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Molecular, Biochemical, and Functional Characterization of a Nudix Hydrolase Protein That Stimulates the Activity of a Nicotinoprotein Alcohol Dehydrogenase*

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The cytoplasmic coenzyme NAD⁺-dependent alcohol (methanol) dehydrogenase (MDH) employed by *Bacillus methanolicus* during growth on C₁-C₄ primary alcohols is a decameric protein with 1 Zn²⁺-ion and 1–2 Mg²⁺-ions plus a tightly bound NAD(H) cofactor per subunit (a nicotinoprotein). Mg²⁺-ions are essential for binding of NAD(H) cofactor in MDH protein expressed in *Escherichia coli*. The low coenzyme NAD⁺-dependent activity of MDH with C₁-C₄ primary alcohols is strongly stimulated by a second *B. methanolicus* protein (ACT), provided that MDH contains NAD(H) cofactor and Mg²⁺-ions are present in the assay mixture. Characterization of the *act* gene revealed the presence of the highly conserved amino acid sequence motif typical of Nudix hydrolase proteins in the deduced ACT amino acid sequence. The *act* gene was successfully expressed in *E. coli* allowing purification and characterization of active ACT protein. MDH activation by ACT involved hydrolytic removal of the nicotinamide mononucleotide NMN(H) moiety of the NAD(H) cofactor of MDH, changing its Ping-Pong type of reaction mechanism into a ternary complex reaction mechanism. Increased cellular NADH/NAD⁺ ratios may reduce the ACT-mediated activation of MDH, thus preventing accumulation of toxic aldehydes. This represents a novel mechanism for alcohol dehydrogenase activity regulation.

Methanol is formed in large quantities in mineralization processes in nature, mostly from degradation of methylesters and -ethers that occur in plants (pectin and lignin). Methylotrophic microorganisms growing on methanol as carbon and energy sources can be isolated readily from soil. They possess a special set of enzymes for generation of energy from methanol oxidation and for synthesis of compounds with carbon-carbon bonds from methanol (1, 2).

Three different type of enzymes catalyze the initial oxidation of methanol to formaldehyde in methylotrophs. Yeasts employ an alcohol oxidase with FAD as cofactor (a flavoprotein); oxygen is used as electron acceptor, resulting in hydrogen peroxide formation. This enzyme is located in peroxi-

somes, an organelle that also contains catalase activity (3). Gram-negative bacteria employ a methanol dehydrogenase (MDH)¹ with pyrroloquinoline quinone as cofactor (a quinoprotein), located in the periplasmic space (4, 5). Gram-positive bacteria (bacilli and actinomycetes) employ cytoplasmic MDH enzymes with NAD(P)⁺ as cofactor (nicotinoproteins), constituting novel NAD(P)⁺-dependent alcohol dehydrogenases with unusual properties (6–8).

Pure cultures of obligately aerobic, thermotolerant *Bacillus methanolicus* strains grow rapidly in methanol mineral medium (doubling times 40–80 min) at temperatures of 35–60 °C and are tolerant to very high methanol concentrations (9, 10). All strains studied employ a coenzyme NAD⁺-dependent MDH for growth on methanol and other primary alcohols (C₁-C₄) (11, 12). MDH from *B. methanolicus* strain C1 belongs to family III of alcohol dehydrogenases (11, 13). It is a decameric enzyme with subunits of 43,000 Da (10, 11, 13–17). Each MDH subunit contains 1 Zn²⁺-ion and 1–2 Mg²⁺-ions (13). Zinc is commonly found in the active site of alcohol dehydrogenases, but the presence of magnesium had not been reported before. Each subunit also contains a tightly (but noncovalently) bound NAD(H) molecule, which is oxidized and reduced by methanol and formaldehyde, respectively, and thus functions as a cofactor (17). NAD⁺ plays two important roles in the MDH-catalyzed reaction: the MDH-bound NAD⁺ cofactor serves as the primary electron acceptor in the alcohol dehydrogenase reaction, and exogenous NAD⁺ coenzyme is responsible for reoxidation of the MDH-bound NADH cofactor. MDH obeys a Ping-Pong type of reaction mechanism, consistent with such a temporary parking of reducing equivalents at the MDH-bound NAD(H) cofactor (17). This is very different from typical NAD⁺ coenzyme-dependent alcohol dehydrogenases, which follow a ternary complex type of reaction mechanism (6).

Studies with purified proteins showed that MDH activity with C₁-C₄ primary alcohols and its affinity for exogenous NAD⁺ and alcohol substrates are strongly increased in the presence of a *B. methanolicus* strain C1-soluble M_r 50,000 activator (ACT) protein. Activation, which takes place within 1 s upon the addition of saturating amounts of ACT, requires the presence of Mg²⁺-ions. Spectral studies showed that Mg²⁺-ions are essential for the formation of an MDH·ACT·NAD⁺·Mg²⁺ complex. At physiological methanol concentrations (0.1–1.0 mM), the methanol turnover rate of MDH *in vitro* was increased up to 40-fold by the ACT protein (16).

Synthesis of the MDH and ACT proteins in *B. methanolicus*

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY128667.

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¹ The abbreviations used are: MDH, methanol dehydrogenase; bMDH, MDH purified from *B. methanolicus* cells; cMDH, MDH purified from *E. coli* cells; Nudix, nucleotide diphosphate group linked to some other moiety (x); ORF, open reading frame; ADPR, ADP-ribose.

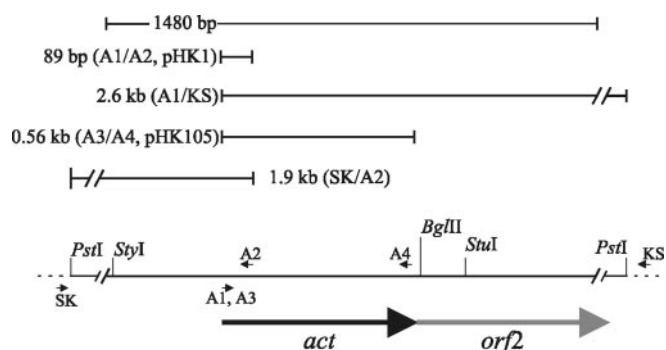


FIG. 1. Schematic representation of the 4.4-kb *Pst*I insert of *B. methanolicus* strain C1 chromosomal DNA in pHK83. The larger arrows indicate the open reading frames (*act* and *orf2*), and the smaller arrows indicate the primers that were used for PCR reactions. The 89-bp fragment shown is the product of the initial DNA amplification reaction on chromosomal DNA of *B. methanolicus* strain C1, which was cloned into pBlueScriptIIKS (pHK1). The fragments of 2.6 and 1.9 kb are DNA amplification products of reactions with pHK83 as template in which, respectively, the primers A1/KS and A2/SK were used to localize the *act* gene on the 4.4-kb insert. The 0.56-kb fragment represents the entire *act* ORF; this fragment was cloned in the expression vector pProk1 (pHK105). The restriction sites *Sty*I, *Bgl*II, and *Stu*I were used for the construction of subclones for nucleotide sequencing. In total, 1480 bp of the 4.4-kb *Pst*I fragment (as shown in this figure) was sequenced in both directions.

is regulated coordinately (14). Here we report a molecular, biochemical, and functional characterization of the ACT protein of *B. methanolicus* strain C1. ACT is a member of the Nudix hydrolase family, that display hydrolytic activity with substrates containing a nucleotide diphosphate group linked to some other moiety (\bar{x}) (18). ACT protein stimulates coenzyme NAD⁺-dependent MDH activity by hydrolytic removal of the NMN(H) part of the NAD(H) cofactor in MDH protein.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

B. methanolicus strain C1 cells were grown as described (11). *Escherichia coli* strains MC1061 and JM109, serving as hosts for genetic modifications and heterologous gene expression, respectively, were grown on LB medium (19), and when appropriate, ampicillin (100 mg.l⁻¹) was added.

