliphatic aldehydes are oxidized preferentially, so that it is unlikely that this enzyme will have a major role in formaldehyde oxidation. In addition, an exclusive role can be excluded by the fact that the enzyme is almost absent in cells grown fed-batch-wise on methanol.

FD-FA1DH (Fig 1; step 2) is specific for formaldehyde and is induced at growth on methanol, not on ethanol. Enzyme and factor also occur in the Gram-positive R. erythropolis [8] when grown on 3,4-dimethoxybenzoic acid (formaldehyde is generated by demethoxylation of the aromatic compound). Therefore, taking also the absence of GSH in Gram-positive bacteria [9], the similarity in enzyme structure, and the mode of operation [10] into account, the combination FD-FA1DH/Factor might be the Gram-positive counterpart of GSH-dependent formaldehyde dehydrogenase (GD-FA1DH)/GSH. However, not all Gram-positive methylo trophs use this pathway. Bacillus C1 and M. gastri MB19 oxidize formaldehyde via the ribulose monophosphate pathway [11, 12].

Both GD-FA1DH and FD-FA1DH can also be regarded as alcohol dehydrogenases since they show activity with higher aliphatic primary alcohols and have significant structural similarity with the long-chain, zinc-containing alcohol dehydrogenases. Since formaldehyde is not a substrate but its hemiacetal, in fact also for this reaction the enzymes act as an alcohol dehydrogenase. Tentative results for FD-FA1DH suggest that this activity has physiological significance and that the dual activity may originate from different active sites. Although the factor can be replaced by high concentrations of methanol (0.6 M) without the addition of a thiol compound (Fig.1; step 5), the high methanol concentrations required will probably not be met in vivo so that this methylformate yielding possibility may not have physiological significance.

A. methanolicum also possesses two dye-linked aldehyde dehydrogenases [7, Chapter 8] able to convert formaldehyde (Fig. 1; step 3): a DCPIP-linked enzyme and a Wurster's Blue-linked enzyme. The DCPIP-linked aldehyde dehydrogenase has not been purified but it might be related to the dye-linked aldehyde dehydrogenases described for Gram-negative bacteria [13 - 15]. The Wurster's Blue-linked enzyme is also able to oxidize formate esters and for reasons mentioned below, it is called formate ester dehydrogenase (FEDH). Since such an enzyme has already been detected in the Gram-negative Comamonas testosteroni [16], molybdoprotein aldehyde dehydrogenases may be widespread (a molybdoprotein aldehyde dehydrogenase in which the flavin is lacking has been detected in the anaerobe Desulfovibrio gigas [17]). Both
the DCPIP-linked and the Wurster's Blue-linked dehydrogenase have a broad substrate specificity and are also present in cells grown on higher alcohols. Therefore, they may have only a marginal role in oxidation of formaldehyde as such. Although MFF shows FAIDL activity, it seems structurally different from formaldehyde dismutase from *Ps. putida* F61 (EC 1.2.99.4) [18] and for reasons discussed below, it is unlikely that MFF has such a role in *A. methanolicum* (as assigned to FAIDL in *Ps. putida* F61 [18]).

The oxidation of formate and formate esters.

A dye-linked formate dehydrogenase activity (Fig. 1; step 6) occurs in cell free extracts of *A. methanolicum*. However, a low specific activity was found and the enzyme is rather labile. Since this excluded purification, no comparison can be made with the dye-linked formate dehydrogenases known from the literature.

It has been claimed that NAD-dependent formate dehydrogenase of *Achromobacter parvulus* is able to oxidize formyl-GSH ester [19]. However, no other esters were tested and in view of the preparation procedure used, contamination of formyl-GSH ester with formate cannot be completely ruled out. Moreover, no attempts were made to detect the putative "GSH-carbonate" product. Therefore, it is still unclear whether this formate dehydrogenase is really able to oxidize formate esters.

