Biochemical and functional characterization of Nudix hydrolase enzymes with novel regulatory roles in Gram positive methylotrophic bacteria

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Chapter 2

Nicotinoprotein methanol dehydrogenase enzymes in Gram-positive methylotrophic bacteria

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A novel type of alcohol oxidoreductase has been characterized from Gram-positive methylotrophic (*Bacillus methanolicus*, the actinomycetes *Amycolatopsis methanolica* and *Mycobacterium gastri*) and non-methylotrophic bacteria (*Rhodococcus* strains). Its *in vivo* role is in oxidation of methanol and other primary alcohols.
***B. methanolicus* displays activity of an NAD-dependent methanol dehydrogenase (MDH), which is strongly stimulated by a specific (activator) protein. *A. methanolica* and *M. gastri* use an N,N'-dimethyl-4-nitrosoaniline (NDMA)-dependent MDH (methanol: NDMA oxidoreductase; MNO).**
MDH (43 kDa subunit) and MNO (49 kDa subunit) possess similar decameric structures with five-fold symmetry. Both proteins contain Zn^{2+}- and Mg^{2+}-ions and tightly (but noncovalently) bound NAD(P)(H) cofactors. These nicotinoproteins share a high degree of sequence similarity; they belong to Family III of NAD(P)-dependent alcohol dehydrogenases (ADH).
***A. methanolica* extracts also possess dye (dichlorophenol indophenol, DCPIP and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide, MTT)-linked ADH activities. These represent the overall activities of multi-enzyme systems. MNO is part of the MTT-ADH complex. The other two proteins and their cofactors most likely participate in transfer of reducing equivalents from the NADPH cofactor in MNO to the respiratory chain.**

Introduction

Methanol is formed in large quantities in mineralization processes in nature, mostly from degradation of the methyl esters and -ethers that occur in plant components such as pectin and lignin. Methylotrophic microorganisms able to grow on methanol as sole carbon- and energy source have been isolated frequently from soil samples. These organisms possess special metabolic pathways allowing generation of energy from methanol oxidation and synthesis of compounds with carbon-carbon bonds from methanol assimilation (Anthony, 1982;Dijkhuizen *et al.*, 1992). The techniques employed for their isolation generally select for the fastest growing organisms and in most cases this has resulted in isolation of pure cultures of Gram-negative methylotrophic bacteria. Little attention has been paid to the large diversity of methylotrophic Gram-positive bacteria that are relatively slow growing (e.g. actinomycetes) or require special growth conditions (e.g. thermotolerant Bacilli). Physiological and biochemical studies of primary alcohol metabolism in actinoycetes and Bacilli have provided evidence for the involvement of novel alcohol dehydrogenase (ADH) enzyme systems. Current knowledge of these enzymes is reviewed.

Enzymes involved in methanol oxidation

Methylotrophic yeasts (e.g. *Hansenula polymorpha*) employ an alcohol oxidase (EC 1.1.3.13) that contains FAD as cofactor and is localized in special cell organelles, the microbody or peroxisome (Harder and Veenhuis, 1989;Woodward, 1990). The enzyme catalyzes the oxidation of methanol into formaldehyde and transfers the electrons derived to oxygen, resulting in hydrogen peroxide formation. The subunit size of the usually octameric alcohol oxidase enzymes is 72-75 kDa and each subunit contains a noncovalently bound FAD cofactor molecule.

Methanol oxidation in Gram-negative bacteria involves a periplasmic quinoprotein methanol dehydrogenase (MDH; EC 1.1.99.8) (Anthony, 1982;Anthony, 1986). This enzyme uses pyrroloquinoline quinone (PQQ) as cofactor (Duine and Frank, Jr., 1980). MDH is directly connected to the electron transport chain. The interactions with the special *c*-type cytochromes

involved are known in much detail, complete with crystal structures. MDH is an $\alpha_2\beta_2$-tetrameric enzyme, with α-subunits of 66 kDa and β-subunits of 8.5 kDa in for instance *Methylobacterium extorquens* AM1. The α-subunit contains one molecule of the PQQ cofactor and one Ca^{2+}-ion in the active site (Anthony *et al.*, 1994).