Primers and DNA Amplification

The primers used were: A1, 5'-GGCGAATTC(A/G)TT(A/G)TT-(T/C)GA(A/G)GA(A/G)AA(A/G)AC-3', and A2, 5'-GGCTGATC(C/T/A)AC(T/C)TG(T/C)AA(T/C)TT(C/T/A) AC(C/T/A)AC-3'. These were based on the N-terminal amino acid sequence of the ACT protein (16). Their degree of degeneration was limited based on codon bias of *B. methanolicus* (11). Primer A3 consisted of an *Eco*RI site followed by the first 19 nucleotides of the *act* gene: 5'-GCGGAATTCATGGGAA-AATTATTTGAGG-3'. The antisense primer A4 contained a *Bam*HI site and the last 18 nucleotides of *act*: 5'-CGCGATCCTCATTTATGTTT-GAGAGC-3'. The KS and SK primers used are available commercially (Stratagene, Westburg, Leusden, The Netherlands). Primer positions are shown in Fig. 1.

DNA amplification reactions were performed with Vent-DNA polymerase (New England Biolabs). Reaction mixtures (100 μ l) contained: enzyme buffer (New England Biolabs), dNTP (50 mM/nucleotide), primers (0.5 mM/primer), target DNA (1–10 ng). Target DNA was incubated for 5 min at 94 °C before adding polymerase. Amplification conditions were: 25 reaction cycles at temperatures for denaturation, primer annealing and primer extension of 94, 45, and 72 °C, respectively. In the last reaction cycle, the primer extension time was set at 5 min.

Plasmids

Plasmid pHK1 was constructed by cloning the PCR product of primers A1/A2, with *B. methanolicus* chromosomal DNA as template, in pBlueScriptIIKS (Stratagene). Plasmid pHK83 contains a 4.4-kb insert of *B. methanolicus* chromosomal DNA in the *Pst*I site of pBlueScriptIIKS. Plasmid pHK105 was constructed by cloning the *act* gene in the

*Eco*RI and *Bam*HI sites of pProk1 (CLONTECH Laboratories, Palo Alto, AC) with *act* expression controlled by the pProk1 *tac* promoter.

DNA Isolation and Manipulation

B. methanolicus strain C1 total chromosomal DNA was isolated as described (11). Methods for DNA handling, modification, and cloning were performed as described previously (19).

Gene Library Construction and Screening

Chromosomal DNA of *B. methanolicus* was digested with *Pst*I, and fragments ranging from 3.7 to 5.1 kb were isolated, ligated in pBlueScriptIIKS, and transformed to *E. coli* MC1061. This partial gene library sequence was screened using primers A1/A2. 40 \times 250 pooled *E. coli* MC1061 colonies were transferred from agar plates to liquid LB medium and incubated for 4 h at 37 °C. Samples from these 40 cultures were lysed by boiling for 5 min and subjected to DNA amplification. Cultures giving a positive amplification signal were plated, and 400 colonies were screened in pools of 20 colonies. Finally, individual colonies were screened for a positive amplification signal.

DNA Sequence Analysis

Nucleotide sequencing was done using dye-primers in the cycle sequencing method (20) with the Thermosequenase kit RPN 2538 from Amersham Biosciences. The samples were run on the ALF-Express sequencing robot. Analysis of the nucleotide sequence (deposited in the GenBank™ data base under accession number AY128667) was done using CloneManager, Version 4.01. Protein sequence comparisons were performed using the facilities of the BLAST server (21) at NCBI (National Library of Medicine, Washington, D. C.).

Preparation of Cell Extracts and Enzyme Assays

Cells were disrupted by two passages through a French pressure cell operating at 1.4 \times 10⁵ kilonewtons.m⁻². Enzyme assays were performed at 50 °C with prewarmed buffer solutions (unless stated otherwise). The NAD⁺-dependent MDH activity and MDH-stimulating activity of ACT were measured as described earlier (12, 16). Assays were performed at 50 °C, and NAD(H) oxidation or reduction was followed at 340 nm in a Hitachi model 100-60 spectrophotometer. The MDH assay, buffered by 100 mM glycine-KOH buffer (pH 9.5), contained 5 mM MgSO₄, 5 mM 2-mercaptoethanol, 1 mM NAD⁺, and enzyme. The reaction was started with 500 mM methanol after a 5-min preincubation. The stimulatory effect of activator protein was analyzed by the subsequent addition of purified activator protein. One unit of MDH-stimulating activity is defined as the amount of ACT that stimulates a fixed quantity of purified MDH (1.0 μ g (2.5 pmol) MDH/ml reaction mixture) to 50% of fully ACT-activated MDH (V_{max}) minus the ACT-independent activity of MDH (V_0) (16).

Nudix hydrolase activity of ACT protein was determined in 300 μ l of 100 mM glycine-KOH buffer (pH 9.5), containing 10 mM MgSO₄ and 250 nmol (di-)nucleotide at 50 °C. Dinucleotide hydrolyzing activity was assayed using an excess amount of calf intestine alkaline phosphatase (EC 3.1.3.1, Roche Molecular Biochemicals), to hydrolyze the nucleoside monophosphates formed to nucleosides and orthophosphate. Alternatively, in assays with mononucleotides as substrate, yeast inorganic pyrophosphatase (EC 3.6.1.1, Roche Molecular Biochemicals) was added to hydrolyze pyrophosphate to orthophosphate. After a 5-min preincubation, reactions were started with 2.5 μ g (59 pmol) of purified activator protein. After 10 min the reactions were terminated with 350 μ l of 1 N H₂SO₄. Orthophosphate was determined by a slight modification of the method of Ames and Dubin (22); 350 μ l of a 2 \times concentrated ascorbate-molybdate mixture was added. After a 20-min incubation at 45 °C, the absorbance of the solution was read in a Hitachi model 100-60 spectrophotometer at 820 nm. 8-oxo-dGTP, which is not commercially available, was prepared as described (23).

Kinetic Studies

Enzyme kinetics was studied using standard assay conditions and varying substrate conditions. Data were fitted with Sigma Plot for Windows, Version 5.0 (Jandell Scientific Software) according to the Michaelis-Menten equation. Data obtained in the MDH-activator protein titration experiments was fitted according to the Hill equation.