Substrate and product analysis proved that FEDH is able to "hydroxylate" formate esters to the corresponding carbonate esters, most probably including formyl-Factor ester (Fig. 1; step 7). This seems not to be an artifact since another molybdoprotein, milk xanthine oxidase, was unable to oxidize methylformate (P.W. van Ophem and J.A. Duine, unpublished results). Molybdoprotein aldehyde dehydrogenase has been isolated from ethanol-grown *C. testosteroni* and the enzyme also shows formate ester dehydrogenase activity. Since this organism is able to grow on methoxylated compounds [20], the formaldehyde formed might be converted into a "formate ester" (containing GSH), so that the enzyme might function as an FEDH also in this organism. On the other hand, FEDH shows excellent dehydrogenase activity for higher aliphatic aldehydes too. Therefore, FEDH might have a dual role, hydroxylating "aldehyde-like" formyl esters as well as genuine aldehydes. There are other examples of enzymes operating in methylotrophic dissimilation pathways which act at two levels of oxidation: methane monooxygenase is also able to oxidize methanol [21]; MDH as well as MFO are able to oxidize formaldehyde; FD-FAIDH and GD-FAIDH are able to oxidize primary alcohols (although not methanol, FD-FAIDH is able to oxidize the hemiketal of methanol and formaldehyde). Whether these dual substrate specificities
are just fortuitous (related to the fact that product can structurally mimic substrate by forming an adduct with a helper compound) or have physiological significance, remains a matter to be elucidated.

A novel pathway in $C_1$-dissimilation involving participation of formate esters?

Formate ester production in methylotrophs is well known since such a compound is found as reaction product of GD-FALDH. However, it has always been assumed that the formyl-GSH ester formed is hydrolysed by a specific hydrolase (EC 3.1.2.12), yielding formate which is oxidized by formate dehydrogenases. Perhaps for that reason, formate esters have not been included in the pathway of $C_1$-dissimilation by yeasts and Gram-negative bacteria.

NMR experiments [22] on whole cells incubated with formaldehyde showed that certain bacteria accumulate methylformate. These authors suggested that formation of the ester was catalyzed by formaldehyde dismutase, converting the hemiacetal of formaldehyde and methanol into this compound. However, the authors did not discuss how methylformate is converted (in this connection it is interesting to note that S-formylglutathione hydrolase is very specific [23] and most probably does not hydrolyse methylformate). Thus, although the absence of methylformate in certain bacteria was ascribed to a low level or the absence of formaldehyde dismutase, it could also be due to a high activity of a formate ester-converting enzyme.

Formate production in A. methanolicus can be catalyzed by the general aldehyde dehydrogenases (NAD- as well as dye-linked) and by the formaldehyde dismutase activity of FFF. However, only low activity of formate dehydrogenase was found in this organism. Moreover, as discussed already, FD-FALDH has a major role in formaldehyde conversion leading to significant formyl-Factor production for which no hydrolase activity was found to exist in cell free extracts. Therefore, it seems very likely that the formate ester oxidizing activity of FEDH is not fortuitous but has physiological significance in the oxidation of the formyl-Factor ester and perhaps also in that of methylformate, formed by oxidation of the methanol/formaldehyde hemiacetal adduct with MFF and FD-FALDH (Fig. 1; step 5). Since the aldehyde dehydrogenase of the Gram-negative C. testosteroni is very similar to FEDH, it would be interesting to search for such a system in non-Gram-positives to see whether this is an alternative for the formyl-GSH hydrolase system in certain organisms or under certain physiological conditions.
Summarizing, it is highly likely that formaldehyde dissimilation by *A. methanocella* follows a novel pathway in which formate esters are oxidatively converted. The occurrence of this pathway in other organisms and the possibility that S-formylglutathione is also converted in this way, remain to be elucidated.

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Summary.

In the Gram-positive methylo troph, *Amycolatopsis methanolica* (Nocardia sp. 239) three methanol-oxidizing activities for the conversion of methanol into formaldehyde have been found: 1. NAD-dependent, DCPIP-linked methanol dehydrogenase (n-MDH); 2. tetrazolium dye-linked alcohol dehydrogenase (TD-ADH); 3. methanol-oxidizing, formaldehyde-reducing, and formaldehyde dismutating enzyme (MFF). Previous work suggested that n-MDH activity resides in a multi-enzyme complex and that it is a quinoprotein. However, n-MDH activity could not reproducibly be detected and was unstable, these facts excluding purification. Two pyrroloquinoline quinone-containing proteins (quinoproteins) could be isolated from methanol-grown cells, not from cells grown on other substrates. Although this suggests that these proteins are involved in methanol dissimilation, no such an activity could be assigned to them (Chapter 2). Methanol-grown cells of *Bacillus* species and *Mycobacterium gastri* do not contain quinoproteins or n-MDH, indicating that these do not play a universal role in methanol dissimilation by Gram-positive bacteria. The relationship between n-MDH and other methanol-oxidizing enzymes and complexes of *A. methanolica* is discussed in Chapter 9.

