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

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# Chapter 4

## Identification of a magnesium-dependent NAD(P)(H) binding domain in the nicotinoprotein methanol dehydrogenase from *Bacillus methanolicus*

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**The *Bacillus methanolicus* methanol dehydrogenase (MDH) is a decameric nicotinoprotein alcohol dehydrogenase (Family III) with one $Zn^{2+}$-ion, 1-2 $Mg^{2+}$-ions and a tightly bound cofactor NAD(H) per subunit. The $Mg^{2+}$-ions are essential for binding of cofactor NAD(H) in MDH. A *B. methanolicus* activator protein strongly stimulates the relatively low coenzyme $NAD^+$-dependent MDH activity, involving hydrolytic removal of the NMN(H)-moiety of cofactor NAD(H) (Kloosterman *et al.*, 2002).**

**Members of Family III of NAD(P)-dependent ADHs contain three unique, conserved sequence motifs (domains A, B and C). Domain C is thought to be involved in metal-binding, while the functions of domains A and B are still unknown. This paper provides evidence that domain A constitutes (part of) a new magnesium-dependent NAD(P)(H) binding domain. Site-directed mutants D100N and K103R lacked (most of the) bound cofactor NAD(H) and had lost all coenzyme $NAD^+$-dependent MDH activity. Also mutants G95A and S97G were both impaired in cofactor NAD(H) binding, but retained coenzyme $NAD^+$-dependent MDH activity. Mutant G95A displayed a rather low MDH activity, whereas mutant S97G was insensitive to activator protein but displayed "fully activated" MDH reaction rates. The various roles of these amino acid residues in coenzyme and/or cofactor NAD(H) binding in MDH are discussed.**

## Introduction

Methanol dehydrogenase (MDH) of *Bacillus methanolicus* belongs to Family III of NAD(P)-dependent alcohol dehydrogenases (ADHs) (de Vries *et al.*, 1992;Vonck *et al.*, 1991), distinct from the zinc-containing medium-chain dehydrogenases/reductases (Family I) and the zinc-lacking short-chain ADHs (Family II) (Jornvall *et al.*, 1987;Reid and Fewson, 1994). The initial members of Family III all were iron-dependent ADHs. In time, with an increasing number of member proteins characterized, it became clear that not all members were iron-dependent. Where investigated, other metals like zinc and magnesium also were found instead of iron (Reid and Fewson, 1994). *B. methanolicus* MDH contains 1 $Zn^{2+}$ and 1-2 $Mg^{2+}$ ions per subunit (Vonck *et al.*, 1991). Identification of members of Family III ADHs increasingly became based on overall sequence similarity. Three unique, conserved amino acid sequence motifs have been defined for this family, aiding in ADH classification (Bairoch, 1992;de Vries *et al.*, 1992) (Table 1). Over 100 fully sequenced members of Family III ADHs are now found in databases. Many of these are putative proteins, with no biochemical data available.

**Table 1**. Conserved amino acid sequence motifs for Family III NAD(P)-dependent ADHs (de Vries *et al.*, 1992;Bairoch, 1992)(http://us.expasy.org/prosite). Positions correspond to the MDH sequence.

| Motif | Position | Sequence |
|---|---|---|
| A | 94-103 | G-G-G-S-$X_2$-D-$X_2$-K |
| B | 171-199 | [STALIV]-[LIVF]-X-[DE]-$X_{6,7}$-P-$X_4$-[ALIV]-X-[GST]-$X_2$-D-[TAIVM]-[LIVMF]-$X_4$-E |
| C | 258-278 | [GSW]-X-[LIVTSACD]-[GH]-$X_2$-[GSAE]-[GSHYQ]-X-[LIVTP]-[GAST]-[GAS]-$X_3$- [LIVMT]-X-[HNS]-[GA]-X-[GTAC] |

$X_N$ indicates an N number of non-discriminating position(s).

The genes encoding MDH of *B. methanolicus*, methanol:NDMA oxidoreductase (MNO) of *Amycolatopsis methanolica*, MNO of *Mycobacterium gastri* MB19, ADH of *Desulfovibrio gigas* and ADH of *Desulfovibrio* HDv enzymes of *B. methanolicus, Amycolatopsis methanolica, Desulfovibrio gigas* and *Desulfovibrio* HDv, have been cloned and characterized by us (de Vries *et al.*, 1992) (unpublished results). Classification of the *Mycobacterium gastri* enzyme was based on N-terminal amino acid sequence analysis (Fig. 1A). Characterization of the 5 purified enzymes revealed that each of the proteins possesses a decameric quaternary structure (Arfman *et al.*, 1989;Bystrykh *et al.*, 1993c;Hensgens *et al.*, 1993;Hensgens *et al.*, 1995). The first three are nicotinoproteins, containing a tightly but non-covalently bound NAD(P)(H) per subunit (Bystrykh *et al.*, 1993c;Arfman *et al.*, 1997). It is unknown whether other members of Family III are nicotinoproteins as well. The bound NAD(P)(H) species of MDH and *A. methanolica* MNO act as cofactors; they become reduced when the enzymes oxidize primary alcohols to the respective aldehydes (Bystrykh *et al.*, 1993c;Arfman *et al.*, 1997). *B. methanolicus* MDH requires a second, exogenous $NAD^+$ for methanol oxidation, serving as a coenzyme and resulting in re-oxidation of the NADH cofactor (Arfman *et al.*, 1997). These two NAD(H) molecules are not exchanged during the reaction (Arfman *et al.*, 1997). *In vitro,* the relatively low coenzyme $NAD^+$-dependent MDH activity is strongly stimulated by a $M_r$ 50 000 activator protein from the same organism, resulting in a 40-fold increase in MDH turnover rate (Arfman *et al.*, 1991;Arfman *et al.*, 1997).

