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Expression of the glyceraldehyde-3-phosphate dehydrogenase gene from the extremely thermophilic archaebacterium *Methanothermus fervidus* in *E. coli*

Enzyme purification, crystallization, and preliminary crystal data

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The gene of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from the extremely thermophilic archaebacterium *Methanothermus fervidus* (growth optimum 82°C) was cloned in vector pJF118EH and expressed in *E. coli* cells. As shown by molecular mass determination, protein sequencing, heat stability, and substrate saturation kinetics, the enzyme synthesized in *E. coli* is identical to the original enzyme from *M. fervidus*. The high thermostability of the *E. coli*-produced *M. fervidus* GAPDH allows rapid purification to homogeneity. From this enzyme protein crystals were grown which proved to be suitable for X-ray analysis. The crystals are of tetragonal space group $P4_122$ and contain a dimer per asymmetric unit.

Methanobacteria; Enzyme expression; Protein thermostability; Protein crystallography

1. INTRODUCTION

Expression systems for enzymes from extremely thermophilic organisms in mesophilic hosts are attractive, since the thermostability of the product favors a simple and effective purification from the heat-unstable host proteins, thus making them easily available for analytical and preparative purposes. While the expression of genes from mesophilic and moderately thermophilic archaebacteria in *E. coli* is well known, and the yield of functional enzymes has already been demonstrated by DNA shotgun cloning followed by screening for complementation of respective *E. coli* mutations [1-3], a successful expression of the genes from extremely thermophilic archaebacteria has not yet been described.

Here we report on the targeted cloning and expression of the *gap* gene coding for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from the extremely thermophilic methanogen *Methanothermus fervidus* in *E. coli*. On the basis of previous structural and functional characterizations [4,5] we provide evidence that the *M. fervidus* GAPDH produced in *E. coli* is identical to the original enzyme. Thus, the expression system provides a basis for extended studies (e.g. protein crystallography, site-directed mutagenesis experiments) on structure and function of the thermophilic protein.

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2. MATERIAL AND METHODS

2.1. *Bacterial strains*

Cells of *M. fervidus* (DSM 2088) were grown by K.O. Stetter (Universität Regensburg). *E. coli* strain JM83 (*ara*, Δ(*lac-proAB*), *rpsL*, φ80, *lacZ*ΔM15) was obtained from D. Kamp (MPI Martinsried); *E. coli* strain WK6 (Δ(*lac-proAB*), *galE*, *strA*, *F'lacI*^{q}, ZΔM15, *pro*A$^+$B$^+$) was a gift from H.-J. Fritz (Max-Planck-Institut für Biochemie, Martinsried).

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2.2. *Plasmids, enzymes and chemicals*

The expression vector pJF118EH [6] was obtained from Professor A. Boeck (Institut für Mikrobiologie, Universität München). The restriction enzyme *Bsm*I was purchased from Serva; all other restriction endonucleases and DNA-modifying enzymes were from Boehringer Mannheim or Gibco/BRL. DNA sequencing was carried out with the sequenase kit from USB. [α-^{35}S]Thio-dATP was obtained from Amersham Buchler, isopropylthiogalactopyranoside (IPTG) from Biomol, ingredients for media from Difco, and the matrex red A gel for affinity chromatography from Amicon. All other chemicals were p.A. grade from Merck (Darmstadt).