TD-ADH activity was reproducibly detected and evidence was obtained that the enzyme may be associated with other redox proteins (Chapter 3). TD-ADH has a broad substrate specificity and it is also present in cells grown on other alcohols. Moreover, the activity is also found in some, but not all, other alcohol-grown Gram-positive bacteria, not in several Gram-negatives tested. Thus, the enzyme responsible for TD-ADH activity is not exclusive for *A. methanolica* or for methanol-conversion, although its distribution is rather restricted.

Using p-nitroso-N,N-dimethylaniline (NDMA) as electron acceptor, two alcohol dehydrogenases could be purified: MFF, able to convert primary alcohols, including methanol (Chapter 4), and NDMA-dependent alcohol dehydrogenase (NDMA-ADH), able to convert alcohols except methanol (Chapter 5). MFF is a decameric enzyme with subunits of $M_r$ 50 kDa and it contains bound NADP as cofactor. It has a broad substrate specificity and it is also present in cells grown on other substrates. The enzyme shows formaldehyde reductase (NADH-dependent) and formaldehyde dismutase activities and it is able to produce methylformate from methanol and formaldehyde. An MFF with very similar properties could be purified from methanol-grown *Mycobacterium gastri*. Methanol dehydrogenase from *Bacillus methanolicus* Cl shows
structural similarity with MFF, but this enzyme is not able to use NDMA as electron acceptor, nor does it show formaldehyde dismutase activity.

Purified NDMA-ADH (Chapter 5) also contains a bound nicotinamide (NAD(H)) as cofactor, but in contrast to MFF it is not able to oxidize methanol, to produce methylformate, nor does it show dismutase or reductase activity. It is a trimeric enzyme and the N-terminal amino acid sequence shows significant homology (56% identical residues) with horse liver alcohol dehydrogenase, an enzyme which uses NAD as coenzyme instead as cofactor.

Four different formaldehyde-oxidizing activities could be demonstrated in A. methanolica (Chapters 6, 7 and 8): 1. NAD-dependent aldehyde dehydrogenase (NA-AlDH); 2. NAD-, and factor-dependent formaldehyde dehydrogenase (FD-FADH); 3. Formate ester dehydrogenase (FEDH); 4. DCPIP-linked aldehyde dehydrogenase. Although the latter enzyme was not purified to homogeneity, a partial purified preparation showed that the enzyme has a broad substrate specificity and a rather low affinity for formaldehyde in vitro (K_m of 7.3 mM), while substrate inhibition was not observed (Chapter 6). NA-AlDH has a M_r of 200 kDa and its subunits of 55 kDa (Chapter 6).

Higher aliphatic aldehydes are better substrates than formaldehyde and substrate inhibition occurs. The level of NA-AlDH produced is strongly affected by the growth conditions: it is present at batch-wise growth on methanol, but almost absent at fed-batch-wise growth. It is also present in cells grown on other alcohols and, therefore, it is probably involved in general aldehyde oxidation. The enzyme is different from NAD-dependent (form)aldehyde dehydrogenases found in Pseudomonas putida C-83 or Hyphomicrobium X.

FD-FADH is specific for formaldehyde (although higher aliphatic alcohols can also be oxidized and, therefore, the enzyme belongs to the class III alcohol dehydrogenases) and the highest levels are observed in methanol-grown cells (Chapter 6). A low activity can be demonstrated in cells grown on higher aliphatic alcohols, which might indicate a physiological role in oxidizing these alcohols (Chapter 7). Activity with formaldehyde requires the presence of a heat stable, low molecular weight compound (Factor), which has to be in a reduced state (Chapter 7). The structure of the Factor is still unknown, but it can not be replaced by common SH-group containing compounds like glutathione (GSH), cysteine, lipoic acid or dithiothreitol. In analogy to GSH-dependent formaldehyde dehydrogenase (GD-FADH), which uses S-hydroxymethylglutathione as substrate, the true substrate for FD-FADH seems to be an adduct of the reduced Factor with formaldehyde. In that view, the reaction product is not free formate, but Factor-formyl adduct. High concentrations methanol can replace Factor and in this case the hemiacetal
of methanol and formaldehyde (CH$_3$-O-CH$_2$OH) is the most likely substrate. A similar or identical factor is present in the Gram-positive *Rhodococcus erythropolis*, grown on 3,4-dimethoxybenzoic acid. FD-FAldH from *A. methanolicus* has a $M_r$ of 120 kDa, consists of three subunits of 40 kDa and contains 6 atoms of Zn per enzyme molecule. This composition is very similar to that of FD-FAldH from *R. erythropolis*. Similarity also exists with GD-FAldH (subunits of $M_r$ 40 kDa; Zn-containing, 30% homology of the N-terminal sequence; the ability to use long-chain aliphatic alcohols). The presence of GSH has never been demonstrated in Gram-positive bacteria and, therefore, FD-FAldH may very well be the Gram-positive equivalent of GD-FAldH. However, the enzyme is absent in *R. methanolicus* C1 and *M. gastri*, organisms lacking a linear route for formaldehyde conversion so that FD-FAldH has no universal role in formaldehyde conversion by Gram-positives.