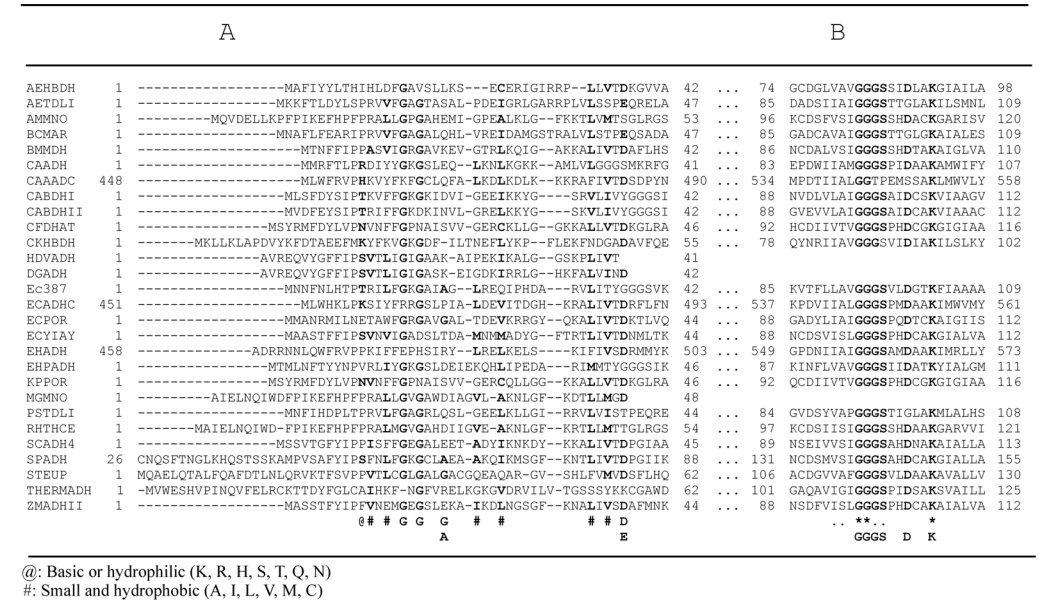
Activator protein mediated activation of MDH is characterized by hydrolytic removal of the NMN(H)-moiety of cofactor NAD(H) and converts the Ping-Pong type of reaction mechanism of MDH to a ternary complex mechanism, implying direct transfer of electrons from methanol to coenzyme $NAD^+$ (Kloosterman *et al.*, 2002). This raises important questions about the binding and proximity of the coenzyme and cofactor NAD(H) species in MDH.

The unique, conserved sequence motifs in MDH and other family III proteins may represent protein domains with important functions in substrate- or metal-binding, or catalysis. Domain C (position 258 - 290) contains several His residues and is thought to be involved in metal-binding (de Vries *et al.*, 1992;Cabiscol *et al.*, 1994;Bairoch *et al.*, 1996;Tamarit *et al.*, 1997). This remains to be confirmed experimentally, however. The functions of the other conserved regions have remained unknown. The characteristic fingerprint of an NAD(P) binding Rossmann fold, GXGXXG/A (Wierenga *et al.*, 1986) (Fig. 1A), is absent in MDH and virtually all other Family III enzymes (de Vries *et al.*, 1992). This dinucleotide-binding consensus motif in the primary structure results in a βαβ-fold in the secondary structure (Wierenga *et al.*, 1986). The three Gly residues involved allow a tight bending between the α-helices and β-sheets. This enables the dinucleotide to position closely, in the correct conformation, to the protein framework. The coenzyme interacts usually with a D or E residue, forming a hydrogen bond with the 2'-OH of the adenosine ribose moiety. In MDH of *B. methanolicus*, and in most other Family III ADHs, only an imperfect fingerprint ($G^{13}XG^{15}$) is found in the N-terminal part of the protein (Fig. 1A). Obviously, these enzymes contain strongly modified or novel NAD(P)(H)-binding domains, allowing binding of $NAD^+$ coenzyme in MDH and tight binding of NAD(P)(H) cofactors in MDH and MNO.

The conserved motif A ($GGGSX_2DX_2K$; position 94-103) in Family III ADHs (Table 1; Fig. 1B) displays similarity with FAD-binding domains, e.g. in alcohol oxidase of *Hansenula polymorpha* ($DIIVVGGGSX_{22}$ E) (Wierenga *et al.*, 1983;de Hoop *et al.*, 1991). FAD also functions as a cofactor and remains bound during catalysis, similar to the NAD(P)(H) cofactors in MDH and MNO enzymes.

Using site-directed mutagenesis, we have modified several amino acid residues in motif A of MDH. The biochemical properties of the purified mutant MDHs provide clear evidence that

residues Gly[95], Ser[97], Asp[100] and Lys[103] of MDH have important roles in binding of NAD(H) coenzyme and/or cofactor. Surprisingly, mutant S97G lacked bound cofactor NAD(H), was insensitive to activator protein, but displayed "fully activated" methanol dehydrogenase reaction rates.




@: Basic or hydrophilic (K, R, H, S, T, Q, N)
#: Small and hydrophobic (A, I, L, V, M, C)

**Figure 1**. Partial alignment of Family III ADHs. **A)** Optimal alignment with the coenzyme $NAD^+$-binding consensus sequence proposed by Wierenga *et al*. (Wierenga *et al.*, 1986). **B)** Alignment with motif A (Table 1). Abbreviations: AEHBDH, Hydroxybutyrate dehydrogenase of *Alcaligenes eutrophus* (Valentin *et al.*, 1995); AETDLI, Trans-dienelactoneisomerase of *A. eutrophus* (Perkins *et al.*, 1990); AMMNO, MNO of *A. methanolica* [Hektor *et al.*, 2000]; BCMAR, Maleylacetate reductase of *Burkholderia cepacia* (Daubaras *et al.*, 1995); BMMDH, MDH of *B. methanolicus* (de Vries *et al.*, 1992); CAADH, ADHI of *Clostridium* sp. NCP262 (Youngleson *et al.*, 1989); CAADHEC, Aldehyde/alcohol dehydrogenase of *Clostridium acetobutylicum* (Nair *et al.*, 1994); CABDHI, Butanol dehydrogenase I of *C. acetobutylicum* (Walter *et al.*, 1992); CABDHII, Butanol dehydrogenase II of *C. acetobutylicum* (Walter *et al.*, 1992); CFDHAT, 1,3-propanediol dehydrogenase of *Citrobacter freundii* (Daniel *et al.*, 1995); CKHBDH, Hydroxybutyrate dehydrogenase of *Clostridium kluyveri* (Sohling and Gottschalk, 1996); DGADH, ADH of *Desulfovibrio gigas* (Hensgens *et al.*, 1993); HDvADH, ADH of *Desulfovibrio* HDv (Hensgens *et al.*, 1995); EC387, gene product *382* of *Escherichia coli* (Accession number U28377); ECADHC, Alcohol/acetaldehyde dehydrogenase of *E. coli* (Goodlove *et al.*, 1989); ECPOR, 1,3-propanediol dehydrogenase of *E. coli* (Chen *et al.*, 1989); ECYIAY, putative gene-product of *E. coli* (Sofia *et al.*, 1994); EHADH, ADH of *Entamoeba histolytica* (Accession number S41377); EHPADH, gene product of *E. histolytica* (Yang *et al.*, 1994); KPPOR, 1,3-propanediol dehydrogenase of *Klebsiella pneumoniae* (Accession number U30903); MGMNO, MNO of *Mycobacterium gastri* MB19 (Bystrykh *et al.*, 1993c); PSTDLI, Trans-dienelactoneisomerase of *Pseudomonas* sp. strain P51 (Van der Meer *et al.*, 1991); RHTHCE, *thc*E gene product of *Rhodococcus* NI86/21 (Nagy *et al.*, 1995); SCADH4, ADH IV of *Saccharomyces cerevisiae* (Williamson and Paquin, 1987); SPADH, ADH of *Schizosaccharomyces pombe* (Accession number Q09669); STEUP, Ethanolamine utilization protein of *Salmonella typhimurium* (Stojiljkovic *et al.*, 1995); THERMADH, ADH of *Thermococcus* strain AN1 (Li and Stevenson, 1997); ZMADHII, ADH II of *Zymomonas mobilis* (Conway *et al.*, 1987).