In contrast to the well-known enzymology and genetics of methanol oxidation in methylotrophic yeasts and Gram-negative methylotrophic bacteria, little is known about the enzymes of methanol oxidation in Gram-positive methylotrophs. These bacteria lack a clear periplasmic space and generally donot possess PQQ. Several papers (Hazeu *et al.*, 1983;Duine *et al.*, 1984;van Ophem and Duine, 1990) provide evidence that methanol induces PQQ synthesis in *Amycolatopsis methanolica*; its physiological role has remained unclear, however. The presence of ADHs active with methanol has been studied in a few Gram-positive isolates only. In *Corynebacterium* sp. XG MDH activity could be detected with phenazine methosulphate as artificial electron acceptor (Bastide *et al.*, 1989). *Brevibacterium methylicum* was reported to possess NAD-dependent MDH activity (Nesvera *et al.*, 1991). In the methylotrophic actinomycete strain 381 a dichlorophenol indophenol (DCPIP)-dependent MDH was detected (Eshraghi *et al.*, 1990). No further characteristics of these systems have been published. Studies with thermotolerant *Bacillus methanolicus* strains (Dijkhuizen *et al.*, 1988;Arfman *et al.*, 1992b), *A. methanolica* (De Boer L. *et al.*, 1990a) and *Mycobacterium gastri* (Kato *et al.*, 1988) have shown that these organisms employ NAD(P)-dependent ADHs. These enzymes display unusual properties, constituting novel ADHs with bound NAD(P)(H) cofactors (previously reviewed by Bystrykh et al. 1993) (Bystrykh *et al.*, 1993a).

Three families of NAD(P)-dependent ADHs (EC 1.1.1.1) have become established (Jornvall *et al.*, 1987;Reid and Fewson, 1994;de Vries *et al.*, 1992). Feature characteristics for members of Family I (medium-chain dehydrogenases / reductases) are: zinc-dependency, di- or tetrameric quaternary structures and usually a subunit size of 43 kDa. Horse liver ADH is a family I enzyme and has been studied in most detail. Family I ADHs show no, or relatively low, activities with methanol. One exception is the methanol-oxidizing ADH of *Bacillus stearothermophilus* strain DSM 2334, a non-methylotrophic bacterium (Sheehan *et al.*, 1988). Members of Family II are metal-independent and possess relatively short primary structures of on average 240 amino acids, and are referred to as SDR** enzymes. Family II ADHs display a broad substrate specificity and have diverse metabolic roles. There are no reports of their involvement in methanol oxidation in methylotrophic bacteria, however. Members of Family III initially were referred to as iron-dependent ADHs. With an increasing number of members of this family identified, the iron-dependency appeared not to be a common property, however. Other metal-ions, such as zinc or magnesium instead of iron, were detected in some of these enzymes. A large number of ADHs were classified as belonging to Family III on the basis of sequence similarity and subunit sizes (325-441 amino acids, on average 391 residues) (de Vries *et al.*, 1992;Hektor, 1997). A recent database screening for Family III ADH enzymes resulted in identification of a total of 27 members, 24 of which have been fully sequenced (Hektor, 1997). These enzymes are found in alcohol-producing and alcohol-consuming micro-organisms, in Gram-negative and Gram-positive bacteria, in aerobic and anaerobic bacteria, in Archaea, in yeasts and amoeba. The various enzymes differ widely in specificity for alcohol substrates. Interestingly, in recent years several members of Family III have been detected in Gram-positive methylotrophic bacteria. These enzymes are active with methanol and *in vivo* catalyze the oxidation of methanol to formaldehyde.

MDH from *B. methanolicus* C1

B. methanolicus C1 is a thermotolerant bacterium able to grow on methanol at temperatures upto 60°C. NAD-dependent MDH constitutes up to 22% of total soluble protein in cells of *B.*

methanolicus strain C1 grown under methanol-limiting conditions in continuous cultures at low dilution rates (Arfman *et al.*, 1989;Arfman *et al.*, 1992a). MDH from *B. methanolicus* oxidizes C_1-C_4 primary alcohols as well as 1,3-propanediol, and also catalyzes NADH-dependent aldehyde reductase reactions. Further biochemical and electron microscopic studies revealed that its structural, kinetic, and mechanistic properties are unique. MDH consists of 10 identical subunits of M_r 43,000, arranged in a 'sandwich' of two pentagonal rings (Vonck *et al.*, 1991). Each subunit contains one zinc, one to two magnesium ions. Zinc is commonly found in the active site of NAD-dependent ADH enzymes but the presence of magnesium had not been reported before. In addition, each MDH subunit contains a tightly (but non-covalently) bound NAD(H) molecule that is not released during catalysis. Such proteins have been labelled nicotinoproteins by van Ophem and Duine (1993) (van Ophem and Duine, 1993).