Hydrolase activity for the conversion of Factor-formyl adduct was not found in *A. methanolicus* and, in addition, formate dehydrogenase activities are low (Chapter 8). Therefore, conversion of the adduct proceeds most likely by the formate ester dehydrogenase (FEDH) to Factor-carbonate ester which will decompose spontaneously to Factor and CO$_2$. FEDH has a $M_r$ of 186 kDa and consists of two subunits of 89 kDa. FEDH contains 1 Mo, 1 FAD, 4 Fe and 3 - 4 S per enzyme molecule. It is not only able to convert formate esters, but aldehydes as well. This may indicate that the enzyme has a dual role, in accordance with the fact that *A. methanolicus* grown on higher aliphatic alcohols contains significant levels of FEDH. All properties investigated so far indicate that the enzyme is very similar to the molybdopterin aldehyde dehydrogenase previously isolated from *Comamonas testosteroni*.

The methanol oxidation step in Gram-positive methanol-utilizers occurs with a variety of enzymes which are dissimilar from PQQ-containing MDH of the Gram-negative ones. MFF is so far the only methanol-oxidizing enzyme purified from *A. methanolicus*. Although it is similar to the methanol dehydrogenase from *M. gastri*, for which no other methanol-oxidizing enzymes have been found, it is not clear whether MFF is the sole methanol oxidizing enzyme in *A. methanolicus* and whether it forms part of n-MDH and/or TD-ADH complexes (Chapters 2 and 3). The significance of all different aldehyde dehydrogenases found in this organism for formaldehyde conversion, is also unclear. Evidence was provided that FD-FAldH is a major formaldehyde-converting enzyme and that the Factor-formyl adduct formed could be oxidatively converted by FEDH. Indications exist that this route may be an alternative one for the GD-FAldH/hydrolase route observed in non-Gram-positives.
Samenvatting.

In de Gram-positieve methylotroof, Amycolatopsis methanolicus (Nocardia sp. 239) zijn drie methanol-oxidereende activiteiten voor de omzetting van methanol naar formaldehyde gevonden: 1. NAD-, en DCPIP-afhankelijk methanol dehydrogenase (n-MDH); 2. tetrazolium dye-afhankelijk alcohol dehydrogenase (TD-ADH); 3. methanol-oxidizing, formaldehyde-reducing, and formaldehyde dismutating enzym (MFF). Uit eerder werk kwam de suggestie voort dat de n-MDH activiteit aanwezig is in een multi-enzym complex en dat het een PQQ-bevattende enzym is. Echter, deze aktiviteit kon niet reproduceerbaar gemeten worden en was bovendien instabiel, zodat opzuivering niet mogelijk was. Twee pyrroloquinoline chinon (PQQ)-bevattende eiwitten (quinoproteïnen) konden worden opgezuiverd uit cellen gegroeid op methanol, maar deze waren niet aanwezig in cellen gegroeid op andere substraten. Dit is een indicatie dat deze eiwitten betrokken zijn bij methanol oxidatie, maar een dergelijke activiteit kon niet aan hen worden toegekend (Hoofdstuk 2). Methanol gegroeide cellen van Bacilli en Mycobacterium gastri bevatten geen quinoproteïnen of n-MDH, wat suggereert dat deze eiwitten geen algemene rol vervullen in methanol dissimilatie door Gram-positieve bacteriën. De relatie tussen n-MDH en andere methanol-oxidereende enzymen en complexen in A. methanolicus wordt bediscussieerd in Hoofdstuk 9.


