Chapter 3

Molecular, biochemical and functional characterization of a Nudix hydrolase protein that stimulates 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 C_1-C_4 primary alcohols is a decameric protein with 1 Zn^{2+}-ion and 1-2 Mg^{2+}-ions plus a tightly bound NAD(H) cofactor per subunit (a nicotinoprotein). Mg^{2+}-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 C_1 - C_4 primary alcohols is strongly stimulated by a second *B. methanolicus* protein (ACT), provided MDH contains NAD(H) cofactor and Mg^{2+}-ions are present in the assay mixture. Characterization of the *act* gene revealed the presence of the highly conserved amino acid sequence motif typical for 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 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.

Introduction

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 source 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 (Anthony, 1982;Dijkhuizen, 1993).

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 peroxisomes, an organel that also contains catalase activity (Harder and Veenhuis, 1989). Gram-negative bacteria employ a methanol dehydrogenase (MDH) with pyrroloquinoline quinone as cofactor (a quinoprotein), located in the periplasmic space (Anthony, 1986;Duine *et al.*, 1986). 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 (Reid and Fewson, 1994;Bystrykh *et al.*, 1993a;Hektor *et al.*, 2000).

Pure cultures of obligately aerobic, thermotolerant *Bacillus methanolicus* strains grow rapidly in methanol mineral media (doubling times 40-80 min) at temperatures of 35-60^{o}C, and are tolerant to very high methanol concentrations (Arfman *et al.*, 1992b;Dijkhuizen *et al.*, 1988). All strains studied employ a coenzyme NAD^+-dependent MDH for growth on methanol and other primary alcohols (C_1-C_4) (de Vries *et al.*, 1992;Arfman *et al.*, 1989). MDH from *B. methanolicus* strain C1 belongs to family III of alcohol dehydrogenases (de Vries *et al.*, 1992;Vonck *et al.*, 1991). It is a decameric enzyme with subunits of 43,000 Da (Dijkhuizen *et al.*, 1988;de Vries *et al.*, 1992;Arfman *et al.*, 1992a;Dijkhuizen and Arfman, 1990;Vonck *et al.*, 1991;Arfman *et al.*, 1991;Arfman *et al.*, 1997). Each MDH subunit contains 1 Zn^{2+}-ion and 1-2 Mg^{2+}-ions (Vonck *et al.*, 1991). 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 non-covalently) bound NAD(H) molecule, which is oxidized and reduced

by methanol and formaldehyde, respectively, and thus functions as a cofactor (Arfman *et al.*, 1997). NAD^+ plays two important roles in the MDH-catalyzed reaction: MDH-bound NAD^+ cofactor serves as primary electron acceptor in the alcohol dehydrogenase reaction and exogenous NAD^+ coenzyme is responsible for re-oxidation 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 (Arfman *et al.*, 1997). This is very different from typical NAD^+ coenzyme-dependent alcohol dehydrogenases, which follow a ternary complex type of reaction mechanism (Reid and Fewson, 1994).
Studies with purified proteins showed that MDH activity with C_1-C_4 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 one second upon addition of saturating amounts of ACT, requires the presence of Mg^{2+}-ions. Spectral studies showed that Mg^{2+}-ions are essential for formation of an MDH.ACT.NAD^+.Mg^{2+} 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 (Arfman *et al.*, 1991).
Synthesis of the MDH and ACT proteins in *B. methanolicus* is regulated coordinately (Arfman *et al.*, 1992a). 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 **nu**cleotide **di**phosphate group linked to some other moiety (**x**) (Bessman *et al.*, 1996). 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.

Bacillus methanolicus strain C1 cells were grown as described (de Vries *et al.*, 1992). *E. coli* strains MC1061 and JM109, serving as hosts for genetic modifications and heterologous gene expression, respectively, were grown on LB medium (Sambrook *et al.*, 1989); when appropriate ampicillin (100 $mg.l^{-1}$) was added.

Primers and DNA amplification.