## Materials and methods

### Bacterial strains and growth conditions

*E. coli* strain DH5α, serving as host for genetic modifications and heterologous gene expression, was grown on LB medium (Sambrook *et al.*, 1989); when appropriate ampicillin (100 $mg.l^{-1}$) was added. Plasmid pMDH was constructed using pBlueScript KS- and the *B. methanolicus* MDH encoding gene, expressed from its own promoter (de Vries *et al.*, 1992).
Expression of MDH (mutant) proteins in *E. coli* DH5α (cMDH) grown on LB medium (LB) or LB medium supplemented with 20 mM $MgSO_4$ (LB+Mg), was performed overnight. Addition of $MgSO_4$ to the growth medium was previously shown to be essential for $Mg^{2+}$ and cofactor NAD(H) binding in MDH protein expressed in *E. coli*, also determining the sensitivity of MDH to the stimulatory effect of activator protein, resulting in hydrolytic removal of the NMN(H)-moiety of cofactor NAD(H) (Kloosterman *et al.*, 2002).

### Secondary structure prediction

The secondary structure of MDH was predicted with the Profile prediction program provided by EMBL Heidelberg (Sander and Schneider, 1991).

### Site-directed mutagenesis

Mutations were introduced with PCR using VENT-DNA polymerase (New-England Biolabs). With plasmid pMDH serving as template DNA, a first PCR was performed with a mutagenesis sense primer and antisense primer R (Fig. 2). The PCR product obtained was used as primer in a second PCR, together with primer L1 (in the case of D88N, G95A, S97G, S97T, D100N and K103R) or L2 (in the case of G13A and G15A) (Fig. 2). The product of the final PCR was digested with *Nsi*I and exchanged with the original *Nsi*I fragment of pMDH, resulting in a plasmid encoding a mutant MDH. Construction of mutants G13A and G15A involved exchange of the *Swa*I - *Nde*I fragment (Fig. 2).

The desired mutants were constructed using the mutagenesis primers listed in Fig. 2. Also depicted in this figure are the new restriction sites introduced by silent mutations, which were used for rapid screening of potential mutants. All mutations were confirmed by determining the full nucleotide sequences of the mutant genes.

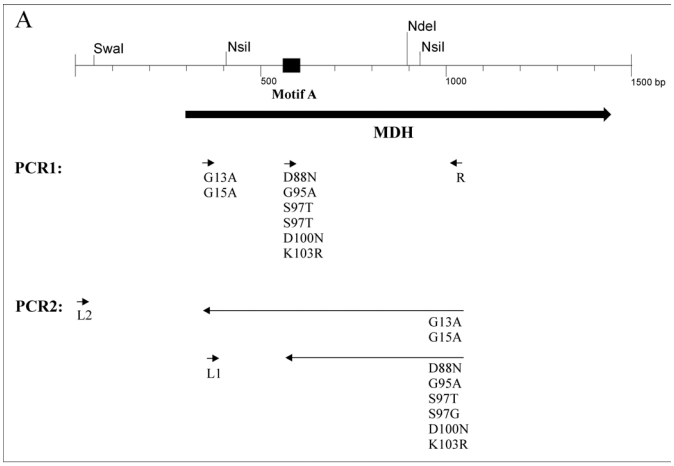
### DNA sequencing.

Nucleotide sequencing was done using dye-primers in the cycle sequencing method (Murray, 1989) with the thermosequenase kit RPN 2538 from Amersham Pharmacia Biotech AB. The samples were run on the A.L.F-Express sequencing robot. Analysis of nucleotide sequence was done using CloneManager version 4.01.

### Enzyme assays

All assays were performed at 50°C, using prewarmed buffer solutions. The oxidation or reduction of NAD(H) was followed at 340 nm. The MDH assay contained enzyme, 100 mM Glycine/KOH, pH 9.5, 5 mM $MgSO_4$, 5 mM β-mercaptoethanol and 1 mM $NAD^+$; after 3 min pre-incubation the reaction was started with 500 mM methanol (Arfman *et al.*, 1989). Saturating amounts of purified *B. methanolicus* activator protein (Kloosterman *et al.*, 2002) were added to analyze its stimulating effect on MDH activity. The low coenzyme $NAD^+$-dependent activity of MDH with $C_1$ - $C_4$ primary alcohols is strongly stimulated by activator protein, provided MDH contains NAD(H) cofactor and $Mg^{2+}$-ions are present in the assay mixture (Arfman *et al.*, 1991) (Kloosterman *et al.*, 2002). The formaldehyde reductase (FoRed) assay contained enzyme, 50 mM potassium phosphate, pH 6.7, and 0.15 mM NADH; after 3 min pre-incubation the reaction

was started with 10 mM formaldehyde (Arfman *et al.*, 1989).




