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Overproduction and one-step purification of the N^5,N^{10}-methenyltetrahydromethanopterin cyclohydrolase (Mch) from the hyperthermophilic *Methanopyrus kandleri*

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Abstract N^5,N^{10}-Methenyltetrahydromethanopterin cyclohydrolase (Mch) is an enzyme involved in methanogenesis from CO_2 and H_2 which represents the energy metabolism of *Methanopyrus kandleri*, a methanogenic Archaeon growing at a temperature optimum of 98°C. The gene *mch* from *M. kandleri* was cloned, sequenced, and expressed in *Escherichia coli*. The overproduced enzyme could be purified in yields above 90% in one step by chromatography on phenyl Sepharose in 80% ammonium sulfate. From 3.5 g cells (250 mg protein), approximately 18 mg cyclohydrolase was obtained. The purified enzyme showed essentially the same catalytic properties as the enzyme purified from *M. kandleri* cells. The primary structure and properties of the cyclohydrolase are compared with those of the enzyme from *Methanococcus jannaschii* (growth temperature optimum 85°C), from *Methanobacterium thermoautotrophicum* (65°C), and from *Methanosarcina barkeri* (37°C). Of the four enzymes, that from *M. kandleri* has the lowest isoelectric point (3.8) and the lowest hydrophobicity of amino acid composition. Besides, it has the highest relative content of glutamate, leucine, and valine and the lowest relative content of isoleucine, serine, and lysine. Some of these properties are unusual for enzymes from hyperthermophilic organisms. They may reflect the observation that the cyclohydrolase from *M. kandleri* is not only adapted to hyperthermophilic conditions but also to the high intracellular concentrations of lyotrophic salts prevailing in this organism.

Key words Hyperthermophilic enzymes · N^5,N^{10}-Methenyltetrahydromethanopterin cyclohydrolase · Tetrahydromethanopterin-dependent enzymes · Methanogenic Archaea · *Methanopyrus kandleri*

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Introduction

In microbial methanogenesis from CO_2, four enzymes are involved. Each is composed of only one type of subunit, lacks a prosthetic group, and is relatively stable under aerobic conditions. Their genes can be expressed in *Escherichia coli* yielding active enzymes. The four enzymes are: formylmethanofuran:tetrahydromethanopterin formyltransferase (Ftr) (DiMarco et al. 1990; Shima et al. 1995; Kunow et al. 1996); N^5,N^{10}-methenyltetrahydromethanopterin cyclohydrolase (Mch) (Vaupel et al. 1996); F_{420}-dependent N^5,N^{10}-methylenetetrahydromethanopterin dehydrogenase (Mukhopadhyay et al. 1995); and F_{420}-dependent N^5,N^{10}-methylenetetrahydromethanopterin reductase (Vaupel and Thauer 1995; Nölling et al. 1995). Production in *E. coli* makes these enzymes available in large amounts, even though they come from methanogens, which are difficult to grow, and also allows structure/function analysis by site-directed mutagenesis.

Recently, the formylmethanofuran:tetrahydromethanopterin formyltransferase from the hyperthermophilic *Methanopyrus kandleri* has been overproduced in *E. coli* (Shima et al. 1995). The purified enzyme was crystallized and the X-ray structure resolved to a resolution of 0.17 nm (Shima et al. 1996; Ermler et al. 1997). The high resolution is probably in part due to the fact that at the temperature of crystallization (4°C), the hyperthermophilic enzyme has a high rigidity and stability. Under the appropriate conditions the enzyme exhibits a temperature activity optimum above 90°C and is completely stable even at the temperature of boiling water (Breitung et al. 1992). These properties are in agreement with the finding that *M. kandleri* can grow at temperatures up to 110°C with a growth temperature optimum at 98°C (Huber et al. 1989; Kurr et al. 1991).

We report here the cloning, sequencing, and expression in *E. coli* of the gene *mch* encoding N^5,N^{10}-methenylte-

trahydromethanopterin cyclohydrolase from *M. kandleri*. The overproduced active enzyme was purified and characterized.

The cyclohydrolase from *M. kandleri* is a soluble monomeric enzyme of an apparent molecular mass of 41 kDa and apparent isoelectric point of 3.5. The enzyme is absolutely dependent on the presence of lyotrophic salts for activity, the concentration dependence being sigmoidal. For half-maximal activity, 1.5 M K_2HPO_4 pH 8.0 or 1.2 M $(NH_4)_2SO_4$ are required. The enzyme is also dependent on the presence of lyotrophic salts for thermostability, the concentration required for maximal thermostability, however, being only 40 mM (Breitung et al. 1991). The salt requirement for catalytic activity is also exhibited by other enzymes from *M. kandleri* (Ma et al. 1991; Rospert et al. 1991; Breitung et al. 1992) and probably reflects the fact that the intracellular concentration of salts in *M. kandleri* is very high, the predominant solutes being cyclic 2,3-diphosphoglycerate and potassium ions, both present in concentrations above 1 M (Huber et al. 1989).