The primers used, A1: 5'-GGCGAATTCAA(A/G)TT(A/G)TT(T/C)GA(A/G)GA(A/G)AA (A/G)AC-3', and A2: 5'-GGCTGATCATC (C/T/A)AC(T/C)TG(T/C)AA(T/C)TT(C/T/A)AC (C/T/A)AC-3', were based on the N-terminal amino acid sequence of the ACT protein (Arfman *et al.*, 1991). Their degree of degeneration was limited based on codon bias of *B. methanolicus* (de Vries *et al.*, 1992). Primer A3 consisted of an *Eco*R1 site followed by the first 19 nucleotides of the *act* gene: 5'-GCGGAATTCATGGGAA-AATTATTTGAGG-3'; anti-sense primer A4 contained a *Bam*H1 site and the last 18 nucleotides of *act*: 5'-CGCGGATC CTCATTTATGTTTGAGAGC-3'. The KS and SK primers used are commercially available (Stratagene, Westburg, Leusden, The Netherlands). Primer positions are shown in Fig. 1.

DNA amplification reactions were performed with Vent-DNA-polymerase (Biolabs, New England). Reaction mixtures (100 ml) contained: enzyme buffer (Biolabs, New England), dNTP (50 mM per nucleotide), primers (0.5 mM per primer), target DNA (1-10 ng). Target DNA was incubated for 5 min at 94°C before adding polymerase. Amplification conditions: 25 reaction cycles at temperatures for denaturation, primer annealing and primer extension of 94, 45 and 72°C, respectively. In the last reaction cycle, 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, Westburg, Leusden). Plasmid pHK83 contains a 4.4 kb insert 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*R1 and *Bam*H1 sites of pProk1 (Clontech Laboratories, Palo Alto), with *act* expression controlled by the pProk1 *tac* promoter.

DNA isolation and manipulation.
B. methanolicus strain C1 total chromosomal DNA was isolated as described (de Vries *et al.*, 1992). Methods for DNA handling, modification and cloning were performed as in (Sambrook *et al.*, 1989).

Gene library construction and screening.
Chromosomal DNA of *B. methanolicus* was digested with *Pst*I and fragments ranging from 3.7 kb to 5.1 kb were isolated, ligated in pBlueScriptIIKS and transformed to *E. coli* MC1061. This partial gene library 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^{o}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 (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 (deposited in the GenBank^{TM} 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 (Altschul *et al.*, 1990) at NCBI (Natl. 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 $1.4*10^5$ kilonewtons.m^{-2}. Enzyme assays were performed at 50^{o}C with prewarmed buffer solutions (unless stated otherwise). NAD^+-dependent MDH activity and MDH stimulating activity of ACT were measured as described earlier (Arfman *et al.*, 1991;Arfman *et al.*, 1989). Assays were performed at 50^{o}C and NAD(H) oxidation or reduction was followed at 340 nm in a Hitachi model 100-60 spectrophotometer. The MDH assay was buffered by 100 mM glycine-KOH buffer (pH 9.5) and contained 5 mM $MgSO_4$, 5 mM 2-mercaptoethanol, 1 mM NAD^+ and enzyme. The reaction was started with 500 mM methanol after 5 min pre-incubation. The stimulatory effect of activator protein was analyzed by 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 μg (2.5 pmol) MDH/ml reaction mixture) to 50% of fully ACT activated MDH (V_{max}) minus the ACT independent activity of MDH (V_o) (Arfman *et al.*, 1991).

Nudix hydrolase activity of ACT protein was determined in 300 μl 100 mM glycine-KOH buffer (pH 9.5), containing 10 mM $MgSO_4$ and 250 nmol (di-)nucleotide at 50^{o}C.

Dinucleotide hydrolyzing activity was assayed using an excess amount of calf intestine alkaline phosphatase (EC 3.1.3.1, Boehringer Mannheim), 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, Boehringer Mannheim) was added to hydrolyze pyrophosphate to orthophosphate. After 5 min pre-incubation, reactions were started with 2.5 µg (59 pmol) purified activator protein. After 10 min the reactions were terminated with 350 µl 1 N H_2SO_4. Orthophosphate was determined by a slightly modified method of Ames and Dubin (Ames and Dubin, 1960): 350 ml of a 2 times concentrated ascorbate-molybdate mixture was added. After 20 min incubation at 45^{o}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 (Mo *et al.*, 1992).

Kinetic studies.
Enzyme kinetics was studied using standard assay conditions and varying substrate conditions. Data were fitted with Sigma Plot for Windows version 5.00 (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 litre batch culture of *E. coli* JM109 (pHK105) (Table 1). Cells were grown to an OD_{595} 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 OD_{595} of 1.35. Cells were harvested by centrifugation (25 min, 6,500 x 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^{o}C. Denatured proteins were precipitated by centrifugation for 10 min at 25,000 x 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: 1st 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 4 separate runs were combined yielding a preparation (12 ml) with 53 mg of protein.

