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Published in:
The Journal of Biological Chemistry

DOI:
10.1074/jbc.M207547200

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Document Version
Publisher’s PDF, also known as Version of record

Publication date:
2002

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Citation for published version (APA):

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Identification of a Magnesium-dependent NAD(P)(H)-binding Domain in the Nicotinoprotein Methanol Dehydrogenase from Bacillus methanolicus*

Harm J. Hektor‡‡, Harm Kloosterman‡, and Lubbert Dijkhuizen¶

From the Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands.

The Bacillus methanolicus methanol dehydrogenase (MDH) is a decameric nicotinoprotein alcohol dehydrogenase (family III) with one Zn$^{2+}$ ion, one or two 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, H., Vrijbloed, J. W., and Dijkhuizen, L. (2002) J. Biol. Chem. 277, 34785–34792). Members of family III of NAD(P)-dependent alcohol dehydrogenases contain three unique, conserved sequence motifs (domains A, B, and C). Domain C is thought to be involved in metal binding, whereas 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) 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 re-action rates. The various roles of these amino acid residues in coenzyme and/or cofactor NAD(H) binding in MDH are discussed.

Methanol dehydrogenase (MDH) of Bacillus methanolicus belongs to family III of NAD(P)-dependent alcohol dehydrogenases (ADHs) (2, 3), distinct from the zinc-containing medium chain dehydrogenases/reductases (family I) and the zinc-lacking short chain 32 ADHs (family II) (4, 5). 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 (5). B. methanolicus MDH contains one Zn$^{2+}$ and one or two Mg$^{2+}$ ions/subunit (3). 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 (2, 6) (Table I). Over 100 fully sequenced members of family III ADHs are now found in data bases. Many of these are putative proteins, with no biochemical data available.

The genes encoding MDH of B. methanolicus, methanol:p-nitroso- N,N$^\text{N}$-dimethylaniline oxidoreductase (MNO) of Amycolatopsis methanolica, MNO of Mycobacterium gastri MB19, ADH of Desulfovibrio gigas, and ADH of Desulfovibrio HDv enzymes of B. methanolicus, A. methanolicas, D. gigas, and Desulfovibrio HDv have been cloned and characterized by us (2). Classification of the M. gastri enzyme was based on N-terminal amino acid sequence analysis (Fig. 1A). Characterization of the five purified enzymes revealed that each of the proteins possesses a decameric quaternary structure (7–10). The first three are nicotinoproteins, containing a tightly but noncovalently bound NAD(P)(H)/subunit (8, 11). 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 (8, 11). B. methanolicus MDH requires a second, exogenous NAD$^+$ for methanol oxidation, serving as a coenzyme and resulting in reoxidation of the NADH cofactor (11). These two NAD(H) molecules are not exchanged during the reaction (11). In vitro, the relatively low coenzyme NAD$^+$-dependent MDH activity is strongly stimulated by a $M_s$ 50,000 activator protein from the same organism, resulting in a 40-fold increase in the MDH turnover rate (11, 12).

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 reactivation mechanism of MDH to a ternary complex mechanism, implying direct transfer of electrons from methanol to coenzyme NAD$^+$ (1). 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 in catalysis.

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¶ To University of Groningen on May 24, 2007
Domain C (position 258–290) contains several His residues and is thought to be involved in metal binding (2, 13–15). However, this remains to be confirmed experimentally. The functions of the other conserved regions remain unknown. The characteristic fingerprint of an NAD(P)-binding Rossmann fold, GXXGXGGA/G/A (16) (Fig. 1A), is absent in MDH and virtually all other family III enzymes (2). This dinucleotide-binding consensus motif in the primary structure results in a βββ fold in the secondary structure (16). The three Gly residues involved allow the dinucleotide to position closely, in the correct conformation, to the α-helix and β-strands of the coenzyme binding sites. Obviously, these enzymes contain strongly modified or novel NAD(P)(H)-binding domains, allowing binding of NAD(P) coenzyme in MDH and tight binding of NAD(P)(H) cofactors in MDH and MNO enzymes.

The conserved motif A (GGGSGKDXK) positions 94–103 in family III ADHs (Table I and Fig. 1B) displays similarity with the coenzyme NAD(P)(H) cofactors in MDH and tight binding of 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 Gly85, Ser97, Asp100, and Lys103 of MDH have important roles in binding of NAD(P)H coenzyme and/or cofactor.