Materials and methods

Methanopyrus kandleri (DSM 6324) was from the Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany). The Archeon was grown at 95°C on 80% H_2/20% CO_2, harvested, and stored at −80°C as described by Kurr et al. (1991). The lambda ZAP Express Vector Kit and *Pfu* DNA polymerase were from Stratagene (Heidelberg, Germany). The expression vector pET3a and *Escherichia coli* BL 21 (DE3) pLysS were from AMS-Biotechnology (Bioggio-Lugano, Switzerland). Synthetic oligonucleotides were obtained from MWG-Biotech (Munich, Germany). For Southern hybridizations the oligonucleotides were labelled by digoxigenin-dUTP. The DIG Luminiscent Detection Kit and the DIG Oligonucleotide Tailing Kit were from Boehringer Mannheim (Mannheim, Germany). All DNA-modifying enzymes and Sequenase version 2.0 were from United States Biochemicals (Bad Homburg, Germany). Nylon membrane Hybond-N and [γ-^{32}P]dATP were from Amersham (Braunschweig). The phenyl Sepharose column was from Pharmacia Biotech (Uppsala, Sweden). N^5,N^{10}-Methenyltetrahydromethanopterin was purified from *M. thermoautotrophicum* (strain Marburg) (Breitung et al. 1992).

Cloning of the *mch* gene

Genomic DNA from *M. kandleri* was isolated as described by Shima et al. (1995) and digested to completion with *Bam*HI. Southern analysis with a heterologous digoxigenin-dUTP labelled 36-base probe for *mch* derived from the N-terminal amino acid sequence (Klein et al. 1993) revealed only one strong hybridization signal at 3.4 kbp when hybridization was performed at 58°C in 0.75 M NaCl, 75 mM sodium citrate, 0.1% sodium dodecyl sulfate (SDS), pH 7. The *Bam*HI fragments of 3–4 kbp size were electroeluted, ligated into the *Bam*HI site of lambda ZAP Express, and packaged and amplified in *E. coli* XL1-Blue-MRF' according to the protocol of the manufacturer. From the lambda phages of one of the positive plaques, the phagemid pBK-CMV carrying a 3.4-kbp *Bam*HI fragment was excised, generating pMV 5.

Sequencing of DNA

The dideoxynucleotide method of Sanger et al. (1977) using Sequenase version 2.0 was employed. Both strands were independently and completely sequenced.

RNA isolation and Northern blot hybridization

A 1-l culture of *M. kandleri* was grown to a cell concentration of 10^7 cells per ml, cooled in an ethanol/dry-ice mixture, and then harvested by filtration (0.45-μm pore size) at 4°C. The cells were transferred to a mortar which had been cooled with liquid nitrogen. The cells (0.1 g wet mass) were ruptured by grounding in liquid nitrogen for 15 min. The RNA was isolated from the ruptured cells using the single-step method for RNA isolation and the RNA (5 μg per lane) was separated by electrophoresis as described by Ausubel et al. (1987). The blot was probed with a homologous ^{32}P-labelled 36-base oligonucleotide complementary in sequence to the 5′ end of the *mch* gene. Hybridizations were performed at 65°C in 0.75 M NaCl, 75 mM sodium citrate, 0.1% SDS, pH 7.

Mapping of the 5′ end of the *mch* mRNA

The primer extension method described by Boorstein and Craig (1989) was employed. The 5′ end of the mRNA encoding the *mch* gene was mapped using an oligonucleotide primer complementary to the 5′ end of the *mch* gene (primer: GTCGATCACGGTGGTTCCGTTCTCGAGCTCCTGGAC). Isolated RNA (5 μg) was incubated with the [γ-^{32}P]dATP labelled primer for 5 min at 70°C in 50 mM Tris/HCl pH 8.5 containing 30 mM KCl, 6 mM $MgCl_2$, 1 mM dithiothreitol, and 20 U RNase inhibitor. Annealing was performed by cooling down to 42°C. The primer RNA hybrid was extended with 10 U avian myeloblastosis virus reverse transcriptase and 0.15 mM dATP, dCTP, dGTP, and dTTP for 30 min at 42°C. Extension products were purified by phenol extraction, subjected to denaturing gel electroctrophoresis, and visualized by autoradiography.