B

Primer L1 ggtgcagtaaaggaagtagg
Primer L2 ggatctagtcgatcagtcg
Primer R gccatgtattgtgcataagc

| Mutation | Primer | Restriction site |
|---|---|---|
| G13A | gcgtaattg**cg**c**g**cggtgcagtaa<br>V I **A** R G A V | *Bss*H II |
| G15A | ttggacgag**cc**g**c**g**g**taaaggaag<br>G R **A** A V K E | *Sac* II |
| D88N | gaaaactgt**a**atgc**a**ct**a**gtttctatcg<br>E N C **N** A L V S I | *Spe* I |
| G95A | ctatcggtg**cc**g**c**agctctcacg<br>I G **A** G S S H | *Nae* I |
| S97G | cggtggaggt**g**g**a**t**c**c**c**acgatacagc<br>G G G **G** S H D T | *Bam*HI |
| S97T | cggtggagg**g**a**cg**tctcacgatacagc<br>G G G **T** S H D T | *Aat* II |
| D100N | cggtggagg**ctcgag**tcac**a**atacagc<br>G G G S S H **N** T | *Xho* I |
| K103R | cgatacagc**ccggg**caatcgg<br>D T A **R** A I | *Sma* I |

**Figure 2**. (A) Outline of site-directed mutagenesis strategy of the MDH gene of *B. methanolicus*. A detailed description is given in Materials and Methods. (B) Sequences of PCR primers used for site-directed mutagenesis of MDH. The mutated nucleotides and amino acids are printed in bold and the resulting restriction sites are underlined.

**Protein purification**

Wild type and mutant MDHs were purified as described (de Vries *et al.*, 1992), with some modifications (Table 2). Overnight cultures of *E. coli* (pMDH) were grown on LB medium or LB medium supplemented with 20 mM $MgSO_4$ (LB+Mg) (Kloosterman *et al.*, 2002), were harvested by centrifugation; cells were disrupted by two passages through a French Pressure cell at 140 MPa. Crude extracts were prepared by centrifugation for 30 min at 40 000 x g. Proteins were partially precipitated by adding ammonium sulphate to 30% saturation followed by incubation on ice for 10 min. After centrifugation (10 min at 25 000 x g) the supernatant was applied on a phenyl Superose (hydrophobic interaction) column equilibrated with 20 % (w/v) $(NH_4)_2SO_4$ in buffer A (50 mM Tris/HCl, 5 mM $MgSO_4$, 5 mM β-mercaptoethanol, pH 7.5). Proteins were eluted with a gradient of 20 - 0 % (w/v) $(NH_4)_2SO_4$. Active fractions were pooled, desalted on PD-10 columns (Sephadex G-25 M; Pharmacia-Biotech), and applied on a Mono Q (anion exchange) column; proteins were eluted with a 1 - 0 M KCl gradient in buffer A.

**Table 2**. Purification of wild type MDH expressed in *E. coli* cells grown on LB+Mg medium.

| Sample | Protein purification (mg) | Specific activity (mU/mg) | Total activity (mU) | Yield (%) | Fold |
|---|---|---|---|---|---|
| Crude extract | 22.8 | 165 | 3760 | 100 | 1.0 |
| AS*-precipitation | 17.3 | 235 | 4050 | 107 | 1.4 |
| Phenyl Superose | 6.7 | 332 | 2240 | 60 | 2.0 |
| PD-10 | 8.4 | 294 | 2460 | 65 | 1.8 |
| Mono Q | 3.4 | 413 | 1380 | 37 | 2.5 |

*: ammonium sulphate

Inactive mutants were purified in the same way; fractions were pooled at the same concentration of the salt gradients as wild type MDH.

**Protein determination**

Protein concentrations were determined with a Bio-Rad protein determination kit using bovine serum albumine as a standard (Bradford, 1976). SDS-PAGE was performed as described by Laemmli and Favre (Laemmli and Favre, 1973). The gel was stained with Coomassie brilliant blue R250.

**Kinetic studies**

Enzyme kinetics were studied using standard assay conditions and varying substrate concentrations. Data were fitted with Sigma Plot for Windows 4.0 (Jandell Scientific Software) according to the Michaelis-Menten equation. The effect of the activator protein was determined by adding 5 μg purified protein (Kloosterman *et al.*, 2002).

**Presence of cofactor NADH**

The presence of NADH cofactor in (mutant) MDH proteins purified from *E. coli* was analyzed by cofactor extraction. Purified MDH protein (1-2 mg) was denatured by boiling for 2 min in the presence of 6 M urea (Arfman *et al.*, 1997), and subsequently denatured protein was separated from NADH on a Pharmacia PD-10 column, equilibrated with 10 mM Tris/HCl, pH 8.0, containing 6 M urea (buffer B). The first ml of the salt-fraction (containing NADH), supplemented with 2 ml of buffer B to decrease salinity, was applied on a Mono Q column equilibrated with buffer B, and eluted in a gradient of 0 - 1 M KCl in buffer B. A solution of 10 nmol NADH was treated in the same way and served as standard.

**Molecular modelling**

Molecular modelling of known protein 3D structures was performed with the Swiss-PdbViewer program, version 3.6b3, Glaxo Wellcome Experimental Research.

## Results

**Selection of MDH mutants**

Analysis of the secondary structure of MDH, using Profile network prediction Heidelberg (Sander and Schneider, 1991), revealed a repetition of α-helices and β-sheets in the first 140 N-terminal amino acids. The C-terminal part of MDH consists mainly of α-helices and lacks β-sheets. The imperfect $NAD^+$-binding fingerprint around G13 and G15 present in the N-terminus of MDH and in many other Family III ADHs (Fig. 1A) does show a βαβ type of fold. Also the

domain with the conserved motif A shows a similar succession of α-helices and β-sheets; this is not the case for the conserved B and C motifs.

The G13A and G15A mutants were constructed to analyze the possible role of the imperfect $NAD^+$-binding fingerprint (GXG). A number of amino acids constituting motif A were selected as targets for mutagenesis, based on the predicted functions for these residues in known binding motifs. Thus, D88 or D100 may have a direct interaction with the 2'-OH of the adenosine ribose moiety of $NAD^+$. G95 may be essential for a proper arrangement of the α-helix and β-sheets, which enables a close approach of the cofactor/coenzyme to the protein framework and catalytic residues. K103 and S97 were chosen because of their high degree of conservation within members of Family III (Fig. 1B). To avoid drastic conformational changes, amino acids were replaced by residues of almost the same size, except for the Gly mutations where spherical hindrance was intended. This resulted in the following mutants: G13A, G15A, D88N, G95A, S97G, S97T, D100N and K103R. The genes encoding these mutant MDHs were completely sequenced, confirming that only the intended, single amino acid substitutions had occurred. Any differences in MDH properties therefore could be attributed to primary or secondary effects of the single amino acid modifications.

