Engineering thermostability in archaebacterial glyceraldehyde-3-phosphate dehydrogenase

Hints for the important role of interdomain contacts in stabilizing protein conformation

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Construction of hybrid enzymes between the glyceraldehyde-3-phosphate dehydrogenases from the mesophilic *Methanobacterium bryantii* and the thermophilic *Methanothermus fervidus* by recombinant DNA techniques revealed that a short C-terminal fragment of the *Mt. fervidus* enzyme contributes largely to its thermostability. This C-terminal region appears to be homologous to the α_3-helix of eubacterial and eukaryotic glyceraldehyde-3-phosphate dehydrogenases which is involved in the contacts between the two domains of the enzyme subunit. Site-directed mutagenesis experiments indicate that hydrophobic interactions play an important role in these contacts.

Thermostability; Glyceraldehyde-3-phosphate dehydrogenase; Site-directed mutagenesis; Chimeric gene; Hydrophobic interaction; Archaebacteria

1. INTRODUCTION

To investigate the factors responsible for thermostability of enzyme proteins of thermophilic archaebacteria growing near and above 100°C we analyzed the phenotypic properties and primary structure of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from different mesophilic and thermophilic species [1–4]. The high sequence similarity of the enzyme homologues from *Methanobacterium bryantii* (optimal growth temperature: 37°C [5]), *Methanothermus fervidus* (optimal growth temperature: 83°C [6]) and *Pyrococcus woesei* (optimal growth temperature: 100°C [7]) provides a good opportunity not only to get hints about the structural features of thermoadaptation by sequence comparison but also to use the similar structure of the homologous enzymes for an experimental determination of thermophily-specific structural elements.

In order to determine the structural elements responsible for thermostability of the protein conformation we constructed chimeric genes using parts of the *gap* gene of the thermophile *Mt. fervidus* together with parts of the respective gene of the mesophile *Mb. bryantii* and studied the properties of the hybrid gene products. To define more precisely the intramolecular interactions stabilizing the protein conformation we use the GAPDH of *Mt. fervidus* as target for engineering lower and higher thermostability by site-directed mutagenesis. For the respective experiments the less stable *Mb. bryantii* GAPDH and the more stable *P. woesei* enzyme served as model.

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Abbreviations: DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, FRG); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *gap*, gene encoding GAPDH; *Mb.*, *Methanobacterium*; *Mt.*, *Methanothermus*

2. MATERIALS AND METHODS

2.1. *Bacterial strains*

Cells of *Methanobacterium bryantii* (DSM 863) were grown in the laboratory of K.O. Stetter (Universität Regensburg, FRG). For cloning and expressing the mutated *gap* genes the *E. coli* K-12 strains JM83 [araΔ(*lac-proAB*)*strA thi* ϕ80d*lacZ* M15] [8] and DH5α [F$^-$ *endA1 hsdR17* ($r_K^-m_K^+$) *supE44 thi-1* λ^- *recA1 gyrA96* relA1Δ(*lacZYA-argF*) *U169* ϕ80d*lacZ* M15] [9] were used. *E. coli* WK6 [Δ(*lac-proAB*) *galE strA*/F'*lacI*^{q} *Z M15 pro A*$^+$ *B*$^+$] and *E. coli* WK6mutS [Δ*lac-proAB*) *galE strA mut*S::Tn10/F' *lacI*^{q} *Z M15proA*$^+$*B*$^+$] [10] were applied in the mutagenesis experiments.

2.2. *Plasmids, phages, enzymes, chemicals*

The vectors for cloning and sequencing were pUC18 and M13mp18/19 [11]. The twin plasmids pMa/c5-8 [12] for mutagenesis were a gift from H.-J. Fritz (Universität Göttingen, FRG). For expression of the mutated genes the vector pJF118EH [13] was used. The expression vector pHK256 was a gift from M. Kröger (Universität Giessen, FRG). M13K07 [14] (gift from H.-J. Fritz, Universität Göttingen, FRG) was the helper phage in preparing single-stranded plasmid derivatives for mutagenesis. The sources of the restriction endonucleases and DNA-modifying enzymes, the material for DNA sequencing and gene expression experiments, the chemicals for enzyme

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Table 1

Purification of GAPDH from 20 g *Mb. bryantii* cells

Purification step	Protein (mg)	Total activity (U)	Spec. act (U/mg)	Purification (-fold)	Recovery (%)
Crude extract	920	48.0	0.06	–	100
Ammonium sulfate fractionation	264	45.6	0.17	2.6	95
Hydroxyapatite chromatography	15.0	45.5	3.0	45.4	94
Blue sepharose CL-6B	0.2	34.4	194.0	2900	72

preparation and enzymatic tests and the ingredients for media were the same as given in [1–4].