Step 5: 2nd Anion-exchange chromatography. Samples of 2 ml 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 6 separate runs were combined and frozen at -80^{o}C. The final preparation (13 ml) contained 36 mg of protein.

Table 1. Purification of ACT protein from *E. coli* JM109 (pHK105)

Purification step	Total Protein (mg)	Specific activity ($U.mg^{-1}$)	Yield	(%)	Purification factor
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

[a] 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_{max}-V_o under reaction conditions described (Arfman *et al.*, 1991)

MDH protein purification.
MDH was purified from *B. methanolicus* and *E. coli* as described (Arfman *et al.*, 1989), with some modifications. Overnight cultures were harvested by centrifugation; 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 x g. Proteins were partially precipitated by 30% saturation with ammonium sulphate and incubating for 10 min. Following 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, 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, while 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 non-activated 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 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 Perkin Elmer 1100B atomic absorption spectrophotometer. Prior to analysis, the enzyme was dialysed extensively against 10 mM Tris-HCl buffer (pH 7.5). The elements magnesium and zinc were analysed 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 (Arfman *et al.*, 1991), 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 *Pst*I DNA fragment that hybridized well with the probe was selected to be cloned. For that purpose a partial gene library of *B. methanolicus* chromosomal DNA was constructed in pBlueScriptIIKS (insert frequency of 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 *Pst*I insert (pHK83).

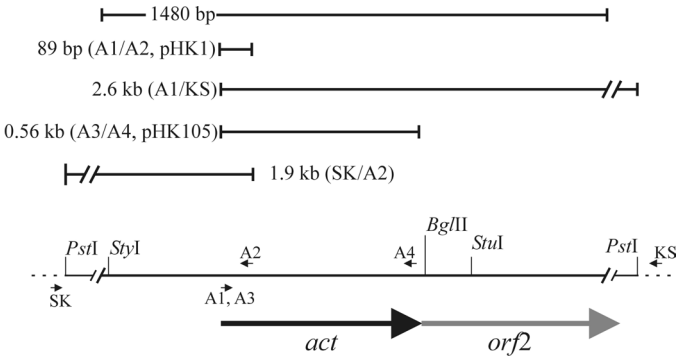

Figure 1. Schematic representation of the 4.4 kb *Pst*I insert of *B. methanolicus* strain C1 chromosomal DNA in pHK83. The large arrows indicate open reading frames (*act* and *orf2*), small arrows indicate the primers that were used for PCR reactions. The 89 bp fragment shown is the product of the initial DNA amplification reaction on chromosomal DNA of *B. methanolicus* strain C1 which was cloned into pBlueScriptIIKS (pHK1). The fragments of 2.6 kb 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 in order 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-*PstI* fragment (as shown in this figure) was sequenced in both directions.

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 ATG^{381} to TGA^{935}; 558 bp in size) encodes a putative protein of 185 amino acids with a 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 databases 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 (Dunn *et al.*, 1999). 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 IPTG, extracts of *E. coli* JM109 (pHK105) displayed high ACT protein activities (425 $U.mg^{-1}$ protein of MDH stimulating activity). ACT activity in its native host, *B. methanolicus*, was estimated as 8.6 $U.mg^{-1}$ (Arfman *et al.*, 1991). 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.