Amplification of the *mch* gene by PCR

The *mch* gene was amplified by polymerase chain reaction (PCR) using pMV 5 as template. For amplification the following two primers derived from the DNA sequence were used: GCGGATAGCATATGGTGAGCGTGAACGAGAACG (sense) and CCGTTCCCGACGGATCCCGACTATAACGTTTACG (antisense). The 100-μl PCR reaction mixture contained: 10 ng DNA of pMV 5 contain-

ing the *mch* gene, 2.5 U *Pfu* DNA polymerase, 100 μM dNTP, 2.5 mM $MgCl_2$, and 500 pM of each of the two primers. The temperature program was 1 × 5 min at 92°C, 35 cycles 1.5 min 92°C/1.5 min 45°C/2.0 min 72°C, and 1 × 5 min at 72°C. The PCR product was purified via adsorption/desorption to a Qia quick spin column (Qiagen, Hilden, Germany) according to the protocol of the manufacturer. The PCR product cloned into pET3a was sequenced from both strands. Sequencing of the amplified *mch* gene revealed one mutation directly after the newly introduced start codon ATG. The original start codon GTG for a valine was mutated to a TTG for a leucine (see Table 2).

Determination of the specific activity of Mch

The standard assay for enzyme activity was carried out at 65°C in 1.5-ml glass cuvettes with N_2 as the gas phase. The 0.7-ml assay mixture contained: 50 mM Tricine/KOH pH 8.0 (adjusted to this pH at room temperature), 1.5 M K_2HPO_4, and 60 μM N^5,N^{10}-methenyltetrahydromethanopterin. The reactions were started by addition of enzyme solution. The disappearance of N^5,N^{10}-methenyltetrahydromethanopterin was monitored by following the decrease in absorbance at 340 nm (ε_{340} = 20.8 $mM^{-1}cm^{-1}$). Protein was quantified by the method of Bradford (1976) using the Bio-Rad microassay (Bio-Rad Laboratories, Hercules, CA, USA). Ovalbumin was used as standard.

Note

The DNA sequence data published here have been submitted to the EMBL sequence data bank and are available under the accession numbers Y08849 (*mch* from *Methanopyrus kandleri*) and Y08843 (*mch* from *Methanosarcina barkeri)*.

Results

The gene encoding the cyclohydrolase from *M. kandleri* was identified on a 3.4-kb *Bam*HI fragment cloned into the Phagemid pBK-CMV via the λ ZAP Express partial genomic library. After recloning into the expression vector pET3a, the *mch* gene was expressed in *E. coli* BL21 (DE3) pLysS yielding an active cyclohydrolase which was subsequently purified and characterized.

Sequence of the *mch* gene

The 3.4-kb DNA fragment cloned into pBK-CMV (pMV5) was sequenced starting from the reverse primer of pBK-CMV. After 250 bp, a nucleotide sequence was found which corresponded to that deduced from the N-terminal amino acid sequence of the cyclohydrolase from *M. kandleri* (Klein et al. 1993). This sequence was part of an open reading frame designated *mch* which was 948 bp long, encoding a protein of 315 amino acids. The calculated molecular mass of the protein was 33.97 kDa which is considerably lower than the 41 kDa determined for the cyclohydrolase by SDS/PAGE (Breitung et al. 1991). However, the amino acid sequence showed a large number of acidic residues which resulted in a net charge of −45.69 at pH 7 and an overall pI of 3.8; highly charged peptides have been found to bind far less SDS per g protein than the calibration proteins (Pitt-Rivers and Impiombato 1968). Thus, the formylmethanofuran:tetrahydromethanopterin formyltransferase from *M. thermoautotrophicum* (pI = 4.4) migrates with a molecular mass of 41 kDa while its actual molecular mass is 31.4 kDa (DiMarco et al. 1990).

The *mch* gene probably starts with the codon GTG for valine, the first amino acid found by Edman degradation. GTG is used frequently as a translation initiation codon in methanogenic Archaea (Reeve 1992). It could be, however, that the TTG just in front of the proposed start codon is the actual start codon. TTG is also used as an alternative start codon in Archaea, and the fact that valine is found by Edman degradation as the first amino acid is compatible with TTG being the start codon and with the resulting methionine being split off during maturation. The start codon is immediately preceded by a sequence GGGGCGGAT which is partially complementary to the 3′ terminal sequence of the 16S rRNA from *M. kandleri* (Burggraf et al. 1991) and is therefore considered to be the ribosome binding site (Brown et al. 1989; Reeve 1992). Downstream of the *mch* gene stop codon TAA, the DNA sequence did not show oligo (T) sequences or inverted repeat sequences, which have been implicated in transcription termination in methanogens (Thomm et al. 1994; Reeve 1992).

