Chapter 7

Summary and concluding remarks
This thesis describes characteristics of the novel enzyme systems involved in oxidation of primary alcohols in Gram-positive bacteria. The studies focussed on two facultatively methylotrophic, methanol-utilizing soil bacteria, the nocardioform actinomycete *Amycolatopsis methanolica* and the thermotolerant bacterium *Bacillus methanolicus*. Methanol is abundantly produced in mineralization processes in nature and used by many micro-organisms as carbon- and energy source for growth. Methylotrophic bacteria possess special metabolic adaptations, allowing generation of energy from methanol oxidation and synthesis of compounds with carbon-carbon bonds from methanol assimilation. Both bacteria studied have the highest growth yields on methanol reported, employing the most efficient pathway for one-carbon assimilation, the Ribulose monophosphate (RuMP) pathway of formaldehyde fixation, and NAD(P)-dependent methanol dehydrogenase enzymes. The initial assimilation of formaldehyde via the RuMP cycle yields hexulose-6-phosphate. Further steps result in the net synthesis of one molecule of glyceraldehyde 3-phosphate from three molecules of formaldehyde (Chapter 1).

Current knowledge of bacterial methanol oxidation is based almost exclusively on studies with Gram-negative methylotrophs; studies of Gram-positive methylotrophs have been fairly limited. The methanol-oxidizing system in Gram-negative bacteria is located in the periplasm and employs PQQ as cofactor. This prompted questions about the nature and location of the comparable systems in Gram-positive bacteria which lack a clear periplasmic space and generally do not possess PQQ. Exceptionally, several papers (Hazeu *et al*., 1983; Duine *et al*., 1984a; Van Ophem and Duine, 1990b) provide evidence that methanol induces PQQ synthesis in *A. methanolica*; its physiological role has remained unclear also in the present study. This thesis focusses on the primary step in methanol oxidation, the oxidation of methanol to formaldehyde, in Gram-positive bacteria. The data show that these organisms employ novel cytoplasmic enzymes that are not only responsible for methanol oxidation but also for oxidation of various other aliphatic primary alcohols.

Crude extracts of methanol-grown cells of *A. methanolica* did not reduce coenzyme NAD(P) upon addition of methanol and other primary alcohols. Artificial electron acceptors were necessary for the *in vitro* oxidation of these alcohols. Three different suitable electron acceptors have been identified over the years, namely DCPIP (n-MDH activity) (Duine *et al*., 1984a; Van Ophem and Duine, 1990b), MTT (MTT-ADH activity) (Van Ophem *et al*., 1991), and NDMA (MNO activity) (Bystrykh *et al*., 1993a, b; Chapter 2). n-MDH activity was the result of an instable complex, specific for methanol and formaldehyde and not for other alcohols, and was stimulated by addition of NAD. Due to its
instability attempts to carry out a detailed characterization failed. MTT-ADH activity also was based on a protein complex, active towards a range of alcohols, and much more stable than n-MDH, allowing more detailed studies (Bystrykh et al., 1997).

The characterization of an *A. methanolica* protein with (a) high formaldehyde dismutase activity (yielding methanol and formate from two molecules of formaldehyde), (b) a relatively low methanol-oxidizing activity with NDMA as electron acceptor (MNO), and (c) NADH-dependent formaldehyde dehydrogenase activity, is presented in Chapter 2 (Bystrykh et al., 1993b). This decameric protein with subunits of 50 kDa not only oxidized methanol but also a range of other alcohols. A similar protein was isolated from *Mycobacterium gastri* MB19, another Gram-positive methylotrophic bacterium (Bystrykh et al., 1993b; Chapter 2). A third example of MNO was identified in *Rhodococcus* NI86/21, most likely oxidizing aldehydes in the degradation of herbicides (Nagy et al., 1995). The data thus indicate a more widespread occurrence of MNO.