![Table I](image)

<table>
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<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
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<td>G-G-G-S-X-D-X-K</td>
</tr>
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**Enzyme Assays**—All of the assays were performed at 50 °C using prewarmed buffer solutions. Partial oxidation or reduction of NADH was followed at 340 nm. The MDH assay contained enzyme, 100 mM glycine/KOH, pH 9.5, 5 mM MgSO4, 5 mM β-mercaptoethanol, and 1 mM NAD+; after 3 min of preincubation, the reaction was started with 500 μM methanol (7). Saturating amounts of purified B. methanolicus activator protein (1) were added to analyze its stimulating effect on MDH activity. When low coenzyme NAD+–dependent activity of MDH with C–C primary alcohols is strongly stimulated by activator protein, provided MDH contains NAD(H) cofactor and Mg2+ ions are present in the assay mixture (1, 12). The formaldehyde reductase (FoRed) assay contained enzyme, 50 mM potassium phosphate, pH 6.7, and 0.15 mM NADH; after 3 min of preincubation, the reaction was started with 10 mM formaldehyde (7).

**Protein Purification**—Wild type and mutant MDHs were purified as described (2), with some modifications (Table II). Overnight cultures of E. coli (pMDH) were grown on LB medium alone or LB medium supplemented with 20 mM MgSO4 (LB-Mg) (1) and were harvested by centrifugation; the cells were disrupted by two passages through a French pressure cell at 140 megapascals. The crude extracts were prepared by centrifugation for 30 min at 40,000 × g. The proteins were partially precipitated by adding ammonium sulfate to 30% saturation followed by incubation on ice for 10 min. After centrifugation (10 min at 25,000 × g), the supernatant was applied on a phenyl Superose (hydrophobic interaction) column equilibrated with 20% (w/v) (NH4)2SO4 in buffer A (50 mM Tris/HCl, 5 mM MgSO4, 5 mM β-mercaptoethanol, pH 7.5). The proteins were eluted with a gradient of 20–0% (w/v) (NH4)2SO4. The active fractions were pooled, desalted on PD-10 columns (Sephadex G-25; Amersham Biosciences), and applied on a Mono Q (anion exchange) column; the proteins were eluted with a 0–1 M KCl gradient in buffer A. Inactive mutants were purified in the same way; the fractions were pooled at the same concentration of the salt gradients as wild type MDH.

**Protein Determination**—The protein concentrations were determined with a Bio-Rad protein determination kit using bovine serum albumin as a standard (22). SDS-PAGE was performed as described by Laemmli and Favre (23). The gel was stained with Coomassie Brilliant Blue R250.

**Kinetic Studies**—Enzyme kinetics were studied using standard assay conditions and varying substrate concentrations. The data were fitted with Sigma Plot for Windows 4.0 (Jandel Scientific Software) according to the Michaelis-Menten equation. The effect of the activator protein was determined by adding 5 μg of purified protein (1). The 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 (11), and subsequently denatured protein was separated from NADH on a Pharmacia PD-10 column, equilibrated with 10 mM Tris/HC1, 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 of NADH was treated in the same way and served as standard.

**Molecular Modeling**—Molecular modeling of known protein three-dimensional 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 (20) 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 Gly13 and Gly14 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 full nucleotide sequences of the mutant genes.

**DNA Sequencing**—Nucleotide sequencing was done using dye primers in the cycle sequencing method (21) with the thermosequenase kit RPN 2538 from Amersham Biosciences. The samples were run on the A.L.F-Express sequencing robot. Analysis of nucleotide sequence was done using CloneManager, version 4.01.
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, Asp\(^{98}\) or Asp\(^{100}\) may have a direct interaction with the 2'-OH of the adenosine ribose moiety of NAD\(^+\). Gly\(^{96}\) 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. Lys\(^{103}\) and Ser\(^{97}\) were chosen because of their high degree of conservation within members of family III (Fig. 1D). 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, because 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), whereas the addition of 20 mM MgSO\(_4\) yields MDH protein containing NAD(H) cofactor, similar to MDH expressed in *B. methanolicus* (bMDH) (1).