**Purification of (mutant) MDH proteins**

Mutant MDH proteins were expressed in *E. coli*, grown on LB and LB+Mg medium, to study the effect of $Mg^{2+}$ on the kinetics of the (mutant) proteins, since it was previously shown that $Mg^{2+}$ has a profound effect on MDH cofactor NAD(H) binding. Purified MDH expressed in *E. coli* (cMDH), in the absence of additional $Mg^{2+}$ in growth medium, lacks cofactor NAD(H), while addition of 20 mM $MgSO_4$ yields MDH protein containing NAD(H) cofactor, similar to MDH expressed in *B. methanolicus* (bMDH) (Kloosterman *et al.*, 2002).

SDS-PAGE analysis of crude extracts revealed the dominant MDH band at 43 kDa in all samples. MDH proteins were purified in three steps. For every mutant the purification fold was about three times, while the yield was approximately 35%. The purity of all purified enzymes, estimated by SDS-PAA gel electrophoresis, ranged from 98% to near electrophoretic homogeneity. The purification of wild type MDH from *E. coli* provides a representative example (Table 2).

**Characterization of mutant MDH enzymes**

The MDH and the FoRed activities of purified (mutant) MDH proteins were kinetically characterized (Table 3). Mutants G15A and D88N showed minor differences with wild type MDH (not shown). These residues thus do not play an important role in MDH or FoRed activities. G13A displayed clearly reduced MDH and FoRed activities, most likely due to reduced protein stability. Mutants D100N and K103R completely lacked MDH and FoRed activities, both in crude extracts and in purified preparations. These proteins were purified following the elution profiles of active enzymes; purification was confirmed by SDS-PAGE. Analysis of crude extracts of both inactive mutants on SDS-PAGE showed a normal level of expression, comparable to other mutants and wild type protein.

Compared to bMDH and cMDH, mutant G95A, expressed in *E. coli* cells grown on LB+Mg, displayed substantially (10-fold) decreased affinity for coenzyme $NAD^+$ and NADH and a strongly reduced Vmax for both MDH and FoRed. Expression of mutant G95A in *E. coli* cells grown on LB medium resulted in an even more reduced substrate affinity. When increasing the assay $NAD^+$ concentration, the very low MDH activity of G95A continued to increase almost proportionally. Kinetic parameters could therefore not be estimated properly in this case. The same was true for the FoRed reaction: increasing amounts of NADH resulted in a

linear increase of activity (not shown) and failure to calculate kinetic parameters.

Compared with cMDH, mutant S97G displayed drastically increased MDH activity. While the $V_{max}$ increased more than 10 times, the affinity for coenzyme $NAD^+$ decreased more than 30 fold (Table 3). Absence of $Mg^{2+}$ in the growth medium of *E. coli* cells producing mutant S97G protein resulted in reduced $V_{max}$ and coenzyme NAD(H) affinity values for both MDH and FoRed. With respect to the effect of $Mg^{2+}$, basically the same observations were made with the S97T mutation, although the changes were less extreme than in mutant S97G. This mutant displayed similar $V_{max}$ values as cMDH, while its affinity constants for coenzyme NADH were intermediate to those of cMDH and S97G.

The activator protein strongly stimulates MDH (but not FoRed) activity of the *B. methanolicus* bMDH protein (Arfman *et al.*, 1991). Activity of cMDH (when purified from *E. coli* cells grown on LB+Mg medium), also was stimulated about 8-fold when adding saturating amounts of activator protein. cMDH purified from *E. coli* cells grown on LB medium was only slightly stimulated in activity by activator protein (Table 3). Regardless of the addition of $Mg^{2+}$ ions, mutants G95A and S97G were completely insensitive to the stimulating effect of the activator protein on MDH activity. Mutant S97G, although insensitive to activator protein, displayed "fully activated" MDH reaction rates, albeit with a clearly reduced $NAD^+$ affinity. The inactive mutants D100N and K103R remained inactive after addition of activator protein. In contrast, the activity of mutant S97T, purified from *E. coli* cells grown on LB+Mg medium, was stimulated again by activator protein (by a factor 5.5). All other MDH mutants (G13A, G15A and D88N) responded to activator protein as cMDH (Table 3; data not shown).

**Table 3**. Kinetic characteristics and cofactor NADH content of wild type *B. methanolica* (bMDH), wild type (cMDH) and mutant MDH enzymes expressed in *E. coli*. Calculations are based on Michaelis-Menten kinetics.

| Mutant | Growth medium | $V_{max}$ (mU/mg) MDH | $V_{max}$ (mU/mg) FoRed | $K_m$ (mM NAD(H)) $NAD^+$ | $K_m$ (mM NAD(H)) NADH | Activation factor [1] | Cofactor Content (%) |
|---|---|---|---|---|---|---|---|
| bMDH | NA | 1310 | 5950 | 0.02[3] | 0.009 | 8.5 | 100 |
| cMDH | - Mg | 390 | 2100 | 0.04 | 0.010 | 1.9 | ND |
| | + Mg | 910 | 4750 | 0.03 | 0.011 | 8.1 | 100 |
| S97G | - Mg | 7780 | 3000 | 2.5 | 0.04 | 1.1 | ND |
| | + Mg | 10400 | 11730 | 0.99 | 0.026 | 1.3 | 4 |
| G95A | - Mg | 220[2] | 2300[2] | ND | ND | 0.7 | ND |
| | + Mg | 100 | 920 | 0.51 | 0.11 | 0.6 | 7 |
| S97T | - Mg | 730 | 2100 | 0.2 | 0.02 | 1.6 | ND |
| | + Mg | 750 | 4170 | 0.04 | 0.017 | 5.5 | 71 |
| D100N | - Mg | 0 | 0 | ND | ND | - | ND |
| | + Mg | 0 | 0 | ND | ND | - | 41 |
| K103R | - Mg | 0 | 0 | ND | ND | - | ND |
| | + Mg | 0 | 0 | ND | ND | - | 1 |

ND = not determined. NA = not applicable.