The physiological importance of MNO in *A. methanolica* remained questionable. The characterization of mutants unable to grow on methanol and/or ethanol provided further insights (Chapter 3). Methanol-negative mutants which could still grow on ethanol all possessed MNO and MTT-ADH activities; they lacked key enzymes of the RuMP cycle, hexulose-6-phosphate synthase (HPS) or hexulose-6-phosphate isomerase (HPI). Mutants unable to grow on methanol as well as on ethanol, however, generally had lost both MTT-ADH and MNO activities. This raised the question whether MNO was one of the components of the MTT-ADH complex. Further support for this was obtained in biochemical experiments (Bystrykh et al., 1997), also demonstrating the involvement of two other components, proteins H and L (Fig. 1). The high molecular weight protein H (>600 kDa) shows weak MTT-dependent NADH dehydrogenase activity and consists of two types of subunits (70 and 45 kDa). No activity could be assigned to protein L (15 kDa). Following purification of these three proteins, MTT-ADH activity could be restored by adding the components together again (Bystrykh et al., 1997). It appears likely that in vivo MNO is oxidizing primary alcohols and transfers the reducing equivalents via proteins L and H, and maybe other unknown proteins, to the electron transport chain. Mutant strain MM49, unable to grow on methanol and ethanol, still possessed MNO activity but lacked MTT-ADH activity and protein H, providing evidence for the essential role of this component in the metabolism of primary alcohols. All mutants unable to grow on ethanol still possessed ethanol:NDMA oxidoreductase (ENO) activity, ruling out the suggested role of ENO in ethanol utilization in *A. methanolica* (Van Ophem, 1993).
Subsequent characterization of the MNO-encoding gene of *A. methanolica* allowed construction of an *mno* disruption mutant (Chapter 4). This mutant failed to grow on C1-C4 primary alcohols. Re-introduction of the wild type *mno* gene restored growth. These data provide conclusive genetic evidence that MNO is essential for the metabolism of methanol and other primary alcohols. The characterization of the *mno* gene confirmed earlier suggestions made on the basis of limited amino acid sequence information that MNO belongs to Family III of NAD(P)-dependent ADHs, initially referred to as the iron-dependent ADHs (Chapter 4). Increasingly, it becomes clear that not all members of this family are iron-dependent and identification of member proteins has become based on overall sequence similarity. MNO contains Zn$^{2+}$- and Mg$^{2+}$-ions instead of iron and carries a tightly bound redox-active NADP in every subunit (Bystrykh *et al.*, 1993a). Database searches identified a total of 24 members of Family III that have been fully sequenced. Three unique conserved sequence motifs were
identified in these proteins, aiding in classification of additional members and in further studies of protein structure and function relations (Chapter 4).

Five members of Family III ADHs show clear resemblances in their quaternary structures. These are MNO of *A. methanolica* and MDH of *B. methanolicus* (fully sequenced) and MNO of *M. gastri*, ADH of *Desulfovibrio gigas* and ADH of *Desulfovibrio HDv* (only amino-terminal protein sequences available). All five are decameric proteins, showing a five-fold symmetry; the first three of these proteins contain tightly bound NAD(P)(H) cofactors (De Vries *et al.*, 1992; Bystrykh *et al.*, 1993a; Hensgens *et al.*, 1993, 1995; Chapter 4). The methanol-oxidizing enzymes identified in methylotrophs thus all possess cofactors, enabling ping-pong type kinetics: FAD in alcohol oxidase of methylotrophic yeasts, PQQ in MDH of Gram-negative methylotrophic bacteria, NAD(P)(H) in MNO and MDH of Gram-positive methylotrophic bacteria. Each of these enzymes displays ping-pong type of kinetics (Bystrykh *et al.*, 1993b; Arfman *et al.*, 1997; Chapters 2, 5). Activity of MDH of *B. methanolicus* requires exogenous NAD (coenzyme) in addition to cofactor NAD(H). The ping-pong type of mechanism of MDH implies that the alcohol substrate and coenzyme NAD bind sequentially to the enzyme. This is consistent with a mechanism involving a temporary deposit of reducing equivalents at the MDH-bound cofactor. In contrast, NAD-dependent ADHs generally lack a cofactor and obey a reaction mechanism which involves simultaneous binding of the alcohol and NAD substrates (formation of a ternary substrate complex). Methanol oxidation indeed reduces the cofactor NAD; coenzyme NAD is responsible for re-oxidation of the NADH cofactor. These two NAD(H) molecules are not exchanged during the reaction (Arfman *et al.*, 1997; Chapter 5). MDH shows a relatively low NAD-dependent MDH activity which is strongly stimulated by a soluble activator protein of 50 kDa; the activation process strictly requires exogenous NAD and Mg^{2+}-ions. These Mg^{2+}-ions appear to be essential for the formation of a MDH-activator protein-NAD complex. This may result in a 40-fold increase in methanol turnover rate of MDH at physiological methanol concentrations (Arfman *et al.*, 1991). Characterization of the gene encoding the activator protein revealed the presence of the conserved sequence motif of the MutT family (Koonin, 1993; Kloosterman *et al.*, 1997). The precise mechanism of the activator protein remains to be elucidated. Interestingly, the activator protein does not affect the formaldehyde reductase activity of MDH.