Met p-nitroso-N,N-dimethylaniline (NDMA) als electron acceptor konden twee alcohol dehydrogenases worden opgezuiverd: MFF, wat primaire alcoholen, inclusief methanol, omzet (Hoofdstuk 4) en NDMA-afhankelijk alcohol dehydrogenase (NDMA-ADH), wat alcoholen, behalve methanol, omzet (Hoofdstuk 5). MFF is een decameer eiwit met subunits met een molecuul massa van 50 kDa en het bevat gebonden NADP als cofactor. Het heeft een brede substraat specificiteit en komt ook voor in cellen gegroeid op andere substraten. Het enzym vertoont formaldehyde reduktase activiteit (NADH-afhankelijk) en formaldehyde dismutase aktiviteit en het is tevens in staat om methanol en formaldehyde in methylformaat om te zetten. Een vergelijkbaar MFF kon worden opgezuiverd uit methanol-gegroeide M. gastri. Methanol dehydrogenase
uit *Bacillus methanolicus* C1 heeft structurele overeenkomsten met MFF, maar dit enzym kan geen NDMA als electron acceptor gebruiken en vertoont tevens geen formaldehyde dismutase activiteit.

Zuiver NDMA-ADH (Hoofdstuk 5) bevat ook een gebonden nicotinamide (NAD(H)) als cofactor, maar, in tegenstelling tot MFF, kan het geen methanol omzetten of methylformiaat produceren en het vertoont tevens geen formaldehyde dismutase of reductase activiteit. Het is een trimeer en de N-terminale aminozuur volgorde vertoont een significante homologie (56 % identieke residen) met paardelever alcohol dehydrogenase. Echter dit laatste enzym gebruikt NAD als co-enzym in plaats van als cofactor.

De aanwezigheid van vier verschillende formaldehyde-oxidereende activiteiten kan worden aangetoond in *A. methanolicum* (Hoofdstukken 6, 7 en 8): 1. NAD-afhankelijk aldehyde dehydrogenase (NA-ALDH); 2. NAD-, en factor-afhankelijk formaldehyde dehydrogenase (FD-FAIDH); 3. Mierenzuur ester dehydrogenase (FEDH); 4. DCPIP-afhankelijk aldehyde dehydrogenase. Ondanks dat dit laatste enzym niet gezuiverd werd, lieten gedeeltelijk opgezuiverde preparaten zien dat het enzym een brede substraat specificiteit heeft en dat de affiniteit voor formaldehyde vrij laag is ($K_m$ van 7.3 mM); tevens vertoonde dit enzym geen substraat inhibitie (Hoofdstuk 6). NA-ALDH heeft een molecuul massa van 200 kDa en bestaat uit vier subunits van 55 kDa (Hoofdstuk 6). Het zet bij voorkeur hogere alifatische aldehydes om en het vertoont substraat inhibitie. Het niveau van de NA-ALDH produktie wordt sterk beïnvloed door de groeiomstandigheden: gedurende batch-groei op methanol is het aanwezig, maar als cellen fed-batch gegroeid worden, is het praktisch afwezig. Het is ook aanwezig in cellen gegroeid op andere alcoholen en het lijkt daarom betrokken te zijn bij de omzetting van aldehydes in het algemeen. Het enzym verschilt van de NAD-afhankelijk (form)aldehyde dehydrogenases uit *Pseudomonas putida* C-83 of uit *Hyphomicrobium* X.