[1] Stimulating effect of activator protein: factor by which MDH activity is changed as a result of addition of activator protein; -: no effect.

[2] MDH and FoRed activities of G95A (- $Mg^{2+}$) increased almost linearly with increasing substrate concentrations; the values given are with 2.5 M methanol and 0.1 M formaldehyde.

[3] Affinity of bMDH for coenzyme $NAD^+$ decreased to 190 μM in the presence of activator protein (Arfman *et al.*, 1991).

**Presence of cofactor in (mutant) MDH proteins**

To establish the presence of cofactor NAD(H) in (mutant) MDHs, urea extracts of MDH proteins purified from *E. coli* cells grown on LB and LB+Mg medium were analysed, using Mono Q anion exchange chromatography (Fig. 3). No, or very little, A260 absorption was detected with wild type and mutant MDH proteins purified from cells grown on LB medium; as previously shown the presence of $Mg^{2+}$ ions in the medium is essential for binding of cofactor NAD(H) in MDH (Kloosterman *et al.*, 2002). Using proteins purified from LB+Mg grown cells, the elution profiles of the cofactor fractions of wild type MDH (2 mg) and mutant S97T (1.6 mg) clearly showed absorbance peaks at 260 nm, corresponding to NADH. A similar amount of S97G protein (1.5 mg) was used for urea treatment, but in this case an A260 absorption peak was nearly absent in the elution profile. The elution profiles of G95A, D100N and K103R (the amounts of protein used were 1.2, 1.0, and 1.1 mg, respectively) revealed a strongly reduced (mutant D100N) or virtually absent A260 absorption (mutants G95A and K103R). The data thus clearly indicate that only S97T and D100N still bind cofactor NADH, while mutants G95A, S97G, and K103R lack bound NADH, or contain only minor amounts. Cofactor NADH was quantified by peak integration (Table 3).

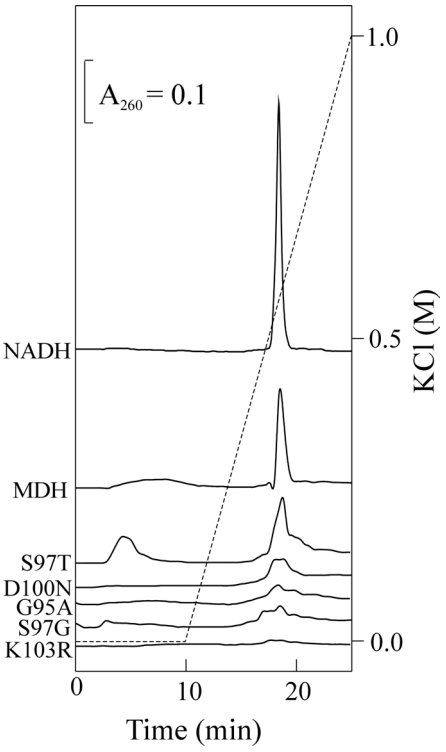



**Figure 3**. Elution profiles of MDH wild type and mutant cofactor NAD(H) fractions on a Mono Q anion exchange column. Amount of protein used for denaturation: MDH (2 mg), G95A (1.2 mg), S97G (1.5 mg), S97T (1.6 mg), D100N (1 mg) and K103R (1.1 mg). The elution profile of NADH (10 nmol) is used as reference. The dotted line represents the KCl concentration in the buffer.

**Steady-state kinetics of cMDH-WT and cMDH-S97G**

MDH purified from *B. methanolicus* (bMDH) obeys a Ping-Pong type of reaction mechanism, in which the NAD(H) cofactor is used as a temporary electron sink (Arfman *et al.*, 1997). Ping-Pong mechanisms are characterized by alternate binding of substrate and release of product. cMDH expressed under conditions in which cofactor NADH binding is poor displays a ternary

complex type of reaction mechanism (Kloosterman *et al.*, 2002). In the absence of a temporary electron sink (cofactor NADH), the reaction will proceed only when both substrates (methanol and coenzyme $NAD^+$) are bound to the enzyme. The kinetics of MDH mutants lacking the capacity to bind NAD(H) cofactor was further analyzed.

Steady-state MDH reaction rates with varying $NAD^+$ concentrations at different methanol concentrations were determined for cMDH and mutant S97G. Hanes plots of cMDH expressed in *E. coli* grown on LB+Mg medium, clearly revealed lines intercepting the vertical axis, indicating uncompetitive cosubstrate inhibition, which is typical for a Ping-Pong type of reaction mechanism. Mutant S97G, however, revealed a mixed-noncompetitive cosubstrate inhibition, indicating either a ternary complex or a Theorell-Chance reaction mechanism (Wong, 1975;Dixon and Webb, 1979). Absence of cofactor NAD(H) binding thus leads to a ternary complex reaction mechanism in mutant S97G.

**Discussion**

The data show that G13 and G15 in the N-terminal part of the protein, containing the imperfect $NAD^+$-binding fingerprint, are not involved in binding of cofactor or coenzyme NADH. These mutant enzymes showed normal characteristics, albeit that mutant G13A displayed decreased $V_{max}$ values for MDH and FoRed, most likely due to reduced protein stability. Also mutant D88N displayed no significant differences with wild type MDH.

Mutants D100N and K103R had completely lost MDH and FoRed activities, both in crude extracts and in purified protein preparations. These proteins displayed a normal mobility on SDS-PAGE with no indications for reduced protein stability. Mutant K103R had completely lost cofactor NADH, while in mutant D100N about 40% of the cofactor binding sites were occupied (Fig. 3; Table 3). However, the cofactor bound to D100N is apparently not functional. In other enzymes with the GXGXXG fingerprint motif, an Asp residue is directly involved in binding of NAD(P)(H) (Wierenga *et al.*, 1986;Branden and Tooze, 1991). Also Lys is a common conserved residue in these NAD(P)(H) binding sites (Branden and Tooze, 1991), but its role is unknown. Our data provide evidence that D100 and K103 are essential residues for MDH cofactor NAD(H) binding.