ACT Protein Expression and Purification

ACT protein was purified from an 8-liter batch culture of *E. coli* JM109 (pHK105) (Table I). Cells were grown to an A₅₉₅ of 0.5, 1 mM isopropyl β -D-thiogalactopyranoside was added, and heterologous gene

expression was allowed for a further 2 h, yielding a final A_{595} of 1.35. Cells were harvested by centrifugation (25 min, $6,500 \times g$).

Step 1: Preparation of Crude Extract—Crude extract (9.8 ml containing 534 mg of protein) was prepared as described above.

Step 2: Heat Treatment—Crude extract was incubated for 30 min at 60 °C. Denatured proteins were precipitated by centrifugation for 10 min at $25,000 \times g$. The pellet was washed thoroughly with buffer A (20 mM Tris-HCl pH 7.5, 5 mM $MgSO_4$, 5 mM 2-mercaptoethanol) to recover any ACT protein present in the pellet. The preparation obtained (6.9 ml) contained 140 mg of protein.

Step 3: First Anion Exchange Chromatography—The sample was loaded onto a Q-Sepharose column equilibrated with buffer A. Proteins were eluted by applying a 40-ml linear 0–1 M KCl gradient at a flow rate of 1 ml.min⁻¹. ACT peak fractions were pooled yielding a preparation (12 ml) containing 67 mg of protein.

Step 4: Gel Filtration Chromatography—3-ml samples were applied onto a Superdex-200 column equilibrated with buffer A at a flow rate of 2 ml.min⁻¹. ACT peak fractions of four separate runs were combined yielding a preparation (12 ml) with 53 mg of protein.

Step 5: Second Anion Exchange Chromatography—2-ml samples were applied onto a Mono-Q column equilibrated with buffer A. Proteins were eluted by applying a 20-ml linear 0–1 M KCl gradient at a flow rate of 0.5 ml.min⁻¹. Pooled fractions with ACT activity of six separate runs were combined and frozen at -80 °C. The final preparation (13 ml) contained 36 mg of protein.

MDH Protein Purification

MDH was purified from *B. methanolicus* and *E. coli* as described (12) with some modifications. Overnight cultures were harvested by centrifugation, and cells were disrupted by two passages through a French Pressure cell at 140 megapascals. Crude extracts were prepared by centrifugation for 30 min at $40,000 \times g$. Proteins were partially precipitated by 30% saturation with ammonium sulfate and incubation for 10 min. Following centrifugation (10 min at $25,000 \times g$) 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, and applied on a Mono-Q (anion exchange) column. Proteins were eluted with a 1–0 M KCl gradient in buffer A.

Separation of ACT, MDH, and Coenzyme NAD(H)

To separate ACT, MDH containing the bound NAD(H) cofactor, and coenzyme NAD(H), reaction mixtures were loaded 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). Free nucleotides (NAD^+ and NADH) appeared in the flow-through, whereas proteins were eluted with a gradient of 20–0% (w/v) $(NH_4)_2SO_4$. ACT eluted at 11% $(NH_4)_2SO_4$ and MDH at 5% $(NH_4)_2SO_4$.

Cofactor Extraction from Activated and Nonactivated MDH

A standard MDH reaction was performed in a total volume of 10 ml. The reaction mixture contained 100 mM glycine-KOH buffer (pH 9.5), 5 mM $MgSO_4$, 5 mM 2-mercaptoethanol, 500 mM methanol, 1 mM NAD^+ , 1.0 nmol (400 μg) of pure MDH (10 nmol subunits), and in the activated MDH reaction, 2.5 nmol (105 μg) of pure ACT. The reaction was started by the addition of MDH protein and terminated in a few seconds by rapid freezing in liquid nitrogen. MDH protein was separated from free nucleotides with a Phenyl-Superose hydrophobic interaction column and desalted on a Pharmacia PD-10 column equilibrated with 10 mM Tris-HCl, pH 8.0, containing 6 M urea (buffer B). The desalted protein fraction was boiled for 2 min and applied onto a Mono-Q anion exchange column equilibrated with buffer B and eluted in a gradient of 0–1 M KCl in buffer B. A solution of AMP and NADH (10 nmol each) prepared in buffer B and boiled for 2 min served as references on the Mono-Q column.

Metal Analyses

The metal composition of purified MDH was determined by atomic absorption spectrophotometry using a PerkinElmer 1100B atomic absorption spectrophotometer. Prior to analysis, the enzyme was dialyzed extensively against 10 mM Tris-HCl buffer (pH 7.5). The elements magnesium and zinc were analyzed in duplicate.

RESULTS

Cloning of the act Gene—Use of the A1/A2 primers based on the N-terminal amino acid sequence of ACT yielded a PCR

fragment of the expected size (89 bp), which was cloned into pBlueScriptIIKS (pHK1) and sequenced. The deduced amino acid sequence was in full compliance with the previously determined N terminus of the ACT protein (16), indicating that the correct DNA fragment had been amplified. Southern hybridization experiments with different digests of chromosomal DNA of *B. methanolicus* strain C1, using the cloned PCR fragment as probe, revealed in all cases only one clear hybridizing signal (results not shown). A 4.4-kb *PstI* DNA fragment that hybridized well with the probe was selected to be cloned. For that purpose a partial gene library sequence of *B. methanolicus* chromosomal DNA was constructed in pBlueScriptIIKS (insert frequency, 60%). Using the primers A1 and A2, a total of 10,000 *E. coli* MC1061 transformants were screened, yielding 16 PCR-positive transformants. Plasmid DNA analysis of the positive transformants showed that all contained a 4.4-kb *PstI* insert (pHK83).

Characterization of the act Gene—In total, 1480 bp of the 4.4-kb *PstI* fragment in plasmid pHK83 was sequenced in both directions, revealing the presence of two open reading frames. The *act* ORF (from ATG³⁸¹ to TGA⁹³⁵, 558 bp in size) encodes a putative protein of 185 amino acids with M_r 21,048. A potential ribosome binding site (AGGA) was identified immediately upstream of *act*. The *act* ORF is immediately followed by ORF2 (at least 545 bp).

Screening of the available data bases revealed a strong similarity between ACT and the YQKG gene product of *Bacillus subtilis* (P54570, 62% identity and 83% similarity with the full-length protein), encoding an ADP-ribose pyrophosphatase (24). The highly conserved sequence motif characteristic for the *E. coli* MutT-related proteins or Nudix hydrolase family was detected in the deduced ACT amino acid sequence (Fig. 2).

Expression of act Gene in E. coli—Extracts of *E. coli* MC1061 containing pHK83 failed to stimulate MDH activity. Clear *act* gene expression in *E. coli* was observed following its introduction in the expression vector pProk1, yielding pHK105. After induction with isopropyl β -D-thiogalactopyranoside, extracts of *E. coli* JM109 (pHK105) displayed high ACT protein activities (425 units.mg⁻¹ protein of MDH-stimulating activity). ACT activity in its native host, *B. methanolicus*, was estimated as 8.6 units.mg⁻¹ (16). SDS-PAGE revealed the presence of a M_r 21,000 protein in strain JM109 (pHK105) that was absent in strain JM109 carrying pProk1. ACT protein was estimated to constitute about 40% of the total soluble protein fraction.