No activator protein was found associated with MNO of *A. methanolica*. Moreover, the *B. methanolicus* activator protein did not stimulate the *A. methanolica* MNO. MNO is part of a three component complex showing MTT-ADH activity; in this case the associated protein H and protein L may be
involved in re-oxidation of the NADPH cofactor in MNO (Bystrykh et al., 1997). MTT-ADHs appear to be more widespread in Gram-positive bacteria. At the moment we can only speculate about the possible advantages of employing the cofactor NAD(H)-dependent MDH and MTT-ADH like enzyme systems for Gram-positive bacteria. Most fundamentally, ADHs of Families I and II generally lack such NAD(H) cofactors and virtually all these enzymes also show very poor activity, if at all, with methanol. Characteristics of the various ADH proteins determining methanol substrate specificity remain to be investigated. Compared to PQQ-MDH or FAD-alcohol oxidase, the NAD(P)-dependent ADHs are clearly more favourable with respect to energy generation from methanol oxidation, reflected in the high growth yields on methanol of the Gram-positive methylotrophs. The situation is more complicated, however, because the product of methanol oxidation, formaldehyde, is already lethal to the cell at relatively low concentrations (1 mM). Methylotrophic Gram-negative bacteria and yeasts have overcome the formaldehyde toxicity by compartmentalizing methanol oxidation in the periplasm and in a special organelle, peroxisomes, respectively. Such compartments are absent in Gram-positive bacteria and they have to solve this problem differently, by accurately tuning the production and consumption of formaldehyde. The presence of multiple aldehyde oxidizing enzymes in A. methanolica (Van Ophem, 1993) and the high activity levels of HPS and HPI in A. methanolica and B. methanolicus (Arfman et al., 1989; De Boer et al., 1990b) for instance may serve as protective mechanisms against formaldehyde accumulation. Alternatively, MDH and MNO may be controlled accurately at their activity levels. Feedback inhibition control at these steps, as reported in Arthrobacter P1 for the conversion of methylamine to formaldehyde by amine oxidase (Levering et al., 1984), has not been observed. Conceivably, interaction of the activator protein with MDH, and the presence of MNO in a complex, provide additional levels of flux control. In case of MDH, evidence for this was obtained when characterizing mutant S97G (see below). In case of MNO at least the associated protein H may be involved in channeling of reducing equivalents to the in vivo electron acceptor. In vivo, additional components may be associated with this complex, also allowing channeling of formaldehyde. The presence of a formaldehyde dehydrogenase in the complex would allow its detoxification in situ. Duine et al. (1984a) reported that the A. methanolica n-MDH complex indeed involves a formaldehyde dehydrogenase. The precise relationships between the n-MDH and MTT-ADH complexes remain to be established.

Multiple alignment of 24 Family III ADHs resulted in identification of three conserved sequence motifs (Chapter 4). Most likely, the amino acids constituting
these motifs are involved in catalysis, or binding of metals, substrates, or product. One of these motifs is rich in His residues and is thought to be involved in metal-binding (De Vries et al., 1992; Cabisco et al., 1994; Tamarit et al., 1997). A second motif is rich in Gly residues (GGGS); Chapter 6 presents experimental data indicating the involvement of several amino acid residues of this motif in the binding of cofactor and/or coenzyme NAD(H) in *B. methanolicus* MDH. In analogy to known binding domains, the Gly-rich motif is part of a Rossmann-fold, an alternation of two β-sheets and one α-helix. In Families I and II of NAD(P)-dependent ADHs such a secondary structure is involved in binding of NAD(P)(H) and can be recognized by the GXGXXG fingerprint motif. Family III ADHs generally do not possess this fingerprint, not even in the Gly-rich motif. Analysis of the secondary structure of MDH, and comparison with known FAD-binding consensi, led to the hypothesis that the Gly-rich motif is involved in binding of NAD(H). Using site-directed mutagenesis, various MDH mutants were constructed and, following their purification, the mutant proteins were biochemically characterized, both for MDH and formaldehyde reductase activity. This resulted in identification of several amino acids involved in binding of NAD(H) cofactor and/or coenzyme. Interestingly, several mutant MDH proteins (G95A, S97G, D100N, and K103R) did not contain cofactor NAD(H) anymore. Mutants D100N and K103R had completely lost both MDH and formaldehyde reductase activity, which may indicate loss of both NAD cofactor/coenzyme-binding. Mutants G95A and S97G were specifically affected in the MDH reaction and displayed normal formaldehyde reductase activity; apparently these mutants were affected only in NAD(H) cofactor-binding. Most interestingly, mutant S97G now displayed very high coenzyme NAD-dependent MDH activity; mutant G95A showed a linear relation between activity and methanol or NAD concentrations. Neither of these mutants was stimulated by the activator protein anymore. These data show that the MDH/activator protein interactions at the NAD(H) cofactor may provide a control mechanism for conversion of methanol into formaldehyde. Mechanistically it makes sense that NAD(H) cofactor and coenzyme are located near to each other, since reducing equivalents are transferred from one to another. More experiments, ideally with crystal structures of MDH (subunits) are needed to identify the precise interactions of this protein with NAD. The present data provide evidence that the Gly-rich motif, conserved in all members of Family III ADHs (Chapter 4), is part of a new dinucleotide-binding domain for a tightly bound NAD(H) cofactor in MDH. Other ADH proteins of Family III also should be analyzed for the presence of cofactor NAD(H).
Tools for the genetic modification of *A. methanolica* have become available in recent years (Vrijbloed, 1996). Unfortunately, such methods are still lacking for *B. methanolicus*. Further work therefore should focus on construction, characterization, and introduction of G95A and S97G mutants of MNO in *A. methanolica* strain MDM2 (with a disrupted *mno* gene; Chapter 4). This should allow a most interesting analysis of the relevance of the NAD(P)(H) cofactors in these decameric proteins on the physiology of growth on methanol and other primary alcohols.

**References**

References are listed on pages 127 - 136.