Nicotinoproteins form a recently recognized class of redox enzymes with NAD(P) as firmly bound cofactor. They catalyze a large variety of reactions, including transhydrogenation, isomerization, dismutation, epimerization of their substrates, and oxidation of primary and secondary alcohols (Hektor, 1997;van Ophem and Duine, 1993;Piersma, 1998). Their mechanistic and functional properties remain to be elucidated. A total of 10 nicotinoproteins, using various substrates, have been described from various sources (Bacteria, Eukarya, Archaea). There is increasing evidence that they occur widespread but they are easily overlooked because of the unusual activity assay conditions required (Hektor, 1997;Piersma, 1998;Li and Stevenson, 1997).

B. methanolicus MDH is the first example of a naturally occurring NAD-dependent ADH containing cofactor NAD(H). UV-spectrophotometry showed that this NAD(H) molecule is redox active and functions as a cofactor; it is oxidized and reduced by formaldehyde and methanol, respectively, while it remains bound to the enzyme (Arfman *et al.*, 1997). Activity of MDH strictly requires exogenous NAD (coenzyme) in addition to bound NAD(H) (cofactor). No incorporation of label in MDH protein was observed in experiments with radioactively labelled coenzyme NAD, indicating that these two NAD(H) species are not exchanged during catalysis (Arfman *et al.*, 1997). NAD thus plays two different and important roles in the MDH reaction, with cofactor NAD acting as primary electron acceptor and coenzyme NAD being responsible for reoxidation of the NADH cofactor. MDH obeys a ping-pong type reaction mechanism, which implies that the alcohol substrate and coenzyme NAD bind sequentially to the enzyme and that the first product is released from the enzyme before binding of the second substrate. This is consistent with a mechanism involving a temporary deposit of reducing equivalents at the MDH-bound cofactor. In contrast, NAD-ADH enzymes lacking a bound NAD(P)(H) cofactor obey a sequential reaction mechanism that proceeds via a ternary enzyme-substrate-nucleotide complex (Sekhar and Plapp, 1990).

The affinities of MDH for alcohol substrates and exogenous NAD, as well as the V_{max} values, are strongly increased by a soluble activator protein (50 kDa, consisting of two 27 kDa subunits). The activator protein only affects the forward reaction of MDH and strictly requires the presence of exogenous NAD and Mg^{2+}-ions. Activation may result in a 40-fold overall increase in the methanol turnover rate of MDH at physiological methanol concentrations (0.1-1.0 mM) (Arfman *et al.*, 1991). The purified activator protein does not contain a cofactor, but is able to bind 1 molecule of NAD(H) per subunit. The activator protein can form a (loose) protein complex with MDH, but the interaction only occurs in the presence of exogenous NAD plus magnesium ions, indicating that these low molecular weight components are part of the MDH-activator protein complex. The activator protein changes the steady state kinetics of the MDH reaction from ping-pong to ternary complex type. These observations, in combination with the structural and kinetic properties of MDH, have led to the postulation that the activator protein facilitates the reoxidation of the bound NADH cofactor (Arfman *et al.*, 1997). This model

implies that the NAD-activator protein complex is a more favourable electron acceptor than free NAD and that the transhydrogenase reaction, resulting in reoxidation of the NADH cofactor, is the rate-limiting step in alcohol oxidation.

The structural MDH gene of *B. methanolicus* strain C1 has been cloned and sequenced (de Vries *et al.*, 1992). The deduced MDH amino acid sequence was found to share significant identity with *Zymomonas mobilis* ADH2, *Saccharomyces cerevisiae* ADH4, *Escherichia coli* ADHE and 1,2-propanediol oxidoreductase and *Clostridium acetobutylicum* ADH1. The latter enzymes do not oxidize methanol and contain zinc or iron. Since the MDH related enzymes clearly differ from the horse liver ADH-type and the SDR ADH-type (Jornvall *et al.*, 1987;Reid and Fewson, 1994), we have adopted the designation Family III for this group of enzymes (de Vries *et al.*, 1992). A new NAD(P)(H) binding domain has been identified in these enzymes (Hektor, 1997).

Recently, we have cloned and characterized the *B. methanolicus* gene encoding the activator protein (Chapter 3). The data show that the activator protein is a member of the family of Nudix hydrolases which all hydrolyze a *nu*cleoside *di*phosphate linked to some other group, (Bessman *et al.*, 1996). The precise mechanism of the activator protein remains to be determined (Chapter 3). The molecular tools that recently have become available for *B. methanolicus* (Cue *et al.*, 1997) may allow disruption of the gene encoding the activator protein. A study of the physiology of growth of such a mutant strain on methanol and other primary alcohols should provide insights in the *in vivo* relevance of the MDH/activator protein couple.