Purification of ACT Protein—Following induction with isopropyl β -D-thiogalactopyranoside, cell extracts were prepared from 8-liter cultures of *E. coli* JM109 (pHK105). *B. methanolicus* is a thermotolerant bacterium, able to grow at temperatures up to 60 °C (9, 15), and we observed that ACT protein in *E. coli* extracts is stable at this temperature for at least 2 h. *E. coli* proteins started to denature after ~10 min at 60 °C. After 30 min of incubation, denatured proteins were collected by centrifugation and discarded. A combination of anion exchange chromatography and gel filtration steps allowed purification of active ACT protein. The M_r of native ACT protein was estimated as 45,000 by gel filtration chromatography on a Superdex-200 column. ACT thus is a homodimer of 21-kDa subunits. SDS-PAGE showed that this procedure yielded pure ACT protein, with a final purification factor of 5.4 and a total yield of 37% (Table I). ACT appears to be a thermostable enzyme; at 100 °C the half-life of its MDH stimulating activity is 1.5 min.

Titration of MDH Activity with ACT Protein—The ACT protein concentration required for maximal stimulation of *in vitro* activity of MDH purified from *B. methanolicus* (bMDH) was determined (Fig. 3). The addition of 1.0 $\mu g.ml^{-1}$ (24 pmol.ml⁻¹) ACT protein stimulated initial MDH activity rates (1.0 $\mu g.ml^{-1}$

Organism	Accession	Partial sequence	gene	substrate
<i>E. coli</i>	P08337	EFPPGKIEMGETPEQAVVRELQEEVIGITPQHFSLFKLEYEFDRH	<i>mutT</i>	8-oxo-dGTP
<i>H. influenza</i>	I64101	EFPPGKVDAGETPEQALKRELEEEIGIVALNAELYERFQFEYPTKI	<i>mutT</i>	8-oxo-dGTP
<i>P. vulgaris</i>	P32090	EFPPGKLEDNETPEQALLRELQEEIGIDVTQCTLLDVAHDFDRH	<i>mutT</i>	8-oxo-dGTP
<i>Str. pneumoniae</i>	P41354	IGVGGKLERGETPQECARREILLEETGLKAKPVLKGV---ITFPEFT	<i>mutX</i>	8-oxo-dGTP
Human	P36639	NGFPGKVOEGETIEDGARRELQEEESGLTVDALHKVG--QIVFEFVG	<i>hmth1</i>	8-oxo-dGTP
<i>E. coli</i>	P36651	EMVAGMIEEGESVEDVARREAIKEEAGLIVKRTKPVLS-FLASPGGT	<i>yqie</i>	ADPR
<i>B. subtilis</i>	P54570	EIPAGKLEKGEPEYTAALRELEEEETGYTAKKLTAKITA-FYTSFGFA	<i>yqkg</i>	ADPR
<i>H. influenza</i>	P44684	ELIAGMVEKGEKPEDVALRESEEEAGIQVKNLTHCLS-VWDSFGGI	<i>yqie</i>	ADPR
<i>B. methanolicus</i>	AY128667	EIPAGKLEKGEDPRVTALRELEEEETGYECEQMEWLIS-FATSPGFA	<i>act</i>	ADPR
<i>E. coli</i>	P45799	GFSKGLIDPGEESVYEAANRELKKEEVGFANDLTFLLK-LSMAPSYF	<i>orf186</i>	Ap3A
Human	P50583	TTPKGFHVEPCEDDLETALRETOEEAGIEAGQLTIEGFKRELNYVA		Ap4A
<i>E. coli</i>	P32664	TVLAGFVEVGETLEQAVAREVMEESGIKVKNLRYVTS--QPWFPPQ	<i>orf257</i>	NADH
<i>E. coli</i>	P24236	QSVTGSVEEGETAPQAAMREVKKEEVTIDVVAEQTLIDCQRTVEFE	<i>orf17</i>	dATP
Human	NP006694	IVPGGMEPEEEPSVAAREVCEEAGVKGTGLGRLVGFIFENQERKHR	<i>dipp</i>	DIPP
		G E R E E E G (P)		

FIG. 2. Partial sequence alignment of selected members of the Nudix hydrolase family of proteins. The Nudix hydrolase consensus sequence (18, 27) is shown in bold below the sequence. Also the conserved Pro residue downstream of the Nudix box, characteristic of the ADPR hydrolase subfamily, is shown in bold.

TABLE I
Purification of ACT protein from *E. coli* JM109 (pHK105)

One unit of MDH-stimulating activity is defined as the amount of ACT that stimulates a fixed quantity of purified MDH (1.0 μg ; 2.5 pmol) in the reaction mixture (1 ml) to 50% of $V_{\text{max}} - V_0$ under reaction conditions described (16).

Purification step	Total protein	Specific activity	Yield	Purification factor
	mg	units.mg ⁻¹	%	
Crude extract	534	425	100	1.0
Heat treatment	140	1232	76	2.9
Q-Sepharose	67	1746	51	4.1
Superdex-200	53	1638	38	3.9
Mono-Q	36	2301	37	5.4

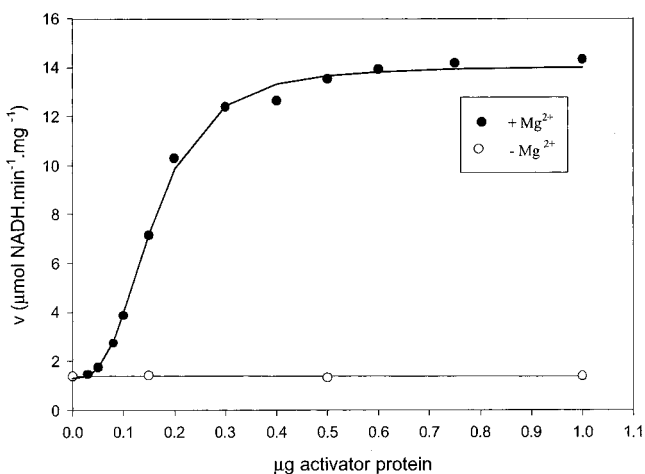


FIG. 3. Titration of ACT protein purified from *E. coli* JM109-pHK105 with purified *B. methanolicus* MDH (1.0 $\mu\text{g}\cdot\text{ml}^{-1}$; 2.5 pmol MDH $\cdot\text{ml}^{-1}$). The assays were performed under conditions described in the presence (●) or absence (○) of 10 mM MgSO_4 .

(2.5 pmol $\cdot\text{ml}^{-1}$) MDH protein) by \sim 10-fold. The presence of Mg^{2+} -ions was a prerequisite for this stimulating effect (Fig. 3).