The G + C content of the *mch* gene is 65.82 mol/100 mol and is thus somewhat higher than the average G + C content of 60 mol/100 mol of *M. kandleri* DNA (Kurr et al. 1991). The codon usage was almost identical to that for other genes in *M. kandleri* (Zirngibl et al. 1992; Shima et al. 1995; Nölling et al. 1995, 1996; Andrä et al. 1996).

In the region 250 bp upstream and 300 bp downstream of the *mch* gene, open reading frames were not found. The localization is thus different from that in *M. thermoautotrophicum* where the 5′ end of the *mch* gene overlaps by 8 bp with the 3′ end of a gene encoding a putative thymidylate synthase (Vaupel et al. 1996).

Transcript of the *mch* gene

A homologous ^{32}P-labelled 36-base oligonucleotide probe for the *mch* gene was used for hybridization against total RNA extracted from *M. kandleri*. Only one strong band at the 1 kb position was observed in Northern blots. The predicted minimal length of the *mch* transcript is 948 bases.

The primer extension experiment using a *mch* specific 19-base ^{32}P-labelled oligonucleotide as primer generated a strong signal corresponding to a transcript initiated at the A located 26 bp upstream of the *mch* translation initiation

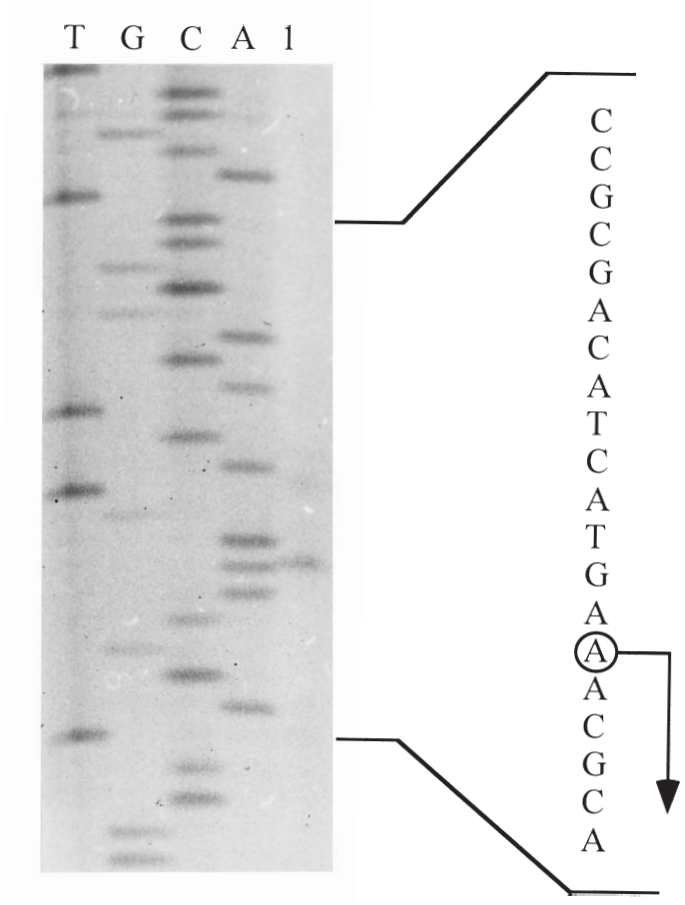

Fig. 1. Mapping of the 5′ end of the *mch* mRNA by primer extension. In *lane 1* the product of the primer extension reaction with reverse transcriptase is shown; *lanes TGCA* show the DNA sequencing ladder obtained by using the same primer. The *circled* nucleotide indicates the putative site of transcription initiation, and the *arrow* indicates the direction of transcription