Organism	Accession	Partial sequence	Gene	Substrate
E. coli	P08337	EFPG**G**KIEMG**E**TPEQAVV**RE**LQ**EE**V**G**ITPQHFSLFEKLEYEFPDRH	*mutT*	8oxodGTP
H. influenza	I64101	EFPG**G**KVDAG**E**TPEQALK**RE**LE**EE**I**G**IVALNAELYERFQFEYPTKI	*mutT*	8oxodGTP
P. vulgaris	P32090	EFPG**G**KLEDN**E**TPEQALL**RE**LQ**EE**I**G**IDVTQCTLLDTVAHDFPDRH	*mutT*	8oxodGTP
Str.pneumoniae	P41354	IGVG**G**KLERG**E**TPQECAV**RE**IL**EE**T**G**LKAKPVLKGV---ITFPEFT	*mutX*	8oxodGTP
Human	P36639	NGFG**G**KVQEG**E**TIEDGAR**RE**LQ**EE**S**G**LTVDALHKVG--QIVFEFVG	*hmth1*	8oxodGTP
E. coli	P36651	EMVA**G**MIEEG**E**SVEDVAR**RE**AI**EE**A**G**LIVKRTKPVLS-FLAS**P**GGT	*yqie*	ADPR
B. subtilis	P54570	EIPA**G**KLEKG**E**EPEYTAL**RE**LE**EE**T**G**YTAKKLTKITA-FYTS**P**GFA	*yqkg*	ADPR
H. influenza	P44684	ELIA**G**MVEKG**E**KPEDVAL**RE**SE**EE**A**G**IQVKNLTHCLS-VWDS**P**GGI	*yqie*	ADPR
B methanolicus	AY128667	EIPA**G**KLEKG**E**DPRVTAL**RE**LE**EE**T**G**YECEQMEWLIS-FATS**P**GFA	*act*	ADPR
E. coli	P45799	GFSK**G**LIDPG**E**SVYEAAN**RE**LK**EE**V**G**FGANDLTFLKK-LSMAPS**Y**F	*orf186*	Ap3A
Human	P50583	TPPK**G**HVEPG**E**DDLETAL**RE**TQ**EE**A**G**IEAGQLTIIEGFKRELN**Y**VA		Ap4A
E. coli	P32664	TVLA**G**FVEVG**E**TLEQAVA**RE**VM**EE**S**G**IKVKNLRYVTS--QPWPFPQ	orf257	NADH
E. coli	P24236	QSVT**G**SVEEG**E**TAPQAAM**RE**VK**EE**VTIDVVAEQLTLIDCQRTVEFE	*orf17*	dATP
Human	NP006694	IVPG**G**GMEPE**E**EPSVAAV**RE**VC**EE**A**G**VKGTLGRLVGIFENQERKHR	*dipp*	DIPP
		G E RE EE G *P Y*		

Figure 2. Partial sequence alignment of selected members of the Nudix hydrolase family of proteins. The Nudix hydrolase consensus sequence (Bessman *et al.*, 1996;Koonin, 1993) is shown in bold and at the bottom. Also the conserved Pro residue downstream of the Nudix box, characteristic for the ADPR hydrolase subfamily, is shown in bold.

Purification of ACT protein.
Following induction with IPTG, cell extracts were prepared from 8 litre cultures of *E. coli* JM109 (pHK105). *B. methanolicus* is a thermotolerant bacterium, able to grow at temperatures up to 60°C (Arfman *et al.*, 1992b;Dijkhuizen and Arfman, 1990) 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 approximately 10 min at 60°C. After 30 min 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 1). ACT appeared 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). Addition of 1.0 μg.ml^{-1} (24 pmol.ml^{-1}) ACT protein stimulated initial MDH activity rates (1.0 μg.ml^{-1} (2.5 pmol.ml^{-1}) MDH protein) approximately 10-fold. The presence of Mg^{2+}-ions was a prerequisite for this stimulating effect (Fig. 3).

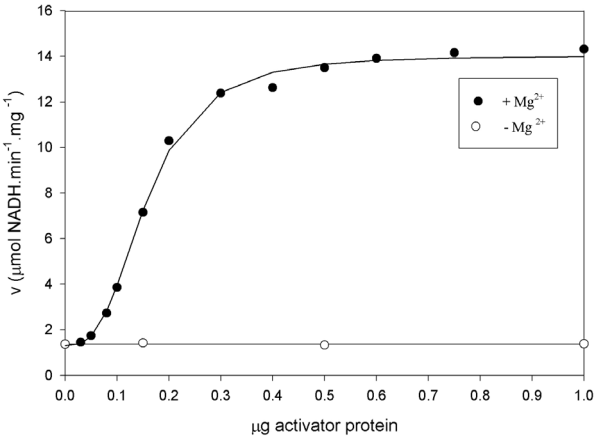

Figure 3. Titration of ACT protein purified from *E. coli* JM109-pHK105 with purified *B. methanolicus* MDH (1.0 μg.ml^{-1}; 2.5 pmol MDH.ml^{-1}). The assays were performed under conditions described in the presence (●) or absence (○) of 10 mM $MgSO_4$.