2.3. *Construction of the* E. coli *clones expressing* M. fervidus *GAPDH*

A 3600 bp DNA fragment from the genomic library of *M. fervidus* known to contain the GAPDH gene [5] was cut with *Bsm*I immediately in front of the ATG start codon. The 5'-overhanging single-stranded ends on both sites of the resulting 1300 bp subfragment were removed by digestion with T_4 DNA polymerase [7]. Expression vector pJF118EH was cut with *Eco*RI, blunt-ended with Klenow fragment of *E. coli* DNA polymerase, and ligated to the blunt-ended 1300 bp subfragment. Transformant *E. coli* JM83 or WK6 cells were selected on nutrient medium plates with ampicillin (5 g yeast extract, 5 g peptone, 5 g NaCl, 0.1 g ampicillin/l). Reculturing the ampicillin-resistant transformants was performed in microtiter wells containing the same medium. The clones bearing the recombinant plasmid pJF-GAP and producing *M. fervidus* GAPDH were differentiated from non-expressing clones by stamping these cultures on nutrient medium plates with ampicillin containing 30 mM IPTG and incubating them for 6 h. Clones which were retarded or failed completely in growing were further analyzed by plasmid restriction mapping.

DNA sequence determination was performed using a synthetic primer oligonucleotide (CAAATAATTTTACCCTTT) complementary to the coding sequence 170 bp downstream of the ATG start codon of the *gap* gene (Applied Biosystems DNA synthesizer 380A in Department of D. Oesterhelt, MPI Martinsried) according to Sanger et al. [8].

The expression of the *M. fervidus gap* gene was tested by enzymatic assay [4].

2.4. *Production of* M. fervidus *GAPDH in* E. coli *cultures*

Cells of *E. coli* containing the recombinant vector pJF-GAP were grown at 37°C to $A_{578} = 1.0$ in nutrient medium with ampicillin. After 200 mg/l IPTG had been added, the culture was shaken over an induction period of 6 h, which proved to be optimal for expression. Then cells were harvested by centrifugation and stored at −80°C.

2.5. *Purification of* M. fervidus *GAPDH from* E. coli

10 g cells were suspended in 30 ml buffer containing 500 mM potassium phosphate (pH 7.5) and 30 mM mercaptoethanol (ME) and pressed 3 times through a French pressure cell. Following 20 min centrifugation at 27 000 × *g* the supernatant was incubated at 90°C for 30 min to heat denature thermolabile proteins. After a further centrifugation the supernatant was diluted 10-fold and loaded on a matrex red A gel column (3.2 × 6 cm; equilibrated with a 25 mM potassium phosphate buffer, pH 7.5 + 30 mM ME [buffer A]). The column was washed with 150 ml of 50 mM potassium phosphate pH 7.5 +