MDHs from *A. methanolica* and *M. gastri*

A. methanolica is a nocardioform actinomycete, showing the characteristic formation of a pseudomycelium on solid media, presence of spores and high GC content of DNA (60-70 mol%) (Hazeu *et al.*, 1983;De Boer L. *et al.*, 1990a;Kato *et al.*, 1975). It is the first methylotrophic actinomycete characterized. For a number of years it has proven to be difficult to identify the enzymes involved in methanol oxidation in this organism. Following its isolation from soil of New Guinea, Kato et al. (Kato *et al.*, 1975) reported the presence of low PMS/DCPIP-dependent MDH activity, which was measured at pH 7.0 in the absence of ammonium ions. Duine et al. (1984) (Duine *et al.*, 1984) subsequently provided evidence for the presence of a DCPIP-dependent MDH, the activity of which could be measured at pH 9.0. This MDH activity strictly required the presence of ammonium ions and NAD, but accumulation of free NADH could not be detected. DCPIP-MDH activity was detected in methanol-grown cells, producing relatively large amounts of PQQ. Several quinoproteins have been detected in *A. methanolica* but thus far no physiological functions could be assigned to any of them (van Ophem and Duine, 1990). It was suggested that this MDH forms part of a loose multi-enzyme complex, together with an NAD-dependent formaldehyde dehydrogenase and an NADH dehydrogenase (Duine *et al.*, 1984). The complex appeared rather unstable, however, and MDH activity could not be reconstituted from its components. A similar MDH activity was demonstrated in extracts of methanol-grown cells of the actinomycete strain 381 (Eshraghi *et al.*, 1990). Only recently, we have been able to develop reproducible assay conditions for the DCPIP-dependent MDH activity in *A. methanolica*. The components involved remain to be identified and characterized.

Further progress became possible following the observation by van Ophem et al. (1991) (van Ophem *et al.*, 1991) of a tetrazolium dye (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide, MTT)-linked ADH activity in extracts of methanol-grown cells of *A. methanolica*. MTT-ADH was stably maintained and could be assayed reproducibly. This enzyme system showed activity with methanol and various other alcohols. MTT-ADH activity strongly increased with increasing ionic strength (at high phosphate and sulphate

concentrations) of the buffer solution. The relationship between the amount of extract and activity was non-linear. MTT-dependent activity was completely lost following chromatographic fractionation of crude extracts. Activity could be restored, however, by combining separate fractions of the eluate, indicating that this MTT-MDH represents the overall activity of a multienzyme system. Biochemical (van Ophem *et al.*, 1991;Bystrykh *et al.*, 1993b;Bystrykh *et al.*, 1993c;Bystrykh *et al.*, 1997) and mutant (Hektor and Dijkhuizen, 1996) evidence has been obtained showing that MTT-ADH constitutes a complex of three different components which have been purified. Component 1 has been identified as methanol:NDMA oxidoreductase (MNO, see below for more details) (Bystrykh *et al.*, 1993b;Bystrykh *et al.*, 1993c). Component 2 is a high molecular weight (>640 kDa) protein with subunits of 44 and 72 kDa, which possesses a low MTT-dependent NADH dehydrogenase activity. The protein contains a yellow chromophore of unknown identity. Component 3 is a low molecular mass (15 kDa) protein (no separate activity identified) containing a 5'-deazaflavin and at least one other low-molecular mass compound with properties similar, but not identical, to those of nicotinamide coenzymes (Bystrykh *et al.*, 1997). MTT-ADH activities have been reported for the methylotrophs *A. methanolica* and *M. gastri*, and the methanol-oxidizing (but non-methylotrophic) bacteria *Rhodococcus erythropolis* and *R. rhodochrous* (van Ophem *et al.*, 1991).