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, whereas the yield was ~35%. The purity of all purified enzymes, estimated by SDS-PAGE, ranged from 98% to near electrophoretic homogeneity. The purification of wild type MDH from *E. coli* provides a representative example (Table II).

Characterization of Mutant MDH Enzymes—The MDH and the FoRed activities of purified (mutant) MDH proteins were kinetically characterized (Table III). 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 because of 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 with other mutants and wild type protein.

Compared with 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 \(V_{\text{max}}\) 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. Although the \(V_{\text{max}}\) increased more than 10 times, the affinity for coenzyme NAD\(^+\) decreased more than 30-fold (Table III). Absence of Mg\(^{2+}\) in the growth medium of *E. coli* cells producing mutant S97G protein resulted in reduced \(V_{\text{max}}\) 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 \(V_{\text{max}}\) values similar to those of cMDH, whereas 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 (12). 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 III). 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 the 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 III; data not shown).

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 analyzed, using Mono Q anion exchange chromatography (Fig. 3). No, or very little, A\(_{260}\) 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 the binding of cofactor NAD(H) in MDH (1). 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 A\(_{260}\) 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 A\(_{260}\) absorption.
**NAD(P)(H) Binding in Nicotinoprotein Alcohol Dehydrogenase**

**TABLE III**

Kinetic characteristics and cofactor NAD content of wild type B. methanolicus (bMDH), wild type (cMDH), and mutant MDH enzymes expressed in E. coli

<table>
<thead>
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<th>Mutant</th>
<th>Growth medium</th>
<th>( V_{\text{max}} ) MDH</th>
<th>FoRed</th>
<th>( K_m ) NAD(^+)</th>
<th>( K_m ) NADH</th>
<th>Activation factor(^a)</th>
<th>Cofactor content</th>
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<td>+Mg</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>—</td>
<td>ND</td>
</tr>
<tr>
<td>K103R</td>
<td>−Mg</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>—</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^{a}\) Stimulating effect of activator protein: factor by which MDH activity is changed as a result of addition of activator protein; —, no effect.  

\(^{b}\) NA, not applicable.  

\(^{c}\) Affinity of bMDH for coenzyme NAD\(^+\) decreased to 190 \(\mu\)M in the presence of activator protein (12).  

\(^{d}\) ND, not determined.

**FIG. 3.** Elution profiles of MDH wild type and mutant cofactor NAD(H) fractions on a Mono Q anion exchange column. The amounts of protein used for denaturation were as follows: 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.

(mutants G95A and K103R). The data thus clearly indicate that only S97T and D100N still bind cofactor NADH, whereas mutants G95A, S97G, and K103R lack bound NADH or contain only minor amounts. Cofactor NADH was quantified by peak integration (Table III).

**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 (11). 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 (1). 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 (24, 25). Absence of cofactor NAD(H) binding thus leads to a ternary complex reaction mechanism in mutant S97G.

**DISCUSSION**

The data show that Gly\(^13\) and Gly\(^15\) 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, although mutant G13A displayed decreased \( V_{\text{max}} \) values for MDH and FoRed, most likely because of 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, whereas in mutant D100N about 40% of the cofactor-binding sites were occupied (Fig. 3 and Table III). 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(H) cofactor (16, 26). Also Lys is a common conserved residue in these NAD(P)(H)-binding sites (26), but its role is unknown. Our data provide evidence that Asp\(^160\) and
Lys\textsuperscript{103} are essential residues for MDH cofactor NAD(H) binding.

Mutants G95A and S97G contained no or very little cofactor NADH, indicating that the Gly\textsuperscript{95} and Ser\textsuperscript{97} residues are also very important for cofactor NAD(H) binding. Speculatively, the hydroxyl group of Ser\textsuperscript{97} may form a hydrogen bond with cofactor NAD(H). Mutant S97T, only slightly affected in binding of cofactor NADH (Table III), apparently is still able to form the same hydrogen bond, whereas mutant S97G, lacking this hydroxyl group, has completely lost this hydrogen bonding ability and thus lacks cofactor NADH binding. A Ser residue is also essential in the (unrelated) cofactor NADP(H)-binding site of glucose:fructose oxidoreductase of \textit{Zymomonas mobilis}. Glucose:fructose oxidoreductase mutant protein S116D was shown to lack tightly bound NADP(H) cofactor (27, 28). Determination of the three-dimensional structure of glucose:fructose oxidoreductase revealed that Ser\textsuperscript{116} (Ser\textsuperscript{64} in the mature protein) is involved in hydrogen bonding with the 2'-phosphate group of the NADP(H) cofactor (29). These results clearly demonstrate that mutational loss of one specific residue, among the several residues involved in cofactor binding, already may result in the complete loss of cofactor NAD(H).