FD-FAIDH is specifiek voor formaldehyde (hoewel ook hogere alifatische alcoholen worden geoxideerd en het daarmee een alcohol dehydrogenase klasse III is) en de hoogste rivo's zijn aanwezig in cellen gegroeid op methanol (Hoofdstuk 6). Een lage activiteit wordt gevonden in cellen gegroeid op hogere alifatische alcoholen, wat misschien te maken heeft met een fysiologische rol in de omzetting van deze alcoholen. Voor activiteit met formaldehyde is het nodzakelijk dat een hitte stabiele, laag moleculaire stof (Factor), welke gereduceerd moet zijn, aanwezig is (Hoofdstuk 7). De structuur van de Factor is nog onbekend, maar het kan niet vervangen worden door bekende SH-groep bevattende stoffen, zoals glutathion (GSH), cysteine, lipoïnezuur of dithiothreitol. Analoog aan GSH-afhankelijk formaldehyde dehydrogenase (CD-FAIDH), wat S-hydroxymethylglutathion als substraat
gebruikt in plaats van formaldehyde, zal het werkelijke substraat voor FD-FALDH waarschijnlijk het adduct van Factor met formaldehyde zijn. Als gevolg hiervan is het reaktieprodukt niet vrij mierezuur, maar een Factor-formyl adduct. Hoge concentraties methanol kunnen de Factor vervangen en in dit geval zal het hemiacetaal van methanol en formaldehyde (CH$_3$O-CH$_2$OH) het waarschijnlijke substraat zijn. Een vergelijkbare of identieke factor is aanwezig in de Gram-positieve Rhodococcus erythropolis, gegroeid op 3,4-dimethoxybenzoëzuur. FD-FALDH uit A. methanolicus heeft een molecularmassa van 120 kDa, bestaat uit drie subunits van 40 kDa en bevat 6 Zn atomen per enzym molecul. Deze samenstelling lijkt sterk op dat van FD-FALDH uit R. erythropolis. Er zijn ook overeenkomsten met GD-FALDH (subunits van 40 kDa; Zn-bevattend; 30 % homologie van de N-terminale aminozuur volgorde; de mogelijkheid om hogere alifatische alcoholen om te zetten). GSH is nooit aangetoond in Gram-positieve bacteriën en daarom lijkt het aannemelijk dat FD-FALDH het Gram-positieve equivalent van GD-FALDH is. Echter, het enzym is afwezig in B. methanolicus GI en M. gastr (organismen die geen lineaire route voor formaldehyde omzetting bezitten) en FD-FALDH heeft dus geen universele rol in formaldehyde omzetting door Gram-positive bacteriën.

Hydrolase activiteit voor de omzetting van Factor-formyl adduct kon niet worden aangetoond in A. methanolicus en mierezuur dehydrogenase activiteiten zijn laag (Hoofdstuk 8). Het lijkt daarom waarschijnlijk dat omzetting van het adduct gebeurt middels een mierezuur ester dehydrogenase (FEDH) wat dan Factor-carbonaat oplevert; dit laatste valt vervolgens spontaan uiteen in Factor en kooldioxide. FEDH heeft een molecularmassa van 186 kDa en bestaat uit twee subunits van 89 kDa. Het bevat 1 Mo, 1 FAD, 4 Fe en 3 - 4 S per enzym molecul. Het enzym zet niet alleen mierezuur esters om, maar ook aldehydes. Dit kan een aanwijzing zijn dat FEDH een dubbelrol vervult, in overeenstemming met het feit dat in A. methanolicus gegroeid op hogere alifatische alcoholen, FEDH duidelijk kan worden aangetoond. Alle tot nu toe gevonden eigenschappen wijzen erop dat het enzym grote gelijkenis vertoont met het al eerder beschreven molybdopterne aldehyde dehydrogenase geïsoleerd uit Comamonas testosteroni.

De omzetting van methanol naar formaldehyde door Gram-positieve methanol gebruikers gebeurt met verschillende enzymen, welke allen verschillen van het PQQ-bevattend MDH uit de Gram-negative bacteriën. MFF is tot op heden het enige gezuiverde methanol-oxyderende enzym uit A. methanolicus. Ondanks dat het vergelijkbaar is met het methanol dehydrogenase uit M. gastr (waarvoor geen andere methanol omzettende enzymen gevonden zijn), is het niet duidelijk of MFF het enige methanol oxyderende enzym in A. methanolicus is en of het deel uitmaakt van de n-MDH en/of TD-ADH complexen (Hoofdstukken 2 en 3). De betekenis voor de omzetting van formaldehyde van alle
verschillende aldehyde dehydrogenases in dit organisme is ook nog onduidelijk. Het is aangetoond dat FD-FA1DH een belangrijk formaldehyde-omzettend enzym is en dat het Factor-formyl adduct wat daarbij gevormd wordt, oxidatief omgezet kan worden door FEDH. Dit zijn aanwijzingen dat een dergelijke route een mogelijk alternatief is voor de GD-FA1DH/hydrolase route, zoals die gevonden is in niet-Cram-positieve bacteriën.
List of publications.

ABSTRACTS:


PAPERS:


Van Ophem, P.W., and Duine, J.A. (1990) Different Types of Formaldehyde-Oxidizing Dehydrogenases in Nocardia Species 239. Purification and


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