(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 (Arfman *et al.*, 1991). 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 (Bessman *et al.*, 1996). However, incubations of ACT with ADP-ribose (ADPR) revealed that this is an outstanding substrate for the enzyme (V_{max}: 348 μmol.min^{-1}.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 x 10^3 versus 2.05 x 10^6 $M^{-1}.s^{-1}$) (Fig. 4, Table 2). 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 ACT hydrolysis products of ADPR and NAD^+, respectively.

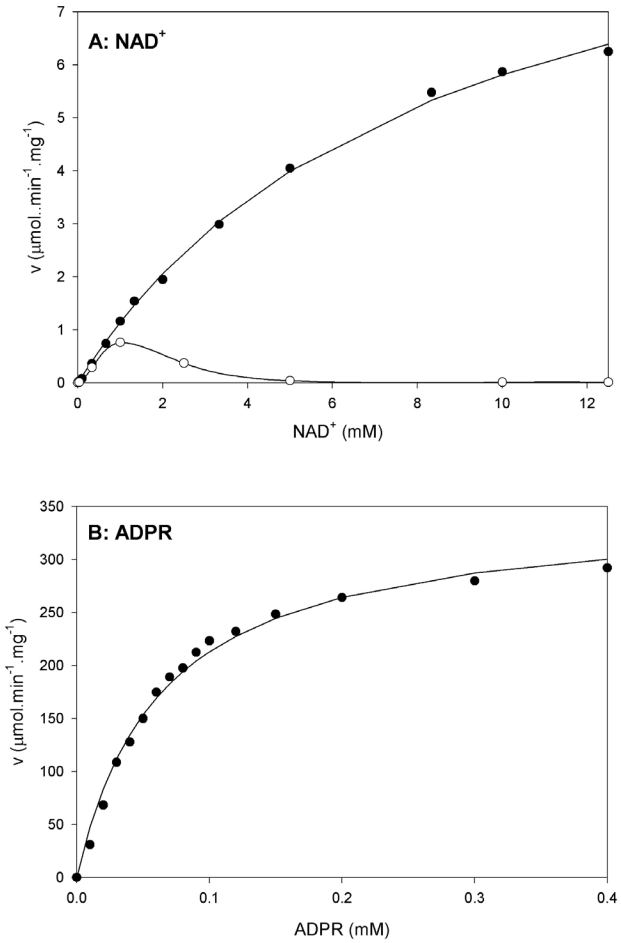

Figure 4. Primary plots of NAD^+ and ADPR hydrolyzing activity of ACT. A: NAD^+ hydrolyzing activity of ACT versus concentration of NAD^+, in the presence (●) or absence (○) of $MgSO_4$ (10 mM). Reactions were performed in a total volume of 300 µl with 1.25 µg (30 pmol) ACT; B: ADPR hydrolyzing activity of ACT versus concentration of ADPR, in the presence of $MgSO_4$ (10 mM). Reactions were performed in a total volume of 300 µl with 15.6 ng (0.40 pmol) ACT.

Table 2. Kinetic constants of dinucleotide hydrolyzing activity of ACT. Kinetic constants were determined under standard assay conditions described in "Methods" with protein concentrations described in Fig. 4. k_{cat} was calculated from V_{max}, assuming that one subunit contains one active site.

Substrate	V_{max} µmol.min^{-1}.mg^{-1}	k_{cat} s^{-1}	K_m	mM	k_{cat}/K_m $M^{-1}.s^{-1}$
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	n.d.		n.d.

n.d.: not determined; ADPR: ADP-ribose

Table 3. Purification of wild type MDH expressed in *E. coli*, without additional $MgSO_4$ in the growth (LB) medium.

Sample	Protein (mg)	Spec. activity mU/mg	Total activity mU	Yield %	Purification 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

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, purification fold was about three times, while the yield was approximately 35% (Table 3). cMDH purified from *E. coli* cells grown in LB medium could not be activated by ACT (de Vries *et al.*, 1992) (Table 4). 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), also displaying a much reduced activity (Table 4). cMDH also contained less than 50% of the Mg^{2+} and Zn^{2+} ions found in bMDH (de Vries *et al.*, 1992;Vonck *et al.*, 1991). 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 further investigated by cultivating *E. coli* expressing MDH in LB media 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 similar V_{max} value as bMDH (Table 4). Activity of this cMDH was stimulated 8-fold by ACT, similar to observations made with bMDH (Table 4). Addition of 10 mM $ZnSO_4$ 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 4).