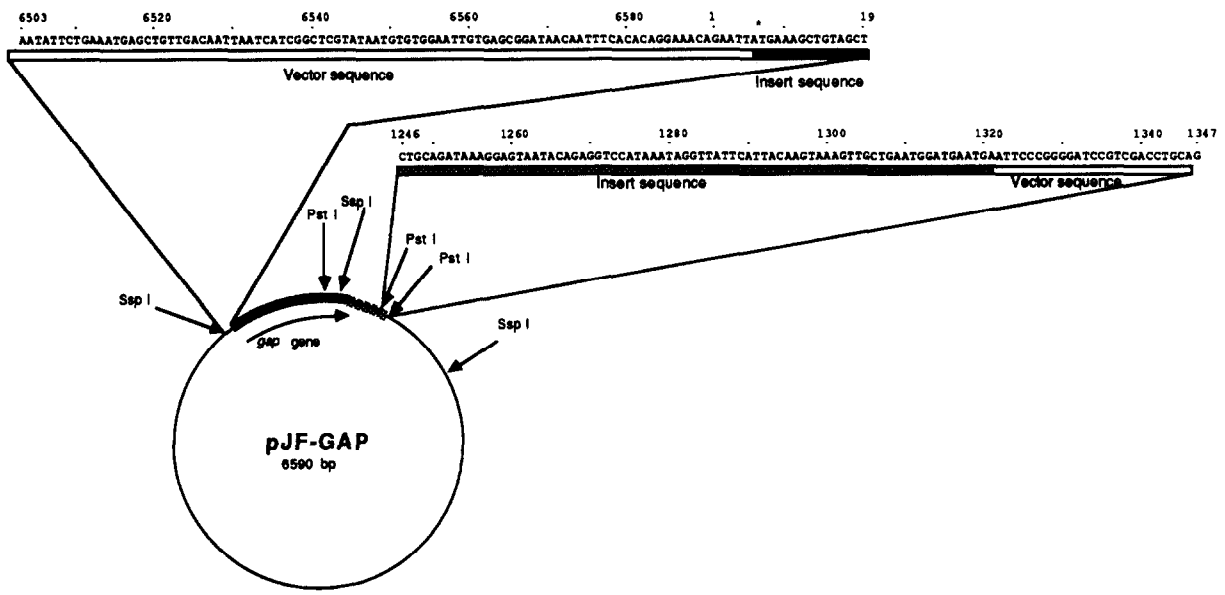

Fig.1. Map of the recombinant plasmid pJF-GAP. The nucleotide sequences from the *Ssp*I site (position 6503) including the first 5 triplets of the coding sequence of *M. fervidus gap* gene and between the *Pst*I sites (positions 1246 and 1341) are shown. Numbering starts with the first base of the vector multiple cloning site. The bold black segment shows the coding region of the *gap* gene, the bold shaded segment the downstream noncoding part of the insert. The asterisk points to the ATG start codon of the *gap* gene.

Table 1

Purification of *M. fervidus* GAPDH from 10 g *E. coli* JM83 containing plasmid pJF-GAP

	Protein (mg)	Activity total (U)	Spec. act. (U/mg)	Purification (-fold)	Recovery (%)
Crude extract	300	150	0.5	-	100
Heat treatment	46	150	3.26	6.5	100
Affinity chromatography	2	90	45.0	90	60

30 mM ME and the *M. fervidus* GAPDH was then eluted with buffer A containing 1 mM NADP.

2.6. *Analysis of the gene product*

The enzymatic and heat stability tests were carried out as reported [4]. N-terminal amino acid sequence determination was done by F. Lottspeich (MPI Martinsried) as described [9].

2.7. *Crystallization and X-ray analyses of GAPDH*

To concentrate the GAPDH, the enzyme was loaded on a hydroxyapatite column (2.5 × 0.5 cm) and eluted with a 0.5 M potassium phosphate buffer, pH 7.5 + 30 mM ME. From the concentrated enzyme (5 mg/ml) crystals were grown at 20°C by the vapor diffusion technique at 2.8 M phosphate (NaH_2PO_4/K_2HPO_4, pH 7.3) and 30 mM ME in the presence of 1 mM NADP. These crystals were transferred to 3.2 M phosphate, sealed in siliconized glass capillaries, and mounted on modified precession rotation cameras.

3. RESULTS AND DISCUSSION

3.1. *Construction of the recombinant expression vector and expression in* E. coli

The correct fusion of vector pJF118EH with the 5′ part of the 1300 bp *Bsm*I subfragment of *M. fervidus* genomic DNA containing the *gap* gene was controlled by DNA sequencing. A physical map of the recombinant vector pJF-GAP including the sequences of both vector-insert linkages is given in fig.1. As shown, the ligation of the *M. fervidus gap* gene-containing fragment in pJF118EH resulted in a construct in which the ATG start codon of the insert was 8 bp apart from the vector ribosome-binding site.

The highest expression level was obtained after the cells were induced in the middle of the log phase (A_{578} = 1.0) for 6 h. Obviously, the composition of the growth medium does not influence the gene expression as shown by comparative expression experiments with *E. coli* strain WK6 grown in M9 medium [7] supplemented with 1 μg/l thiamine, 0.1 mg ampicillin, and 10 ml/l glycerol instead of glucose.