2.3. *Standard enzyme assay*

The activity of the GAPDH of *Mt. fervidus* and *Pc. woesei* was measured as described [1,4]. The assay mixture for the *Mb. bryantii* GAPDH contained 80 mM Tris-HCl, 120 mM potassium arsenate, 1 mM D-glyceraldehyde 3-phosphate and 0.1 mM $NADP^+$ (pH 7.5). The activity was measured by following the reduction of $NADP^+$ at 45°C. The test conditions for the mutant GAPDHs were the same as applied for the *Mt. fervidus* enzyme.

2.4. *Purification of the* Mb. bryantii *GAPDH*

20 g frozen cells of *Mb. bryantii* were thawed in 20 ml of 20 mM potassium phosphate buffer, pH 7.5, containing 30 mM mercaptoethanol (buffer A) and pressed 3 times through a French pressure cell at 1200 bar. The homogenate was then centrifuged 30 min at 37 000 × *g*. During the following ammonium sulfate fractionation the GAPDH precipitated at 65–90% saturation. After centrifugation (20 min at 10 000 × g) and resuspension in buffer A the GAPDH fraction was dialyzed against buffer A overnight and then applied to a 70 ml hydroxyapatite column equilibrated with buffer A. The column was washed with buffers of increasing phosphate concentration from 50 mM, 70 mM, 100 mM to 150 mM. The eluted enzyme fractions were pooled, dialyzed against buffer A and loaded on a 100 ml Blue Sepharose CL-6B column (3.2 × 6 cm) equilibrated with the same buffer. The column was washed first with 2000 ml buffer A containing 140 mM NaCl and then with 150 ml buffer A containing 3 mM NAD^+. Finally, the pure enzyme was eluted by addition of 0.6 mM $NADP^+$ to buffer A. A representative purification protocol is given in Table 1.

2.5. *Mutagenesis of the* Mt. fervidus gap *gene*

Oligonucleotide-directed mutagenesis was performed using the gapped duplex method [12,15]. The *gap* gene of *Mt. fervidus* was excised from a recombinant pUC plasmid and inserted between the *Eco*RI and *Pst*I sites of the multiple cloning site of the plasmid pMa5-8 [12]. The following mutagenic deoxyoligonucleotides (synthesized on the DNA synthesizer 380 A from Biosystems) were used: 5′-GCACTTATAAGTATAACCCTAGGCGTATC-3′ (creation of a new *Sty*I site) 5′-GTTAGTTTTATTTATAGATTTCCATTTATC-CTCTTCCATCTC-3′ (construction of the $Y_{321}W$ mutant) 5′-TTG-TTAGTTTTATTTATAGATTCGATTTATCCTCTTCCATCTC-3′ (construction of the $Y_{321}S$ mutant).

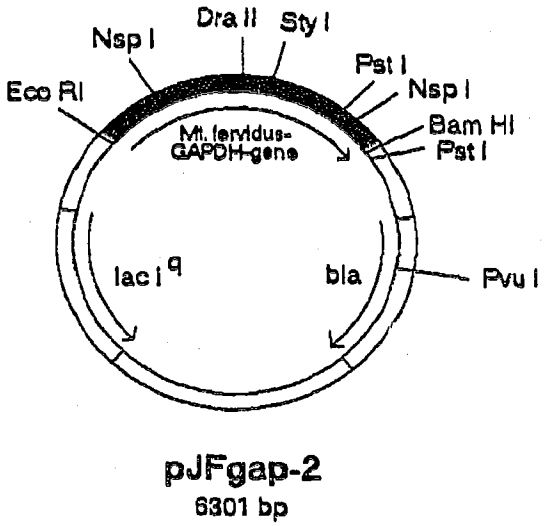
The whole nucleotide sequence of the mutated genes were confirmed by the dideoxy chain termination method [16] prior to insertion into the pJF118EH expression vector.

2.6. *Construction of the recombinant* gap *genes*

All recombinations between the *gap* genes of *Mt. fervidus* and *Mb. bryantii* were performed using the plasmids pJFgap-2 and EX-6 (Fig. 1) which contain the respective wild-type *gap* genes. For the construction of the chimeric genes *rec 42*, *rec 94* and *rec 160* 3′-fragments of the *Mt. fervidus gap* gene were substituted by homologous fragments of the *Mb. bryantii* gene. The hybrid *gap* gene *revrec* represents a derivative of *rec 160*, in which a 129 bp fragment at the 3′-end was exchanged by the homologous region of the *Mt. fervidus* gene.

The chimeric gene *rec 42* was constructed by ligating the 857 bp *Eco*RI-*Nsp*I fragment of pJFgap-2 containing the 5′-part of the *Mt. fervidus gap* gene with the 129 bp 3′-terminal fragment of the *Mb. bryantii* gene prepared from EX-6 by a *Nsp*I/*Hin*dIII digest.