Table 4. Kinetic characteristics and metal composition of MDH purified from *B. methanolicus* (bMDH) and MDH purified from *E. coli* (cMDH), expressed either in the presence or absence of additional $MgSO_4$ in the growth medium. Calculations are based on Michaelis-Menten kinetics.

Sample	[$MgSO_4$] in LB medium (mM)	Metal/MDH subunit ratio	Activation fold[1]	V_{max} of MDH activity[2] (mU/mg)	K_m NAD^+ (mM)	NADH cofactor/MDH subunit ratio
bMDH	n.a.	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	n.d.
cMDH	20	1.81	8.1	910	0.03	0.8

[1] 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.
[2] Determined in the absence of ACT protein n.a.: not applicable; n.d.: not determined.

ACT mediated hydrolysis of MDH-bound NAD(H) cofactor.

The data thus show that ACT hydrolyses free NAD^+ and only stimulates 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 (Arfman *et al.*, 1997) (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); 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 ACT hydrolyses the MDH cofactor NAD(H) into AMP and NMN(H), with only AMP remaining bound in MDH protein.

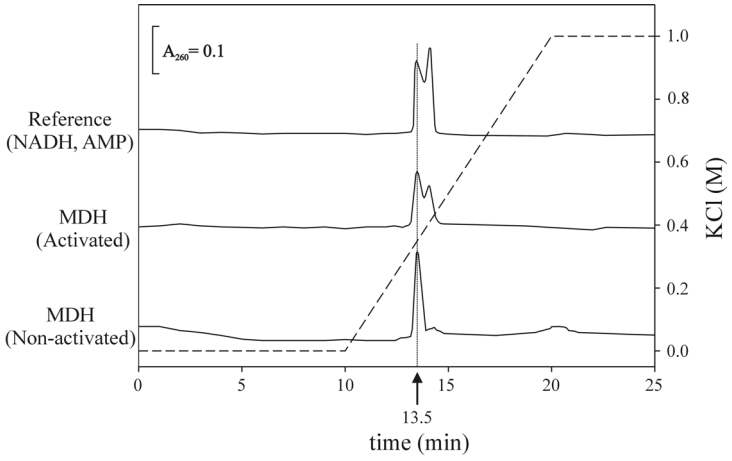

Figure 5. Mono-Q anion exchange chromatography analysis of urea extracts of MDH (432 µg; 1.08 nmol) incubated with and without ACT (112 µg; 2.67 nmol) for 5 sec. Detailed conditions are given under Materials and Methods. The elution profiles of NADH (10 nmol) and AMP (10 nmol) are used as references.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

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 uncompetitive cosubstrate inhibition, typical for a Ping-Pong type of reaction mechanism (Fig. 6). The MDH bound cofactor NAD(H) thus functions as 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 Mg^{2+}-ions in the growth medium (resulting in a strongly decreased NAD(H) cofactor content; Table 4) 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 (Wong, 1975;Dixon and Webb, 1979) (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 $MgSO_4$ supplemented LB-medium (resulting in a NADH cofactor/MDH subunit ratio close to 1.0; Table 4), again obeyed a Ping-Pong type of reaction mechanism.

The presence of ACT, resulting in hydrolysis of 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 reaction mechanism.