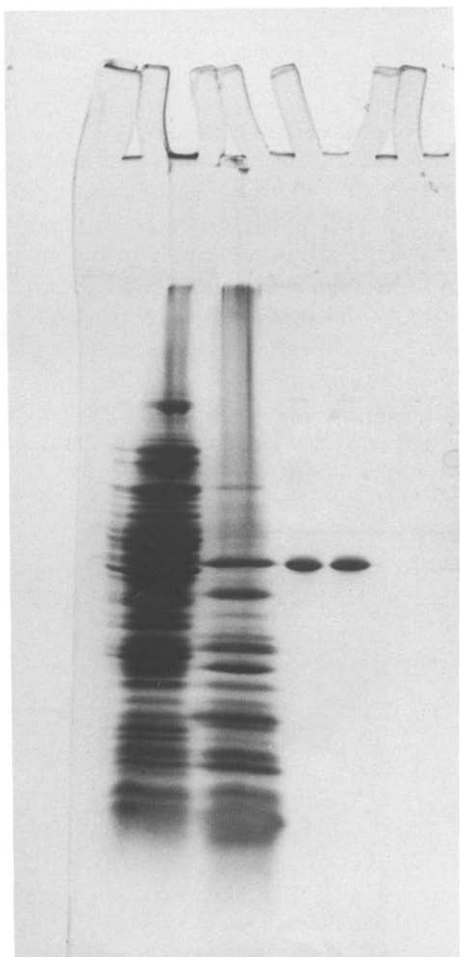

Fig.2. SDS-polyacrylamide gel showing the purification steps of *E. coli*-produced *M. fervidus* GAPDH. From left to right: crude extract; heat precipitation, affinity chromatography; original *M. fervidus* GAPDH (control).

The activity of the *M. fervidus* GAPDH per g wet cells of *E. coli* transformants was 4-times higher than that of equal amounts of *M. fervidus* cells. However, considering the total protein con-

Table 2

Kinetic properties of the *M. fervidus* GAPDH produced in *E. coli:* comparison to the original enzyme

Variable substrate	K_m (mM)		V_{max} (U/mg protein)	
	Enzyme from *E. coli*	Original	Enzyme from *E. coli*	Original
Arsenate	44	44	15	14
Phosphate	35	35	9	9
NAD^+	0.5	0.5	14	13.5
$NADP^+$	0.003	0.003	7.0	7.0

Concentration of fixed substrates: arsenate, 150 mM; 3-phosphoglycerate, 2 mM; NAD^+, 2 mM, assay temperature, 40°C

Table 3

Effectiveness of several salts in stabilizing *M. fervidus* GAPDH produced in *E. coli* against heat denaturation: comparison to the original enzyme

Salt additive (concentration = 250 mM)	Residual GAPDH activity (%)	
	Enzyme from *E. coli* transformants	Original enzyme
Potassium salt of cyclic 2,3-diphosphoglycerate	95	95
Potassium phosphate	70	72
Sodium phosphate	58	60
Potassium chloride	5	5
Sodium chloride	2	2

Heat incubation at 90°C for 30 min

tent of *E. coli*, the GAPDH production amounted to maximally 1%, which is rather low compared to other systems [10]. With respect to (i) the distance between vector ribosome-binding site and start codon of the *gap* gene [10,11] and (ii) the base triplet (AAA) following ATG [12], suitable conditions for high expression in *E. coli* are present. Therefore, the relatively low efficiency must be due to other constraints of the gene structure or just to the strongly differing codon usages between *M. fervidus* and *E. coli* (not shown).