MNO constitutes the first single protein with MDH activity identified in *A. methanolica* (and in *M. gastri*) (Bystrykh *et al.*, 1993b;Bystrykh *et al.*, 1993c). Also the ThcE enzyme of *Rhodococcus* sp. NI86/21 is very similar to these two MNO proteins, but the latter organism is unable to grow on methanol; ThcE is induced by atrazine and thiocarbamate herbicides (Nagy *et al.*, 1995). NDMA is known to reoxidize pyridine nucleotides which are tightly bound to the active centers of dehydrogenases (Dunn and Bernhard, 1971;Kovar *et al.*, 1984). The MNO proteins of *A. methanolica* and *M. gastri* use methanol as well as formaldehyde as substrates (Bystrykh *et al.*, 1993b;Bystrykh *et al.*, 1993c;Bystrykh *et al.*, 1997). The *in vivo* electron acceptor of these enzymes remains unknown. MNO failed to oxidize methanol in the presence of the artificial electron acceptors DCPIP or MTT. No evidence was obtained for the presence of an activator-like protein in these organisms and addition of *B. methanolicus* activator protein to crude extracts did not result in appearance of NAD-dependent MDH activity. The enzymatic properties of the MNO enzymes differ from *B. methanolicus* MDH in various other aspects as well (Bystrykh *et al.*, 1993b;Bystrykh *et al.*, 1993c). The MNO enzymes not only catalyze the NDMA-linked oxidation of methanol and formaldehyde but also NADH-dependent formaldehyde reductase and formaldehyde dismutase (yielding formaldehyde and formate from methanol (Kato *et al.*, 1986)) reactions. *B. methanolicus* MDH on the other hand displayed NAD-dependent MDH and NADH-dependent formaldehyde reductase activities but was inactive with NDMA; it also failed to catalyze the formaldehyde dismutase reaction.

Analysis of the quaternary protein stuctures of the purified *A. methanolica* and *M. gastri* MNO enzymes (subunit M_r 49,000-50,000) by electron microscopy and image processing (Bystrykh *et al.*, 1993c) revealed that these MNO proteins are strikingly similar to *B. methanolicus* MDH (i.e. decameric structures displaying five-fold symmetry). The three proteins are also similar with respect to their metal composition (Zn^{2+}- and Mg^{2+}-ions) and the presence of a bound pyridine nucleotide cofactors (NADPH in case of both MNO enzymes). Characterization of the *mno* gene of *A. methanolica* and the deduced amino acid sequence of MNO, and N-terminal amino acid sequence analysis of the *M. gastri* MNO protein, allowed further comparison with *B. methanolicus* MDH. The data show that the three enzymes also share a high degree of amino acid sequence similarity (42%) and all three clearly belong to the Family III ADH enzymes.

The mutant data obtained (Hektor and Dijkhuizen, 1996) provided clear evidence that

at least the MNO and component 2 of the protein complex with MTT-ADH activity play a crucial role in utilization of primary alcohols (C1-C4) in *A. methanolica*. MNO may play a role in the initial oxidation of these alcohols. Since component 2 displays low NADH dehydrogenase activity with MTT, it is tempting to speculate that it functions as an electron acceptor for MNO, with component 3 acting as mediator in this. How and whether the further redox components present (5'-deazaflavin, the nicotinamide-like compound, and the yellow chromophore) act as cofactors in this complex remains to be elucidated. Also the coupling of the MTT-ADH to the electron transport chain awaits investigation.

Tools for the genetic modification of *A.methanolica* have become available in recent years (Vrijbloed *et al.*, 1995d). Further work will focus on the construction of an *A.methanolica* strain expressing a mutant MNO protein lacking bound cofactor NADPH (Hektor, 1997). This will allow an interesting analysis of the relevance of the NADPH cofactor in MNO on the physiology of growth on methanol and other primary alcohols.

Concluding remarks

The identification and characterization of structurally similar, NAD(P)H-containing MDHs from *B. methanolicus*, *A. methanolica* and *M. gastri* shows that a new type of methanol-oxidizing enzymes is employed by these Gram-positive methylotrophs. All enzymes involved in methanol oxidation investigated thus possess a tightly bound cofactor (FAD in yeast alcohol oxidase; PQQ in quinoprotein MDH; NAD in *Bacillus* MDH; NADP in the MNO enzymes), suggesting that a temporary deposit for reduction equivalents is a prerequisite for methanol-converting ADHs. Under *in vivo* conditions both MDH of *B. methanolicus* and MNO of *A. methanolica* and *M. gastri* appear to be associated with additional proteins. Our current knowledge indicates that these proteins participate in the reoxidation of the NAD(P)H cofactors, most likely resulting in transfer of reducing equivalents to NAD coenzyme and/or to the electron transport chain. The DCPIP-MDH and MTT-ADH complexes in *A.methanolica* may share one or more components, although DCPIP-MDH is highly specific for methanol and MTT-ADH shows much broader substrate specificity.

Further biochemical investigations are required in order to obtain a full understanding of the nature of the methanol oxidation pathways in Gram-positive, methanol-utilizing bacteria.