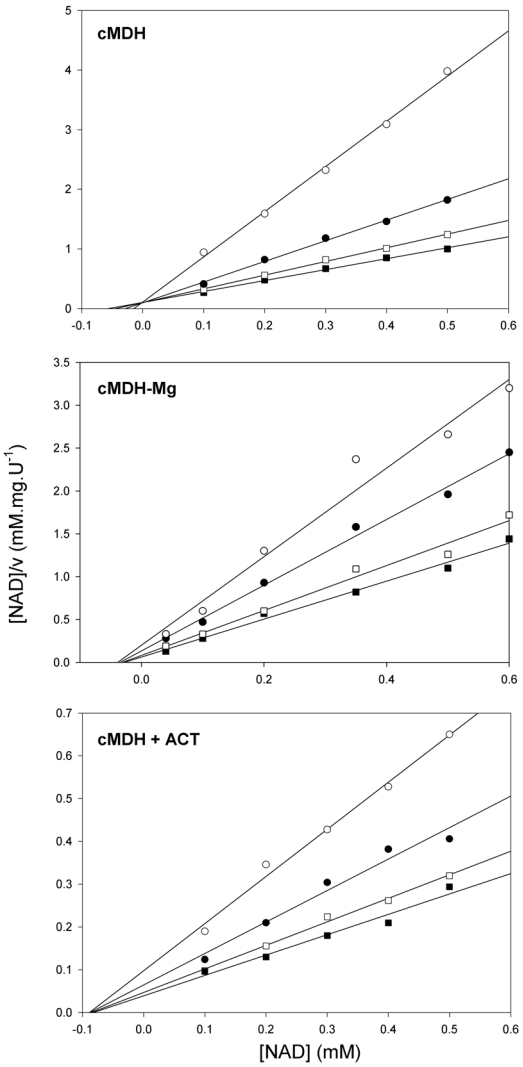

Figure 6. Steady-State kinetics of cMDH expressed in *E. coli* grown on LB medium with (cMDH) and without (cMDH-Mg) additional $MgSO_4$, and of activated cMDH. MDH and ACT concentrations were 25 and 50 pmol, respectively. The following methanol concentrations (mM) were applied: 62.5, ○; 125, ●; 250, □; 500, ■.

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 (Arfman *et al.*, 1991), also was observed in the present study with ACT protein purified from *E. coli* JM109 (pHK105). Both ACT protein preparations are capable to stimulate bMDH activity *in vitro* up to a factor 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 (Arfman *et al.*, 1991).

MDH and ACT protein expression is under co-ordinate control (methanol induced) in

B. methanolicus (Arfman *et al.*, 1992a). The *act* gene was not detected, however, during characterization of the cloned *B. methanolicus mdh* gene and its flanking regions (de Vries *et al.*, 1992). Southern hybridization experiments (data not shown) and nucleotide 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 immediately followed 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™ database 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 remains to be elucidated.

A database search for proteins with amino acid sequence similarities revealed that ACT carries the highly conserved sequence motif characteristic for MutT-related proteins (Koonin, 1993) or Nudix hydrolases (Bessman *et al.*, 1996) (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 (Bessman *et al.*, 1996;Koonin, 1993;Maki and Sekiguchi, 1992). Other members of this family subsequently were found to possess hydrolytic activity with a wider variety of substrates, containing a common **nu**cleotide **di**phosphate moiety linked to some other group (**x**) (Bessman *et al.*, 1996;Frick and Bessman, 1995;O'Handley *et al.*, 1998). Analysis of Nudix hydrolase activity of ACT revealed that it is an ADPR hydrolase. Similar to other ADPR hydrolase enzymes (Dunn *et al.*, 1999), 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) (Dunn *et al.*, 1999). ADPR, a major product of NAD^+ glycohydrolase, can be transferred enzymatically onto acceptor proteins in order to regulate their biological activity, or is attached nonenzymatically to nucleophilic amino acid residues such as lysines and cysteines (Ziegler, 2000). These random ADPR attachments may be detrimental for cellular processes (Dunn *et al.*, 1999).

ACT also hydrolyses free NAD^+, albeit with a very low catalytic efficiency (Table 2). 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 MDH-bound NAD(H) cofactor as a substrate than for free NAD^+ (K_m 8.3 mM), since ACT is capable to hydrolyse at least 50% of the MDH cofactor within seconds, while 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 3D 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 (Ruzheinikov *et al.*, 2001). 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* (Hektor *et al.*, 2002). 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 properties of cMDH and bMDH (de Vries *et al.*, 1992): Firstly, cMDH activity could not be stimulated by activator protein; secondly, its metal content (Zn^{2+} and Mg^{2+}) was considerably lower; thirdly, cMDH lacked bound cofactor NAD(H). The results presented in this paper demonstrate that all three differences disappeared when adding $MgSO_4$ 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 $MgSO_4$, showed a positive correlation between the amount of $MgSO_4$ present in the growth medium and the Mg^{2+} and NAD(H) cofactor composition of cMDH. It is likely that Mg^{2+} 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) (Arfman *et al.*, 1997). cMDH, purified from cells grown under conditions in which NAD(H) cofactor binding is poor, displays a ternary complex reaction mechanism (Wong, 1975;Dixon and Webb, 1979).

MDH cofactor analysis studies subsequently provided conclusive evidence that ACT hydrolyses the MDH-bound NAD^+ cofactor, yielding AMP (and NMN^+). This resulted in a 50% decrease in extractable NAD(H) cofactor, and also showed 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 catalyses 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 (Vonck *et al.*, 1991).