Mutants G95A and S97G contained no or very little cofactor NADH, indicating that also the G95 and S97 residues are very important for cofactor NAD(H) binding. Speculatively, the hydroxyl group of S97 may form a hydrogen bond with cofactor NAD(H). Mutant S97T, only slightly affected in binding of cofactor NADH (Table 3), apparently is still able to form the same hydrogen bond, whereas mutant S97G, lacking this hydroxyl group, has completely lost this hydrogen bonding ability, thus lacking in cofactor NADH binding. A Ser residue is also essential in the (unrelated) cofactor NADP(H) binding site of glucose:fructose oxidoreductase (GFOR) of *Zymomonas mobilis*. GFOR mutant protein S116D was shown to lack tightly bound NADP(H) cofactor. (Kanagasundaram and Scopes, 1992;Wiegert *et al.*, 1997). Determination of the 3-dimensional structure of GFOR revealed that S116 (S64 in the mature protein) is involved in hydrogen-bonding with the 2'-phosphate group of the NADP(H) cofactor (Kingston *et al.*, 1996). These results clearly demonstrate that mutational loss of one specific residue, amongst the several residues involved in cofactor binding, already may result in complete loss of cofactor NAD(H).

Coenzyme $NAD^+$ affinity was drastically decreased in mutant S97G. Loss of cofactor NAD(H) thus also strongly affects coenzyme $NAD^+$ binding in mutant S97G. The same phenomenon was observed in mutant S97T, purified from *E. coli* cells grown in the absence of $Mg^{2+}$. However, expression of mutant S97T in *E. coli* grown on LB+Mg medium restored cofactor NAD(H) binding and sensitivity to activator protein; this protein displayed a similar

coenzyme $NAD^+$ affinity as the cMDH and bMDH proteins (Table 3). In conclusion, (1) the presence of $Mg^{2+}$ ions in the growth medium (and in MDH protein) is crucial for cofactor NAD(H) binding; this involves a new magnesium-dependent NAD(H) binding domain with the G95, S97, D100 and K103 amino acid residues. (2) The stimulatory effect of activator protein on MDH activity is dependent on the presence of cofactor NAD(H). (3) The cofactor NAD(H) binding site is (only mildly) affected by mutation S97T, but not the coenzyme $NAD^+$ binding site; mutants G95A and S97G have entirely lost cofactor NAD(H) binding and display reduced affinity for coenzyme $NAD^+$ (Table 3). These data are taken to indicate that in wild type MDH, cofactor NAD(H) and coenzyme $NAD^+$ bind in close proximity, and that mutations strongly reducing cofactor NAD(H) binding also affect coenzyme $NAD^+$ binding (mutants G95A and S97G). Conceivably, in MDH mutants G95A and S97G, the cofactor NAD(H) binding site still has (a decreased) affinity for NAD(H), and now functions as a coenzyme $NAD^+$ binding site. With the cofactor NAD(H) binding site positioned ideally towards the active site, reducing equivalents may be rapidly transferred to coenzyme $NAD^+$, now present in the cofactor NAD(H) binding site. This results in a low MDH activity in mutant G95A, with a very low affinity for coenzyme $NAD^+$ (Table 3). In contrast, a much higher MDH reaction velocity is observed for mutant S97G (Table 3), most likely due to proper binding of coenzyme $NAD^+$ in the cofactor NAD(H) binding site and because no transfer of reducing equivalents from cofactor NAD(H) to coenzyme $NAD^+$ is necessary, previously shown to be the rate limiting step in the overall MDH reaction cycle (Arfman *et al.*, 1997).

The hypothesis that in mutants devoid of tightly bound cofactor NAD(H), coenzyme $NAD^+$-dependent MDH activity proceeds via coenzyme $NAD^+$ binding in the cofactor NAD(H) binding site, is supported by several further observations: Firstly, the results show that $Mg^{2+}$ has a profound effect on cofactor binding of cMDH and mutant S97T, emphasizing its function in the cofactor NAD(H) binding site. In mutants S97G and G95A the presence of $Mg^{2+}$ leads to an increased coenzyme $NAD^+$ affinity (Table 3), indicating that coenzyme $NAD^+$ is bound in the $Mg^{2+}$-dependent cofactor NAD(H) binding site. Secondly, (mutant) proteins equipped with cofactor (cMDH and S97T expressed in LB+Mg, and bMDH) share equivalent coenzyme $NAD^+$ affinities, indicating that the coenzyme site is unaffected by the S97T mutation. Residue S97 thus is not part of the coenzyme-binding site. In mutant enzymes devoid of cofactor NAD(H) the coenzyme $NAD^+$ affinity has become reduced by at least one order of magnitude, which indicates that an alternative binding site is employed. Thirdly, it is more plausible to assume that mutants D100N and K103R are unable to bind coenzyme $NAD^+$ in the cofactor NAD(H) binding site, yielding inactive and (partly) cofactor NAD(H) deficient mutants, than to speculate that in both mutants a single amino acid change leads to binding deficiency in both cofactor and coenzyme binding sites.

Wild type MDH displays a Ping-Pong type of reaction mechanism, with the cofactor functioning as temporary electron sink. MDH expressed in LB medium and MDH mutants lacking the capacity to bind cofactor NAD(H) thus are expected to change from a Ping-Pong type of reaction mechanism to a ternary complex reaction mechanism. This is clearly confirmed by the cosubstrate inhibition patterns of cMDH containing cofactor NAD(H) and mutant S97G lacking cofactor NAD(H), the first displaying uncompetitive cosubstrate inhibition typical for a Ping-Pong type reaction mechanism, the latter showing mixed-noncompetitive cosubstrate inhibition, indicating either ternary complex or a Theorell-Chance reaction mechanism (Wong, 1975;Dixon and Webb, 1979).