(Di-)nucleotide Hydrolyzing Activity of ACT—In view of the presence of the highly conserved Nudix hydrolase sequence motif in ACT, its (di-)nucleotide hydrolyzing activity was evaluated. Experiments were performed in a glycine-KOH buffer (pH 9.5) at 50 °C, the pH and temperature values optimal for MDH activity and for the stimulatory effect of ACT on MDH (16). No activity was detected with the canonical (deoxy-)nucleoside triphosphates, 8-oxo-dGTP, or with diadenosine tri- or tetraphosphates, previously shown to be substrates for members of the Nudix hydrolase family (18). However, incu-

bations of ACT with ADP-ribose (ADPR) revealed that this is an outstanding substrate for the enzyme (V_{max} , 348 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$; K_m , 63 μM). ACT also showed a clear NAD^+ hydrolyzing activity, although the substrate affinity and V_{max} for NAD^+ are much lower, resulting in a catalytic efficiency (k_{cat}/K_m) of 3–4 orders of magnitude lower than for ADPR (0.48×10^3 versus 2.05×10^6 $\text{M}^{-1}\cdot\text{s}^{-1}$) (Fig. 4, Table II). The presence of Mg^{2+} -ions is a prerequisite for ACT catalyzed ADPR and NAD^+ hydrolysis. ACT did not show any NADP(H) hydrolyzing activity. Only a very low NADH hydrolyzing activity was detected, at least a factor 100 lower than the NAD^+ hydrolysis rate. AMP plus ribose 5'-phosphate and AMP plus NMN^+ , were identified as the ACT hydrolysis products of ADPR and NAD^+ , respectively.

Heterologous Expression and Purification of MDH Protein—MDH protein was purified from *B. methanolicus* (bMDH) (12) and from *E. coli* (pHK105) cells (cMDH) (this paper). For cMDH, the purification fold was about three times, whereas the yield was \sim 35% (Table III). cMDH purified from *E. coli* cells grown in LB medium could not be activated by ACT (11) (Table IV). This allowed analysis of the mechanism by which ACT activates the alcohol oxidation reaction of MDH. cMDH was found to contain considerably less NAD(H) cofactor than bMDH and also to display a greatly reduced activity (Table IV). cMDH also contained less than 50% of the Mg^{2+} and Zn^{2+} ions found in bMDH (11, 13). The addition of Mg^{2+} and/or Zn^{2+} to the assay mixture did not affect the reaction rate. These discrepancies between bMDH and cMDH were investigated further by cultivating *E. coli* expressing MDH in LB medium with additional MgSO_4 and/or ZnSO_4 . Surprisingly, LB medium supplemented with 20 mM MgSO_4 yielded cMDH protein with strongly increased NAD(H) and Mg^{2+} content, similar to bMDH. cMDH from such cultures displayed a V_{max} value similar to bMDH (Table IV). Activity of this cMDH was stimulated

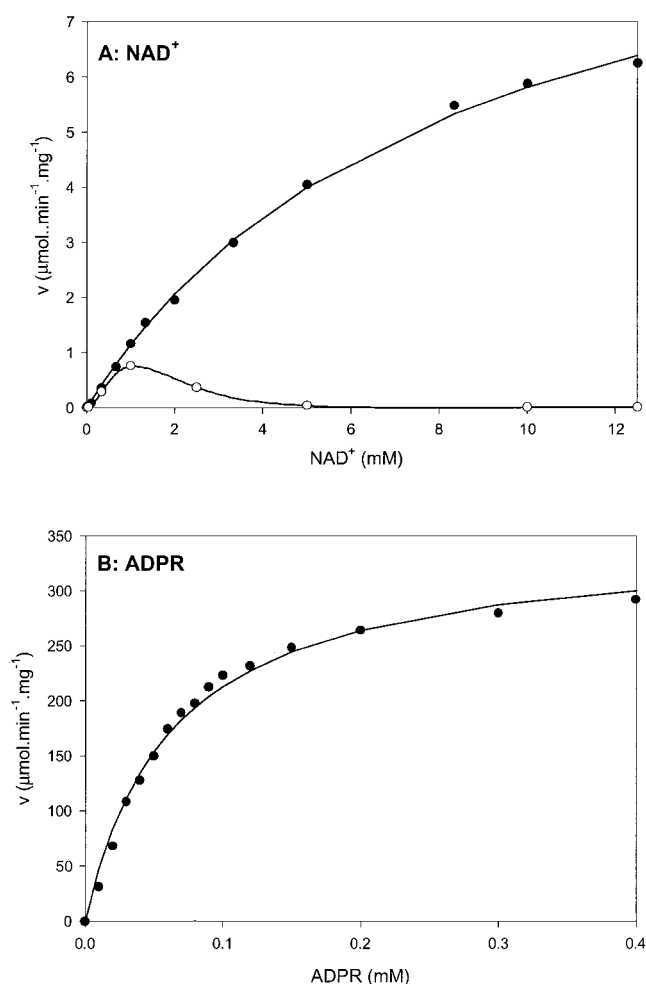


FIG. 4. Primary plots of NAD⁺ and ADPR hydrolyzing activity of ACT. A, NAD⁺ hydrolyzing activity of ACT versus concentration of NAD⁺ in the presence (●) or absence (○) of MgSO₄ (10 mM). Reactions were performed in a total volume of 300 μl with 1.25 μg (30 pmol) of ACT. B, ADPR hydrolyzing activity of ACT versus concentration of ADPR in the presence of MgSO₄ (10 mM). Reactions were performed in a total volume of 300 μl with 15.6 ng (0.40 pmol) of ACT.

8-fold by ACT, similar to observations made with bMDH (Table IV). The addition of 10 mM ZnSO₄ to the medium had no effect on cMDH activation and reaction rate. The data thus clearly show that a positive correlation exists between the NAD(H) cofactor content of the cMDH protein and the ability of ACT to stimulate MDH activity (Table IV).

ACT-mediated Hydrolysis of MDH-bound NAD(H) Cofactor—The data thus show that ACT hydrolyzes free NAD⁺ and only stimulates the activity of MDH protein that carries NAD(H) cofactor. The effects of ACT on the bMDH-bound NAD(H) cofactor during the initial (activation) phase of the reaction therefore were studied in more detail. Following incubations of reaction mixtures for a few seconds, bMDH containing bound NAD(H) cofactor was separated from ACT and from free coenzyme NAD(H) by Phenyl-Superose hydrophobic interaction column chromatography. Subsequently, bound nucleotides were extracted from the bMDH protein preparations obtained via urea extraction.