Coenzyme NAD\textsuperscript{+} affinity was drastically decreased in mutant S97G. Loss of cofactor NAD(H) thus also strongly affects coenzyme NAD\textsuperscript{+} binding in mutant S97G. The same phenomenon was observed in mutant S97T, purified from \textit{E. coli} cells grown in the absence of Mg\textsuperscript{2+}. However, expression of mutant S97T in \textit{E. coli} grown on LB+Mg medium restored cofactor NAD(H) binding and sensitivity to activator protein; this protein displayed a coenzyme NAD\textsuperscript{+} affinity similar to that of the cMDH and bMDH proteins (Table III). In conclusion, 1) the presence of Mg\textsuperscript{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 Gly\textsuperscript{95}, Ser\textsuperscript{97}, Asp\textsuperscript{100}, and Lys\textsuperscript{103} 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\textsuperscript{+}-binding site; mutants G95A and S97G have entirely lost cofactor NAD(H) binding and display reduced affinity for coenzyme NAD\textsuperscript{+} (Table III). These data are taken to indicate that in wild type MDH, cofactor NAD(H) and coenzyme NAD\textsuperscript{+} bind in close proximity and that mutations strongly reducing cofactor NAD(H) binding also affect coenzyme NAD\textsuperscript{+} 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\textsuperscript{+}-binding site. With the cofactor NAD(H)-binding site positioned ideally toward the active site, reducing equivalents may be rapidly transferred to coenzyme NAD\textsuperscript{+}, now present in the cofactor NAD(H)-binding site. This results in low MDH activity in mutant G95A, with a very low affinity for coenzyme NAD\textsuperscript{+} (Table III). In contrast, a much higher MDH reaction velocity is observed for mutant S97G (Table III), most likely because of proper binding of coenzyme NAD\textsuperscript{+} in the cofactor NAD(H)-binding site and because no transfer of reducing equivalents from cofactor NAD(H) to coenzyme NAD\textsuperscript{+} is necessary, previously shown to be the rate-limiting step in the overall MDH reaction cycle (11).

The hypothesis that in mutants devoid of tightly bound cofactor NAD(H), coenzyme NAD\textsuperscript{+}-dependent MDH activity proceeds via coenzyme NAD\textsuperscript{+} binding in the cofactor NAD(H)-binding site is supported by several further observations. First, the results show that Mg\textsuperscript{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\textsuperscript{2+} leads to an increased coenzyme NAD\textsuperscript{+} affinity (Table III), indicating that coenzyme NAD\textsuperscript{+} is
bound in the Mg$^{2+}$-dependent cofactor NAD(H)-binding site. Second, (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 Ser$^{97}$ 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 1 order of magnitude, which indicates that an alternative binding site is employed. Third, 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 a 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), with the former displaying uncompetitive cosubstrate inhibition typical for a Ping-Pong type reaction mechanism and the latter showing mixed noncompetitive cosubstrate inhibition, indicating either a ternary complex or a Theorell-Chance reaction mechanism (24, 25).

The results presented above combined with our studies on the mechanism of activator protein mediated activation of MDH (1) 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 nonactivated state and the activated state. Nonactivated 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 NMM(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$^+$. In this situation the NMM$^+$ 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 small molecules) hydrolases (30). 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 (31). The action of activator protein on 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; modeling of the K103R in the GlyDH structure showed that Arg$^{103}$ causes steric hindrance for the Asp$^{100}$ residue, interfering with binding of Asp$^{100}$ to the nicotinamide group.

The availability of E. coli-B. methanolicus shuttle vectors and a suitable transformation system for B. methanolicus strains (33) 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.

Acknowledgments—We thank R. K. Wierenga (Heidelberg) and P. Terpstra (Groningen) for valuable suggestions for targets of site-directed mutagenesis.

REFERENCES
NAD(P)(H) Binding in Nicotinoprotein Alcohol Dehydrogenase