3.2. *Purification of* M. fervidus *GAPDH from induced* E. coli *cells*

The thermostability of the archaebacterial enzyme in the presence of high ionic strength [13] provided a basis for a rapid and simple purification by removing thermolabile host proteins. Thus, after heat treatment of the crude extract at 90°C and the following affinity chromatography on matrex red A gel homogeneous *M. fervidus*

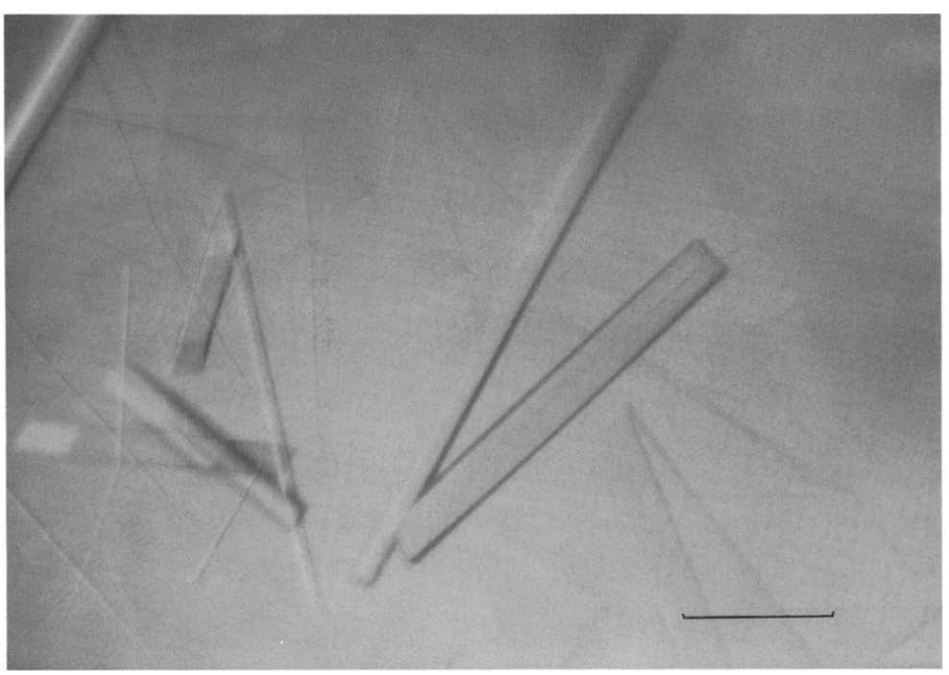

Fig.3. Crystals of *M. fervidus* GAPDH. Bar, 300 μm.

GAPDH was obtained. A representative purification protocol is given in table 1. Purity of the enzyme is demonstrated by SDS gel electrophoresis (fig.2).

3.3. *Characterization of the* M. fervidus *GAPDH synthesized in* E. coli

As shown in fig.2, the molecular masses of the *E. coli*-produced GAPDH and the original enzyme isolated from *M. fervidus* are identical. No processing occurred at the enzyme's amino terminus in the *E. coli* system as proved by sequence determination of the first 3 amino acids of the enzyme. Differences in substrate saturation kinetics or the characteristic ion dependency of heat stability of the enzyme could not be found between the two GAPDHs (tables 2,3). Thus, the *M. fervidus* GAPDH produced in *E. coli* appears to be phenotypically identical with the original enzyme from *M. fervidus*, indicating that the folding of the thermophilic enzyme is not affected during its biosynthesis in the mesophilic *E. coli* environment.

3.4. *Enzyme crystallization and preliminary X-ray data*

The *M. fervidus* GAPDH synthesized in *E. coli* crystallizes in prismatic needles mostly of rectangular cross-section (of maximal length and diameter 1.5 and 0.1 mm) (fig.3). According to diffraction symmetry and systematic extinctions these crystals belong to the tetragonal space group $P4_122$ or to its enantiomorph $P4_322$. The cell constants evaluated from rotation photographs are $a = b = 74.8$ Å, $c = 272$ Å, $\alpha = \beta = \gamma = 90°$. The crystals diffract to beyond 3.0 Å resolution. According to density measurements with a modified Ficoll 400 gradient method [14] the crystals contain four tetramers per unit cell, i.e. a dimer of molecular mass 75 kDa per asymmetric unit. Thus two dimers of a given tetramer will be related by an exact crystallographic 2-fold rotation axis. A native X-ray data set is currently being collected in our laboratory.

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