Urea extracts of bMDH protein preparations, from incubations with or without ACT and/or methanol plus coenzyme NAD⁺, were analyzed on a Mono-Q anion exchange chromatography column. Cofactor elution profiles of bMDH preparations from ACT-independent incubations (with or without methanol plus coenzyme NAD⁺) showed one clear 260 nm

absorbance peak co-eluting with the urea-treated NADH reference (Fig. 5). MDH cofactor NAD(H) occurs mainly (>95%) in the reduced state (Ref. 17 and this study). Also bMDH preparations from incubations with ACT, but without coenzyme NAD⁺ or alcohol substrate, resulted in a single NADH peak. These results clearly show that ACT has no effect on the bMDH NAD(H) cofactor when in the reduced form. A major change occurred during ACT-dependent incubations of MDH in the presence of methanol plus coenzyme NAD⁺ (Fig. 5); on average, a 47% (±7%) decline in NADH cofactor content was observed in the elution profile of MDH (peak integration of the elution profiles), and a second peak was present co-eluting with urea-treated AMP. The AMP content in MDH was calculated to occupy on average 43% (±6%) of the NAD(H) cofactor binding sites in MDH, suggesting a stoichiometric conversion of MDH cofactor NAD(H) into MDH-bound AMP. No bound NAD⁺ or NMN⁺ was found in activated MDH preparations. The data thus show that only under these conditions will ACT hydrolyze the MDH cofactor NAD(H) into AMP and NMN(H), with only AMP remaining bound in MDH protein.

The combined data thus show that ACT stimulates MDH activity by hydrolyzing the MDH cofactor NAD(H) when occurring in the oxidized state (NAD⁺). This oxidized state occurs as an obligate step in the alcohol oxidation reaction cycle, when MDH is presented with its substrates methanol and coenzyme NAD⁺. Under the incubation conditions used, with MDH-bound cofactor NAD⁺ present at very low concentrations, ACT rapidly hydrolyzed MDH-bound cofactor NAD⁺, indicating that ACT has a much higher affinity for NAD⁺ bound to MDH than for free NAD⁺ (K_m 8.3 mM).

MDH Reaction Kinetics—In the absence of ACT, bMDH obeyed a Ping-Pong type of reaction mechanism; Hanes plots of the initial reaction rates of bMDH clearly revealed lines intercepting the vertical axis, indicating noncompetitive cosubstrate inhibition, typical for a Ping-Pong type of reaction mechanism (Fig. 6). The MDH-bound cofactor NAD(H) thus functions as a temporary electron sink, with alternate binding of substrate and release of product. In contrast, cMDH purified from *E. coli* cells grown in the absence of additional Mg²⁺-ions in the growth medium (resulting in a strongly decreased NAD(H) cofactor content; Table IV) displayed a different type of reaction mechanism; Hanes plots of the initial reaction rates of cMDH revealed mixed noncompetitive cosubstrate inhibition, indicating a ternary complex type of reaction mechanism (25, 26) (Fig. 6). This indicates that, in the absence of a temporary electron sink, the reaction can proceed only when both substrates are bound to the enzyme. Steady-state kinetics of cMDH purified from *E. coli* cells grown on MgSO₄-supplemented LB medium (resulting in a NADH cofactor/MDH subunit ratio close to 1.0; Table IV) again obeyed a Ping-Pong type of reaction mechanism.

The presence of ACT, resulting in hydrolysis of the MDH-bound cofactor, NAD⁺ (see above), strongly affected bMDH kinetics, resulting in a ternary complex type of reaction mechanism (Fig. 6). Similar to cMDH with a low NADH cofactor content, ACT-treated MDH apparently has to bind both methanol and coenzyme NAD⁺ before the reaction can proceed. The absence of a temporary electron sink excludes a Ping-Pong type of reaction mechanism.

DISCUSSION

Heterologous expression of active ACT protein in *E. coli* provided clear evidence that the *B. methanolicus* C1 *act* gene had been cloned successfully in the present study. The strong stimulation of bMDH activity by ACT, as observed previously with both proteins purified from *B. methanolicus* strain C1 (16), also was observed in the present study with ACT protein

TABLE II
Kinetic constants of dinucleotide hydrolyzing activity of ACT

Kinetic constants were determined under standard assay conditions as described under "Materials and Methods," with protein concentrations as described in the legend for Fig. 4. k_{cat} was calculated from V_{max} , assuming that 1 subunit contains one active site. ND, not determined.

Substrate	V_{max} $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$	k_{cat} s^{-1}	K_m mM	k_{cat}/K_m $\text{M}^{-1}\cdot\text{s}^{-1}$
ADPR	347.8	129.1	0.063	2.05×10^6
NAD ⁺	10.6	3.9	8.28	0.48×10^3
NADH	<0.1	<0.04	ND	ND

TABLE III
Purification of wild type MDH expressed in *E. coli* without additional MgSO_4 in the growth (LB) medium

Sample	Protein <i>mg</i>	Specific activity <i>milliunits/mg</i>	Total activity <i>milliunits</i>	Yield <i>%</i>	Purification <i>-fold</i>
Crude extract	22.8	165	3760	100	1.0
Ammonium sulfate 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

TABLE IV
Kinetic characteristics and metal composition of bMDH and cMDH

bMDH and cMDH were expressed either in the presence or absence of additional MgSO_4 in the growth medium. Calculations are based on Michaelis-Menten kinetics. NA, not applicable; ND, not determined.

Sample	$[\text{MgSO}_4]$ in LB medium <i>mM</i>	Metal/MDH <i>subunit ratio</i>	Activation ^a <i>-fold</i>	V_{max} of MDH activity ^b <i>milliunits/mg</i>	K_m NAD ⁺ <i>mM</i>	NADH cofactor/MDH <i>subunit ratio</i>
bMDH	NA	1.30	8.5	1310	0.02	1.0
cMDH	0	0.32	1.5	390	0.04	0.09
cMDH	10	1.15	3.8	820	0.03	ND
cMDH	20	1.81	8.1	910	0.03	0.8

^a Stimulating effect of activator protein-factor by which V_{max} of MDH activity is changed as a result of addition of a saturating amount of ACT protein.

^b Determined in the absence of ACT protein.

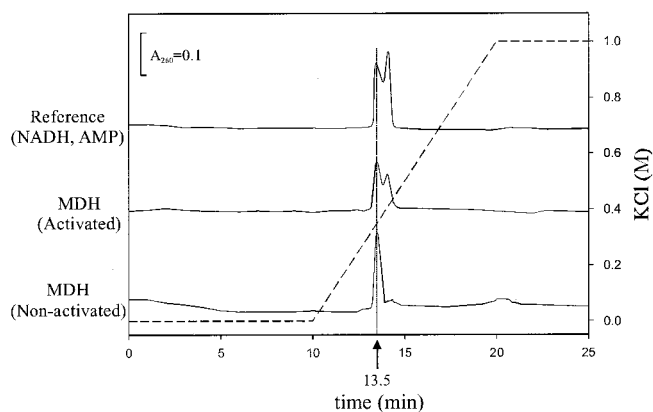


FIG. 5. Mono-Q anion exchange chromatography analysis of urea extracts of MDH (432 μg ; 1.08 nmol) incubated with and without ACT (112 μg ; 2.67 nmol) for 5 s. Detailed conditions are given under "Materials and Methods." The elution profiles of NADH (10 nmol) and AMP (10 nmol) are used as references. NADH and AMP eluted at 13.5 (dashed line) and 13.9 min, respectively.

purified from *E. coli* JM109 (pHK105). Both ACT protein preparations are capable of stimulating bMDH activity *in vitro* up to a factor of 10 provided that Mg^{2+} -ions are present in the assay mixtures. The estimated M_r of native ACT protein (45,000) purified from *E. coli* is similar to that of the *B. methanolicus* strain C1 homodimeric ACT protein (16).