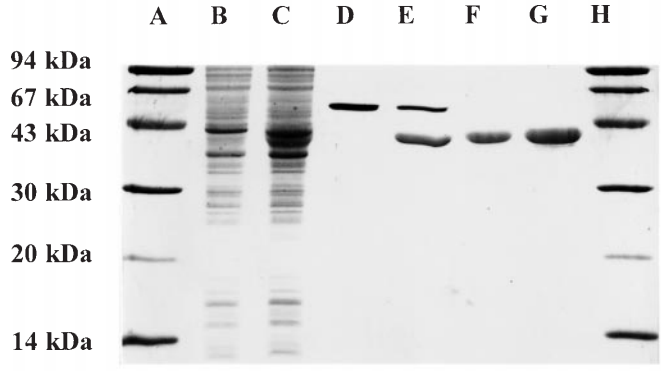

Fig. 2. Expression of the *mch* gene from *M. kandleri* in *E. coli* BL 21 (DE3) pLysS as evidenced by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE). Protein was separated on a 16% polyacrylamide gel subsequently stained with Coomassie brilliant blue (Laemmli 1970). Except where indicated, the protein was heated for 5 min to 100°C in the presence of SDS (4%) and dithiothreitol (15 mM) prior to SDS/PAGE. *Lanes A* and *H*, 4 μg molecular mass markers; *lane B*, 4 μg of cell extract of *E. coli* transformed with pMV6 and before induction with isopropyl β-D-thiogalactopyranoside; *lane C*, 6 μg of cell extract of *E. coli* transformed with pMV6 and 1.5 hours after induction with isopropyl β-D-thiogalactopyranoside (0.1 mM); *lane D*, 1.6 μg of the protein present in the 27000 × *g* supernatant of the 80% ammonium sulfate precipitation step (Table 1); *lane E*, 3.2 μg of purified cyclohydrolase after chromatography on phenyl Sepharose (Table 1); *lanes F* and *G*, 3.2 μg and 6.4 μg, respectively, of purified cyclohydrolase heated for 30 min in the presence of SDS (4%) and dithiothreitol (15 mM) prior to SDS/PAGE. pMV6, expression vector pET3a carrying the *mch* gene

GTG codon (Fig. 1). Upstream of the transcription start site, the DNA sequence did not show similarities to the box A (TATA-box) of methanogenic promoters (Reeve 1992; Thomm 1996).

Expression of the *mch* gene in *E. coli*

For incorporation of the *mch* gene into an expression vector, it was amplified by PCR using pBK-CMV containing the 3.4-kbp insert as template. The sense primer was designed such that the amplified gene started with ATG placed directly before the GTG start codon and that a *Nde*I restriction site was located one nucleotide upstream of the ATG codon. The antisense primer was designed such that the *Bam*HI restriction site was located five nucleotides downstream of the *mch* gene stop codon. After purification of the PCR product and digestion with *Nde*I and *Bam*HI, it was ligated into the pET3a expression vector previously digested with *Nde*I and *Bam*HI. The construct thus obtained, which was designated pMV6, was used to transform *E. coli* BL21 (DE3) pLysS. In this strain the gene cloned into pET3a is transcribed by T7 RNA polymerase whose gene resided on the prophage DE3 integrated into the chromosome of the *E. coli* BL21 under the control of the *lac*UV5 promoter. This promoter can be induced by addition of isopropyl β-D-thiogalactopyranoside (Studier et al. 1990).

For expression of the *mch* gene in *E. coli* BL21 (DE3) pLysS the cells transformed with pMV6 were aerobically grown at 37°C in 2 l minimal medium M9 (Sambrook et al. 1989) supplemented with ampicillin (100 μg/ml) and chloramphenicol (20 μg/ml). When the absorbance at 578 nm of the culture had reached 0.5, isopropyl β-D-thiogalactopyranoside was added to the culture to a final concentration of 0.1 mM; 1.5 h later, the A_{578} had increased to 0.7, and the cells (approximately 3.5 g wet mass) were harvested by centrifugation. The cells were suspended in 5 ml 50 mM Tris/HCl pH 7 and a cell extract was prepared by ultrasonication followed by centrifugation for 30 min at 12000 × *g*. SDS/PAGE of the cell extract revealed the presence of a major protein of apparent molecular mass 41 kDa (Fig. 2, lane c). This protein was not dominant in cells not induced with isopropyl β-D-thiogalactopyranoside (Fig. 2, lane b). The induced cells exhibited cyclohydrolase activity which was absent in *E. coli* BL21 (DE3) pLysS not transformed with pMV6.

When *E. coli* BL21 (DE3) rather than *E. coli* BL21 (DE3) pLysS was used to express the *mch* gene, the cultures were found to synthesize cyclohydrolase already before

Table 1. Purification of cyclohydrolase from *M. kandleri* overproduced in *E. coli*

Step	Volume	Protein	Activity	Specific activity	Yield
	ml	mg	U	U/mg	%
Cell extract	10	255	105200	413	100
80% $(NH_4)_2SO_4$-supernatant	50	83	100500	1210	96
Phenyl Sepharose HiLoad (26/10)	10	18	97400	5410	93

The purification procedure started from 3.5 g cells (wet mass) as described in the Results section. Cyclohydrolase activity was determined at 65°C under standard assay conditions. 1 U = 1 μmol/min.