The results presented above combined with our studies on the mechanism of activator protein mediated activation of MDH (Kloosterman *et al.*, 2002) enabled us to develop a model of the cofactor NAD(H) binding domain of MDH (Fig. 4). Basically, a MDH molecule can occur in two states: the non-activated state and the activated state. Non-activated MDH exhibits

a Ping-Pong type of reaction mechanism, in which reducing equivalents are transferred from methanol via cofactor NAD(H) to coenzyme $NAD^+$, requiring both sites to be in close proximity of each other. Activator protein mediated activation of MDH is characterized by hydrolytic removal of the NMN(H)-moiety of cofactor NAD(H) and converts the reaction mechanism of MDH to a ternary complex mechanism, implying direct transfer of electrons from methanol to coenzyme $NAD^+$ (Kloosterman *et al.*, 2002). In this situation the $NMN^+$-moiety of coenzyme $NAD^+$, being in close proximity of the cofactor NAD(H) binding site, moves into the partially vacant cofactor NAD(H) binding site. Activator protein belongs to the family of Nudix (Nucleotide diphosphate linked to some other moiety X) hydrolases (Bessman *et al.*, 1996). One member of this protein family -MutT of *E. coli*- requires two divalent cations for catalysis, both positioned toward the pyrophosphate moiety of its substrate (8-oxo-)dGTP (Frick *et al.*, 1994). The action of activator protein on cofactor NAD(H) most likely proceeds via an analogous mechanism, requiring two $Mg^{2+}$-ions positioned towards the pyrophosphate group of cofactor NAD(H): the first being the MDH-bound $Mg^{2+}$-ion (Fig. 4), and the second an external $Mg^{2+}$-ion, since MDH activation by activator protein requires external $Mg^{2+}$-ions.

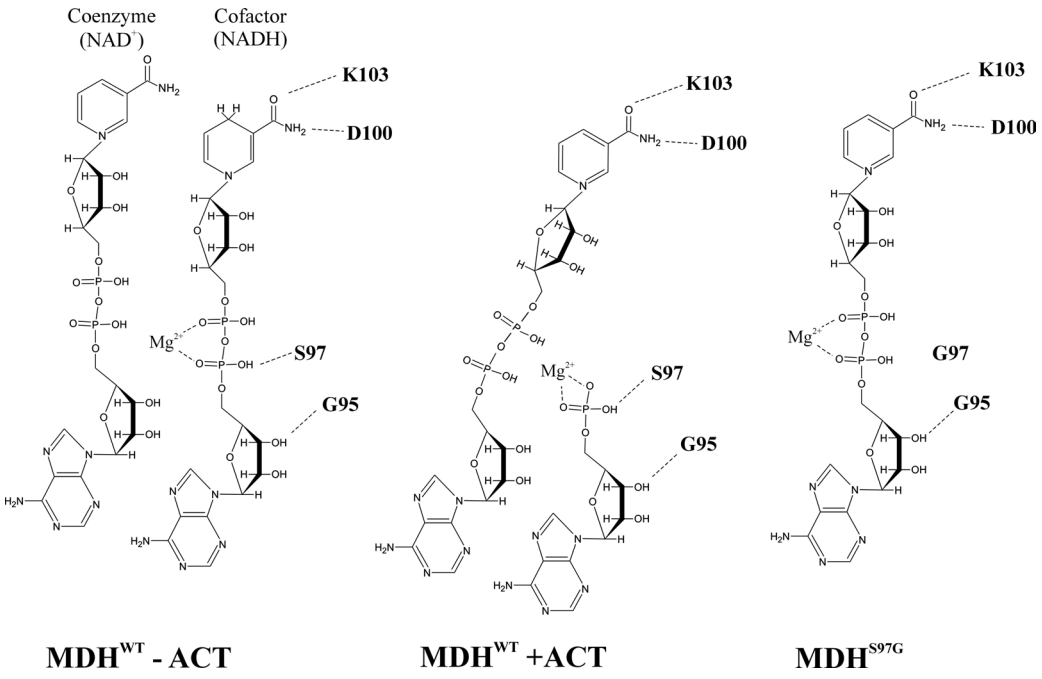



**Figure 4.** Model of the coenzyme $NAD^+$ and cofactor NAD(H) binding site of MDH.

Due to the loss of high affinity NAD(H) binding in the cofactor binding site of mutant S97G, it may now function as a coenzyme $NAD^+$ binding site. Although binding of coenzyme $NAD^+$ in mutant S97G is different from that in activated MDH (Fig. 4), the (ternary complex) reaction mechanism is similar, also leading to a "fully activated" MDH, with a very high coenzyme $NAD^+$-dependent MDH activity. Although mutant G95A is also cofactor deficient and is characterized by the same reaction mechanism, the additional methyl group may

introduce sterical hindrance, thereby preventing the coenzyme to position well in the cofactor binding site, leading to a decrease in $V_{max}$ of the $NAD^+$-dependent MDH activity.

The inactivity of cofactor deficient mutants D100N and K103R could indicate that residues D100 and K103 are involved in binding of the important redox active NMN(H)-group of cofactor NAD(H), or in case of MDH in the activated state, with the NMN(H) moiety of the coenzyme $NAD^+$ (Fig. 4).

Recently, the crystal structure of coenzyme $NAD^+$-dependent glycerol dehydrogenase (GlyDH) of *Bacillus stearothermophilus* was solved (Ruzheinikov *et al.*, 2001). This protein, which is 23% identical and 40% similar to MDH, partly contains the conserved motif A of Family III ADHs, but with a Lys residue at the position of the S97 residue of MDH. In GlyDH it was shown that the three Gly residues interact with the pyrophosphate moiety of coenzyme $NAD^+$. K97 of GlyDH interacts with the nicotinamide ribose, while D100, together with 8 other residues, forms a deep binding pocket for the nicotinamide ring. D100 forms hydrogen bonds with the carboxyamide nitrogen atom of the nicotinamide ring of NAD(H) (see Fig. 4).

It thus appears that the cofactor NAD(H) binding site of MDH, described in this study is similar to the coenzyme $NAD^+$ binding site of GlyDH, adding further support for our suggestion that mutant S97G uses the cofactor NAD(H) binding site as a coenzyme $NAD^+$ binding site. The structure of GlyDH also provided an explanation for the deleterious effect of the K103R mutation: modelling of the K103R in the GlyDH structure, showed that R103 causes steric hindrance for the D100 residue, interfering with binding of D100 to the nicotinamide group.

The availability of *E. coli* - *B. methanolicus* shuttle vectors and a suitable transformation system for *B. methanolicus* strains (Cue *et al.*, 1997), yields interesting opportunities for further *in vivo* analysis of mutant MDHs lacking cofactor NAD(H).

Several residues of the conserved motif in domain A of Family III ADHs thus are indeed involved in binding of cofactor/coenzyme NAD(H). It remains to be studied whether tight binding of NAD(P)(H) cofactors is a lot more widespread in Family III ADHs.

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