MDH and ACT protein expression is under co-ordinate control (methanol induced) in *B. methanolicus* (14). The *act* gene was not detected, however, during characterization of the cloned *B. methanolicus mdh* gene and its flanking regions (11). Southern hybridization experiments (data not shown) and nu-

cleotide sequence analysis (1.48 kb) of the cloned 4.4-kb *Pst*I DNA fragment in pHK83 (this study) also did not provide any evidence for clustering of *mdh* and *act* genes. The *act* gene is followed immediately by ORF2, encoding a putative protein with unknown function. The deduced amino acid sequence of ORF2 does not show any similarity with MDH or any other known proteins. The start codon of ORF2 and the stop codon of *act* overlap (data not shown; GenBank™ data base accession no. AY128667), suggesting translational coupling of both genes. Neither an *E. coli*-like promoter nor a clear termination motif could be found in the regions adjacent to the *act* ORF, which may explain the failure to obtain ACT protein expression in *E. coli* (pHK83) transformants. The regulation of *mdh* and *act* gene expression and their respective chromosomal locations thus remain to be elucidated.

A data base search for proteins with amino acid sequence similarities revealed that ACT carries the highly conserved sequence motif characteristic for MutT-related proteins (27) or Nudix hydrolases (18) (Fig. 2). The *E. coli* MutT protein possesses (8-oxo)-dGTPase activity, resulting in conversion of (8-oxo)-dGTP into (8-oxo)-dGMP and pyrophosphate (18, 27, 28). Other members of this family subsequently were found to possess hydrolytic activity with a wider variety of substrates, containing a common nucleotide diphosphate group linked to some other moiety (x (18, 29, 30). Analysis of Nudix hydrolase activity of ACT revealed that it is an ADPR hydrolase. Similar to other ADPR hydrolase enzymes (24), ACT also contains the conserved proline residue downstream of the Nudix box, which appears characteristic for this subfamily of the Nudix hydrolase enzymes (Fig. 2) (24). ADPR, a major product of NAD⁺ glycohydrolase, can be transferred enzymatically onto acceptor proteins to regulate their biological activity or is attached non-

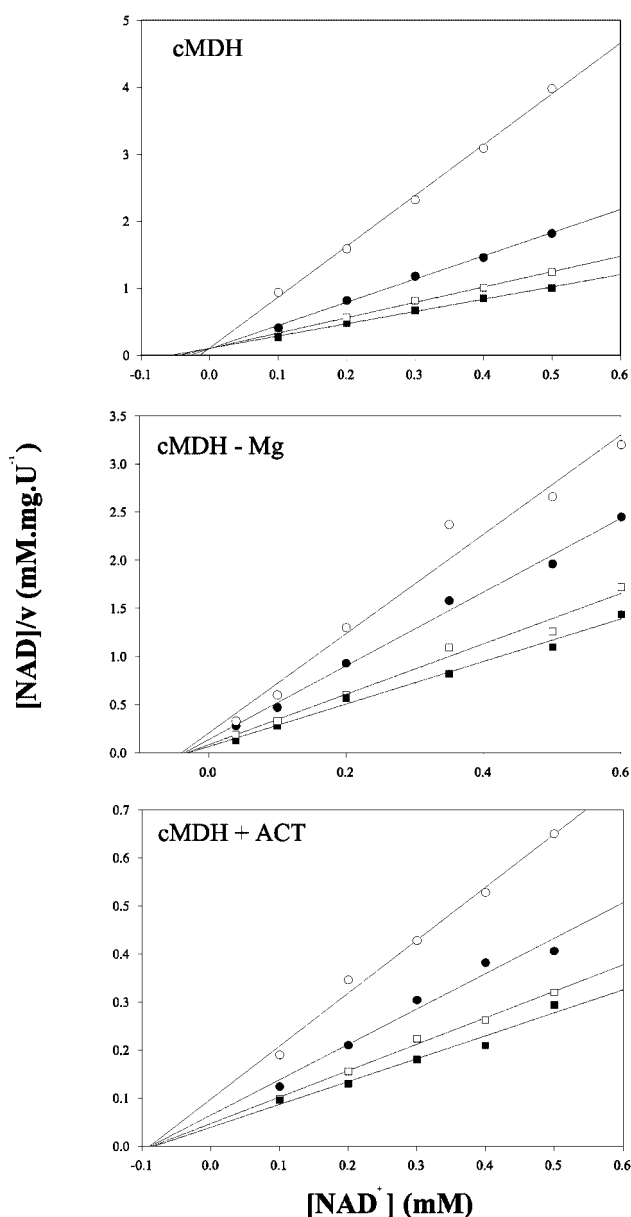


FIG. 6. Steady-state kinetics of cMDH expressed in *E. coli* grown on LB medium with (cMDH) and without (cMDH-Mg) additional MgSO₄ and of activated cMDH (cMDH + ACT). MDH and ACT concentrations were 25 and 50 pmol, respectively. The following methanol concentrations (mM) were applied: ○, 62.5; ●, 125; □, 250; ■, 500.

enzymatically to nucleophilic amino acid residues such as lysines and cysteines (31). These random ADPR attachments may be detrimental to cellular processes (24).

ACT also hydrolyzes free NAD⁺, albeit with a very low catalytic efficiency (Table II). The occurrence of ACT protein with a high affinity for free coenzyme NAD⁺ in fact is undesirable; activity of such an ACT protein might severely reduce the cellular NAD⁺ pool, interfering with NAD⁺ requiring metabolic processes in *B. methanolicus*, including the reaction catalyzed by MDH. The data presented in this paper show that ACT must have a much higher affinity for the MDH-bound NAD(H) cofactor as a substrate than for free NAD⁺ (K_m 8.3 mM), because ACT is capable of hydrolyzing at least 50% of the MDH cofactor within seconds, whereas the MDH cofactor concentration is very low (1 μM). Furthermore, a large part of the NAD(H) will be in the reduced state, which is not a substrate of ACT.

The three-dimensional structure of the glycerol dehydrogenase protein of *Bacillus stearothermophilus* revealed that the nicotinamide group of its coenzyme NAD⁺ binds in a deep pocket formed by nine amino acid residues (32). In MDH, which is 23% identical and 40% similar to glycerol dehydrogenase, this pocket is also present; of these nine amino acid residues, six residues are identical and three are similar. We have clear evidence from site-directed mutagenesis experiments that the NAD(H) cofactor binding site of MDH resembles the NAD⁺ binding site of glycerol dehydrogenase of *B. stearothermophilus*.² Assuming a similar positioning of the nicotinamide group of cofactor NAD(H) in MDH, only the ADPR moiety of the NAD(H) cofactor thus may be exposed, which is an excellent substrate for ACT, yielding AMP and NMN(H).