Table 2. Comparison of the properties of the *M. kandleri* cyclohydrolase overproduced in *E. coli* with the enzyme purified directly from *M. kandleri*

Parameter	Properties of enzyme purified from:	
	E. coli (pMV6)	*M. kandleri*[a]
Apparent molecular mass	41 kDa	41 kDa
N-Terminal amino acid sequence	MLSVNENALPLVE	VSVNENALP?VE
Specific activity	5400 U/mg	5000 U/mg
K_m for N^5,N^{10}-methenyltetrahydromethanopterin	30 μM	40 μM
Optimal salt concentration for activity	1.5 M	1.5 M
Thermostable in 40 mM K_2HPO_4 pH 8.0 up to	>90°C	>90°C

"Thermostable", no inactivation after a 30-min incubation at the temperature indicated.
[a] From Breitung et al. (1991).

induction with isopropyl β-D-thiogalactopyranoside and to lyse at very low cell concentrations ($\Delta A_{578} < 0.3$). *E. coli* carrying the plasmid pLysS accumulates substantial levels of T7 lysozyme, which binds to the T7 RNA polymerase, inhibiting transcription of the cloned genes before induction with isopropyl β-D-thiogalactopyranoside (Studier 1991).

Purification of cyclohydrolase overproduced in *E. coli* BL21 (DE3) pLysS

Most of the *E. coli* proteins were separated from the cyclohydrolase simply by precipitation in 80% ammonium sulfate. To 10 ml extract cooled to 0°C in ice water, 40 ml saturated ammonium sulfate in 100 mM Tris/HCl pH 7.0 was added. After 30 min stirring, the precipitated proteins were removed by a 30-min centrifugation at 27000 × *g*. The supernatant contained more than 96% of the cyclohydrolase activity and essentially only a 55-kDa protein as revealed by SDS/PAGE (Fig. 2, lane d). Removal from contaminating nucleic acids was achieved by adsorption of the 55-kDa protein to phenyl Sepharose (HiLoad 26/10 high performance column from Pharmacia) followed by elution with decreasing concentrations of ammonium sulfate in 50 mM Tris/HCl pH 7.0: 60 ml 0.5 M $(NH_4)_2SO_4$; 60 ml 0.25 M $(NH_4)_2SO_4$; and 60 ml 0 M $(NH_4)_2SO_4$. The cylcohydrolase activity eluted at 0 M $(NH_4)_2SO_4$ in two 5-ml fractions. Using this simple procedure 18 mg of the purified cyclohydrolase was obtained from only 3.5 g cells (wet mass). During purification the specific activity increased from 413 U/mg in the cell extract to 5400 U/mg in the phenyl Sepharose eluate. The activity yield was 93% (Table 1).

SDS/PAGE of the purified enzyme revealed the presence of two different migrating polypeptides with apparent molecular masses of 55 kDa and 41 kDa (Fig. 2, lane e). The relative amount of the 41 kDa form increased when the salt concentration in the sample analyzed was decreased, and vice versa. When the samples were heated for 30 min rather than for the usual 3 min prior to analysis by SDS/PAGE, only the 41-kDa migrating form was observed (Fig. 2, lane f); without heating, only the 55-kDa form was found. This finding indicates that binding of SDS to the cyclohydrolase is strongly affected by salts and temperature and probably reflects the fact that the enzyme is a highly acidic protein (pI = 3.8) which binds much less SDS than normal proteins (Pitt-Rivers and Impiombato 1968).

Catalytic properties

The cyclohydrolase purified as described in Table 1 was analyzed with respect to the K_m value and the dependence of activity and thermostability on the presence of lyotropic salts. The properties were compared with those determined for the enzyme purified from *M. kandleri* (Table 2). The comparison reveals that the cyclohydrolase overproduced in *E. coli* has essentially the same catalytic properties as the enzyme purified from *M. kandleri*, despite the fact that the two enzymes differ in two amino acid residues at the N-terminus. The overproduced enzyme was intentionally designed to start with a methionine. The second amino acid was changed from a valine to a leucine by mutation.

Table 3. Properties of the cyclohydrolase from different methanogens as deduced from the amino acid sequence and properties of the organisms

	M. kandleri	*M. thermautotrophicum*[a]	*M. barkeri*[b]	*M. jannaschii*[c]
Cyclohydrolase				
Calculated molecular mass (Da)	33 972	34 246	34 889	34 903
Amino acid sequence identity (%)	100	57.0	50.3	60.4
Isoelectric point	3.8	4.2	4.3	5.4
Hydrophobicity	+1.8	+5.2	+11.0	+21.7
Acidic amino acids	63	52	45	41
Basic amino acids	17	29	26	37
Organism				
G + C content (mol/100 mol)	60	48	42	31
Growth temperature optimum (°C)	98	65	37	85
Intracellular salt concentration (M)	>1.1	≈0.7	≈0.3	n.d.
Intracellular concentration of cyclic 2,3-diphosphoglycerate (M)[d,e]	1.1	0.065	0.001	0.001

Hydrophobicity of amino acid composition equals the sum of amino acid hydropathies as determined by Kyte and Doolittle (1982).
n.d., not determined.
[a] Vaupel et al. (1996).
[b] Vaupel unpublished.
[c] Bult et al. (1996).
[d] Huber et al. (1989).
[e] Tolman et al. (1986).