The data obtained so far allow us to propose a model for the ACT-MDH interaction (Fig. 7). In this model an activated and a non-activated state of MDH are distinguished: MDH in the non-activated 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 catalyses 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 by ACT. MDH activation is a reversible process (Arfman *et al.*, 1991): MDH can change from the activated state to the cofactor dependent state and vice versa by either binding of NADH to available cofactor binding sites or, conversely, removal of 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 $\mu mol.min^{-1}.mg^{-1}$), compared to the catalytic constants of non-activated MDH (V_{max} and K_m for methanol: 1.3 $\mu mol.min^{-1}.mg^{-1}$ and 230 mM, respectively) (Arfman *et al.*, 1991). 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 for *B. methanolicus*, resulting in 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) (Arfman *et al.*, 1997), but it very poorly hydrolyses this substrate (Table 2). 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 towards growth at varying methanol concentrations in its natural habitat (in soil) (Arfman *et al.*, 1992b;Dijkhuizen *et al.*, 1988). 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 de-activated state.

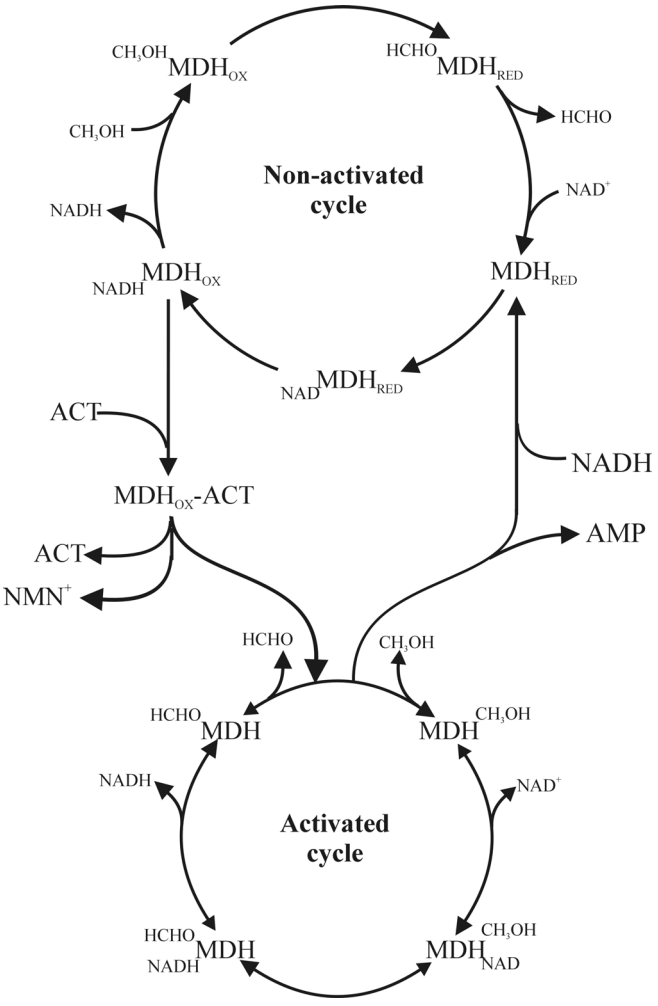

Figure 7. Model of the effects of ACT on the MDH reaction cycles. In this model two types of reaction mechanisms are distinguished: in the upper, non activated reaction cycle the NAD(H) cofactor functions as a temporary electron deposit and the reaction thus proceeds via a Ping-Pong reaction mechanism. 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 characterized by a ternary complex mechanism. Conversely, an activated MDH molecule can reenter the non-activated reaction cycle by binding NADH in the cofactor binding site. Return of activated MDH to the de-activated state may be stimulated by increasing cellular concentrations of NADH (or increased NADH/NAD^+ ratios).

The activator protein of a nicotinoprotein alcohol dehydrogenase

ACT is a member of the mutT proteins or Nudix hydrolases. All members of this family characterized so far are able to hydrolyse a pyrophosphate bond in different (di)nucleotides. This protein family has been referred to as a family of "housecleaning" enzymes (Bessman *et al.*, 1996). More recently Nudix hydrolase proteins were studied exhibiting a clear regulatory function (Moreno-Bruna *et al.*, 2001;Perraud *et al.*, 2001). 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.