The molecular mechanism of the stimulating effect of ACT on the reaction rate of MDH was elucidated by analysis of differences in the properties of cMDH and bMDH (11): first, cMDH activity could not be stimulated by activator protein; second, its metal content (Zn²⁺ and Mg²⁺) was considerably lower; and third, cMDH lacked bound cofactor NAD(H). The results presented in this paper demonstrate that all three differences disappeared when MgSO₄ was added to the growth medium of the MDH-expressing *E. coli* host. Metal and cofactor analyses of cMDH protein purified from *E. coli* cells grown on LB medium with varying concentrations of MgSO₄ showed a positive correlation between the amount of MgSO₄ present in the growth medium and the Mg²⁺ and NAD(H) cofactor composition of cMDH. It is likely that Mg²⁺ has a profound effect on NAD(H) cofactor binding by MDH and consequently on the ability of ACT protein to stimulate MDH activity. bMDH displays a Ping-Pong type of reaction mechanism with the cofactor functioning as temporary electron sink (this paper) (17). cMDH purified from cells grown under conditions in which NAD(H) cofactor binding is poor displays a ternary complex reaction mechanism (25, 26).

MDH cofactor analysis studies subsequently provided conclusive evidence that ACT hydrolyzes the MDH-bound NAD⁺ cofactor, yielding AMP (and NMN⁺). This resulted in a 50% decrease in extractable NAD(H) cofactor, also showing that AMP is present in activated MDH protein fractions (Fig. 5). Hydrolysis of MDH cofactor NAD⁺ resulted in a switch from a Ping-Pong type of reaction mechanism to a ternary complex mechanism as observed in co-substrate inhibition patterns (Fig. 6). Activated MDH thus catalyzes a cofactor-independent reaction with direct transfer of electrons from the methanol substrate to coenzyme NAD⁺. Conceivably, a conformational change occurs in MDH protein to position the NAD⁺ coenzyme binding site closer to the methanol binding site, allowing such a direct electron transfer. If a conformational change occurs, it may be triggered by hydrolysis of the NAD(H) cofactor, yielding an activated MDH molecule. The bound AMP may serve as a stabilizing ligand for the activated state. Image analysis experiments with MDH protein indeed revealed two distinct projections, indicating two conformational states (13).

The data obtained so far allow us to propose a model for ACT-MDH interaction (Fig. 7). In this model an activated and a nonactivated state of MDH are distinguished: MDH in the nonactivated state displays a Ping-Pong type of reaction mechanism in which the redox-active cofactor functions as a temporary electron deposit; MDH in the activated state catalyzes a cofactor-independent reaction, displaying a ternary complex reaction mechanism, due to the absence of the NMN(H) moiety of the NAD(H) cofactor, which has been hydrolytically removed

² H. Kloosterman, H. J. Hector, and L. Dijkhuizen, manuscript in preparation.

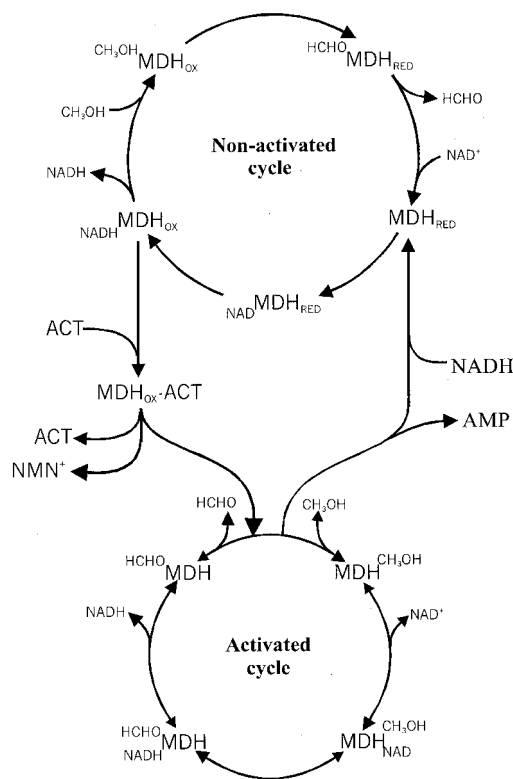


FIG. 7. Model of the effects of ACT on MDH reaction cycles. In this model two types of reaction mechanisms are distinguished. In the nonactivated reaction cycle (top), the NAD(H) cofactor functions as a temporary electron deposit, and the reaction thus proceeds via a Ping-Pong reaction mechanism. On cleavage of the NAD(H) cofactor by the action of ACT, the NMN(H) moiety diffuses out of the cofactor binding site, and MDH enters the activated reaction cycle (top) characterized by a ternary complex mechanism. Conversely, an activated MDH molecule can re-enter the nonactivated reaction cycle by binding NADH in the cofactor binding site. The return of activated MDH to the deactivated state may be stimulated by increased cellular concentrations of NADH (or increased NADH/NAD⁺ ratios).

by ACT. MDH activation is a reversible process (16): MDH can change from the activated state to the cofactor-dependent state and *vice versa* by either binding NADH to available cofactor binding sites or, conversely, removing the cofactor NMN(H) moiety by the action of ACT.

Activated MDH has a higher affinity and V_{\max} for methanol (140 mM and 12 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$), compared with the catalytic constants of nonactivated MDH (V_{\max} and K_m for methanol: 1.3 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ and 230 M, respectively) (16). ACT thus stimulates the methanol turnover rate and thus the catalytic efficiency of MDH. A high methanol turnover rate without proper feedback control might be detrimental to *B. methanolicus*, resulting in the accumulation of toxic intracellular levels of formaldehyde. Free NADH may serve as an inhibitor of ACT activity (hydrolysis of MDH-bound NAD(H)): ACT strongly (but noncovalently) binds free NADH (1 mol/mol ACT subunits) (17), but it very poorly hydrolyzes this substrate (Table II). ACT activity may be controlled by the cellular NADH/NAD⁺ ratio.

B. methanolicus employs a highly sophisticated catalytic mechanism for methanol oxidation, suggesting that this organism is very well adapted to growth at varying methanol concentrations in its natural habitat (in soil) (9, 10). Under conditions in which methanol is scarce, the great majority of MDH protein will be in the activated state, allowing a high catalytic efficiency because of a high methanol affinity and a high V_{\max} .

Under these conditions the rate-limiting step most likely is the binding of methanol to MDH rather than the reoxidation of NADH cofactor. Growth under carbon limitation may result in a low intracellular NADH/NAD⁺ ratio. Therefore, activated MDH will not readily return to the deactivated state.

ACT is a member of the MutT proteins or Nudix hydrolases. All members of this family characterized so far are able to hydrolyze a pyrophosphate bond in different (di-)nucleotides. This protein family has been referred to as a family of "house-cleaning" enzymes (18). More recently Nudix hydrolase proteins were studied and exhibited a clear regulatory function (33, 34). The latter description is more appropriate for ACT. ACT is the first Nudix hydrolase family member that hydrolyzes a Nudix substrate bound to another enzyme and thereby regulates its catalytic activity. This represents a novel mechanism for alcohol dehydrogenase activity regulation.

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