Discussion

In Table 3, the properties of the cyclohydrolase from *M. kandleri* are compared with those of the enzyme from *M. jannaschii*, *M. thermoautotrophicum*, and *M. barkeri*. The Table also contains information on the G + C content, the growth temperature optimum, and the intracellular concentrations of salts and cyclic 2,3-diphosphoglycerate (cDPG) in the organisms from which the enzymes were isolated.

The cyclohydrolase from *M. kandleri* has almost the same molecular mass as the enzymes from *M. jannaschii*, *M. thermoautotrophicum*, and *M. barkeri*. Comparison of the amino acid sequence of the four cyclohydrolases (Fig. 3) reveals a high degree of sequence similarity, the sequence identity being 60.4% between the enzyme that from *M. kandleri* and that from *M. jannaschii*, 57.0% between the enzymes from *M. kandleri* and *M. thermoautotrophicum*, and 50.3% between the enzymes from *M. kandleri* and *M. barkeri*. The four enzymes differ in their isoelectric point, hydrophobicity of amino acid composition, temperature activity optimum, thermostability, and the dependence of the enzyme activity on the presence of lyotropic salts (Table 3).

The cyclohydrolase from *M. kandleri*, as other proteins from this organism (Kunow et al. 1996), has the lowest isoelectric point and the lowest hydrophobicity in its amino acid composition (Table 3). The latter finding is surprising for a hyperthermophilic enzyme since generally, as in the case of the enzyme from *M. jannaschii*, the hydrophobicity of an enzyme increases with increasing growth temperature of the organism from which the enzyme is derived. It has to be considered, however, that the enzyme from *M. kandleri* is not only adapted to a high temperature but also to a high intracellular concentration of lyotropic salts. In general, the hydrophobicity of enzymes decreases upon halophilic adaptation (Zaccai and Eisenberg 1990; Jaenicke and Závodszky 1990; Jaenicke 1987, 1991, 1996).

The cyclohydrolases from *M. kandleri*, *M. jannaschii*, *M. thermoautotrophicum*, and *M. barkeri* differ considerably in amino acid composition. The most pronounced differences were found in the relative content of glutamate, leucine, valine, asparagine, isoleucine, serine, and lysine. The enzyme from *M. kandleri* has the highest relative content of glutamate, leucine, and valine and the lowest relative content of asparagine, isoleucine, serine, and lysine. Some of the differences in the amino acid composition may be reasonably viewed as a side effect of the differences in the genome G + C content rather than as adaptation to an unusual environment (Oshima 1988). Thus, the relatively high glutamate and valine content and the relatively low lysine and isoleucine content of the cyclohydrolase from *M. kandleri* (G + C content = 60 mol/100 mol) is in part the result of changes from lysine to glutamate and from isoleucine to valine by mutation in the first letter of the respective codons from A to G. In turn, the relatively high lysine content of the cyclohydrolase from *M. jannaschii* may have been influenced by the fact that the DNA of the organism has a low G + C content of 31 mol/100 mol and that lysine is encoded by AAA or AAC, codons low in G + C (Shima et al. 1995).

All four cyclohydrolases have 36% of the amino acid sequence in common (Fig. 3). This is noteworthy since the four organisms from which the cyclohydrolases are derived, not only grow at different temperatures but also are phylogenetically only very distantly related, the phylogenetic distance being largest between *M. kandleri* and *M. barkeri* (Boone et al. 1993). The high degree of similarity therefore indicates that a major part of the sequence of the cyclohydrolase is determined by the function of the enzyme, in particular the need to interact with the substrate N^5,N^{10}-methenyltetrahydromethanopterin and the product N^5-

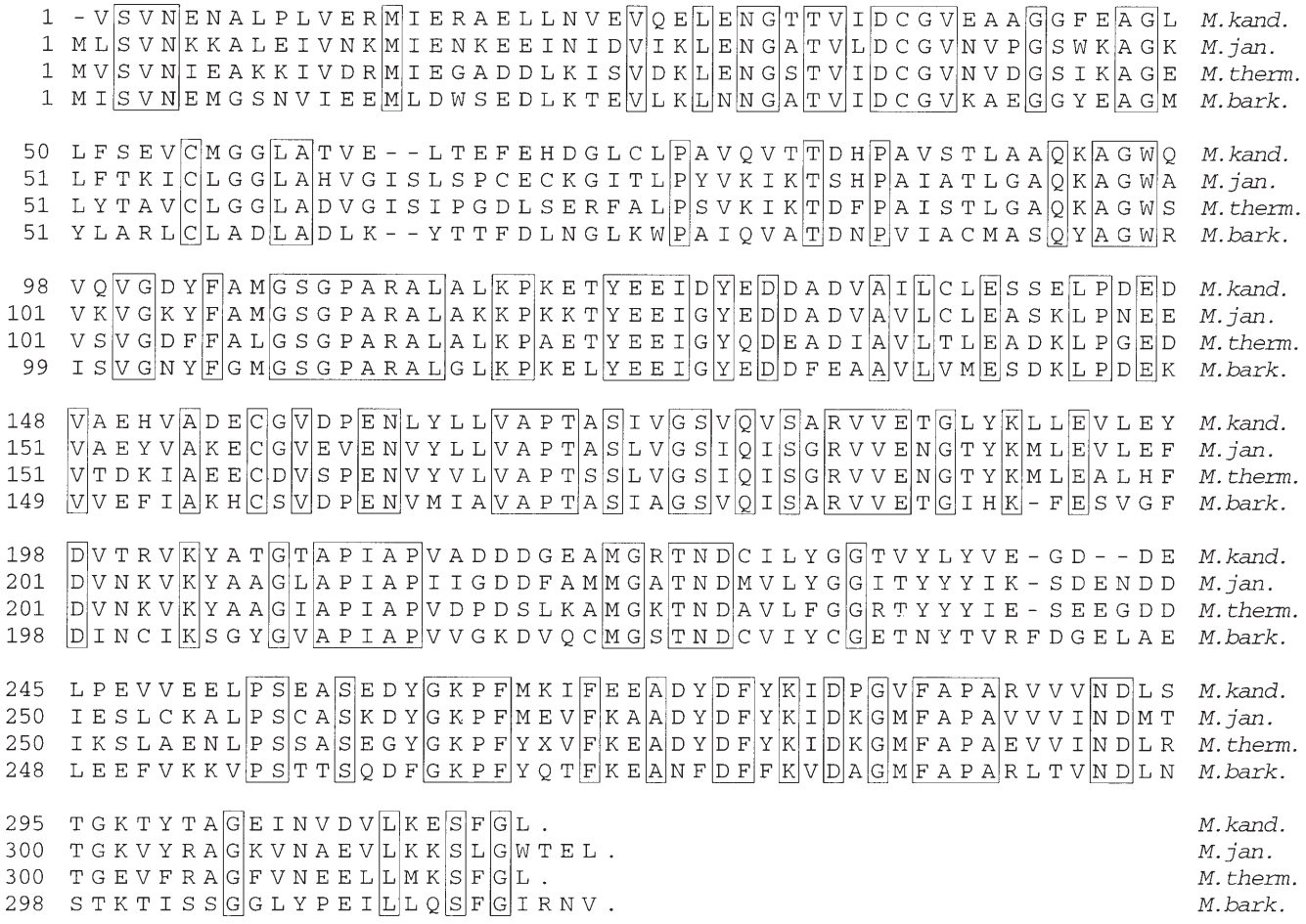

Fig. 3. Alignment of the amino acid sequence of cyclohydrolase from *Methanopyrus kandleri (M. kand.), Methanococcus jannaschii (M. jan.), Methanobacterium thermoautotrophicum (M. therm.)*, and *Methanosarcina barkeri (M. bark.)*. The sequences were aligned according to the Clustal method using the Lasergene computer program (DNA Star, London, UK). Gaps are marked by *dashes*. Identical amino acid residues occurring in all four proteins are *boxed*. The sequence for the *M. jannaschii* enzyme is from Bult et al. (1996) and that for the *M. thermoautotrophicum* enzyme from Vaupel et al. (1996). The sequence for the *M. barkeri* enzyme has not yet been published and is available under accession number Y08843 from the EMBL sequence data bank

formyltetrahydromethanopterin formed in the reaction catalyzed by the enzyme. An understanding of the structure/function relationship will have to await the elucidation of the tertiary structure by X-ray analysis. Crystallization studies of the cyclohydrolase from *M. kandleri* overproduced in *E. coli* are in progress.

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