For generating *rec 94* first a *Sty*I restriction site had to be created



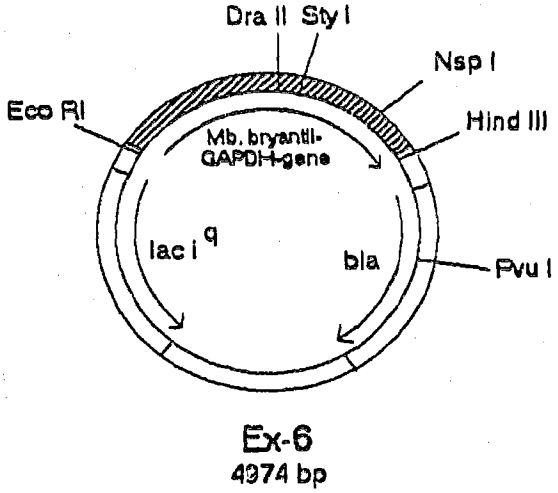


Fig. 1. Structure of the recombinant plasmids pJFgap-2 and Ex-6 used for the construction of the chimeric *gap* genes. pJFgap-2 and EX-6 represent derivatives of pJF118EH and pHK256, respectively, which itself is derived from pJF118EH by deletion of 29.2% of the sequence (M. Kröger, personal communication). *lacI*^{q}, lac repressor gene; *bla*, β-lactamase gene.

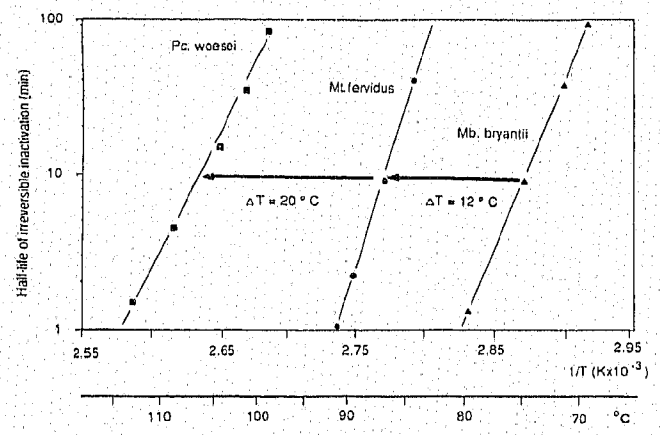


Fig. 2. Temperature dependence of irreversible inactivation of the GAPDH from *Mb. bryantii, Mt. fervidus* and *Pc. woesei*. The logarithms of the half-life of inactivation were plotted against the reciprocal of absolute temperature. The difference in thermostability is given as difference in temperature at which the half-life of inactivation is equal to 10 min.

in the *Mt. fervidus* gene by site-directed mutagenesis. The 753 bp *Eco*RI-*Sty*I fragment of the mutated gene was then recombined with the 314 bp *Sty*I-*Hin*dIII fragment of EX-6.

Rec 160 was constructed by combining the 538 bp *Eco*RI-*Dra*II fragment from pJFgap-2 with the 515 bp *Dra*II-*Hin*dIII fragment from EX-6.

The chimeric gene *revrec* was constructed by recombination of the 538 bp *Eco*RI-*Dra*II and 186 bp *Nsp*I-*Hin*dIII fragments of pJFgap-2 and the 357 bp *Dra*II-*Nsp*I fragment of EX-6.

2.7. *Production of the mutated GAPDHs in* E. coli *and enzyme purification*

The conditions for production of the GAPDHs in *E. coli* as well as the purification protocol for the mutant enzymes $Y_{323}S$ and $Y_{323}W$ were the same as described previously for the *Mt. fervidus* wild-type enzyme [2]. The heat treatment of the less thermostable hybrid enzymes was performed at 70°C. To compensate the lower purification efficiency, a two-step ammonium sulfate fractionation (0–65%, 65–90% saturation) was included in the procedure prior to the affinity chromatography on matrix red A gel. All enzyme preparations were homogeneous as proven by SDS-polyacrylamide gel electrophoresis.

The heat stability tests were conducted as reported previously [4].

3. RESULTS AND DISCUSSION

3.1. *Thermostability of the wild-type enzymes of* Mb. bryantii, Mt. fervidus *and* Pc. woesei

As shown previously, the GAPDH of *Mt. fervidus* and *Pc. woesei* are efficiently expressed in *E. coli* [2,4]. For preparative reasons the heterologously expressed enzymes of both thermophilic organisms were used in the following experiments. Lacking a suitable expression system for the *Mb. bryantii* GAPDH, however, this enzyme has to be isolated from the original organism yet.

The heat stability of the enzymes was determined by following the velocity of irreversible inactivation. The half-lives of inactivation were deduced from semilogarithmic plots of the inactivation kinetics. The plots showed linearity up to 40% residual activity but deviated from linearity at lower values. This deviation may be due to superposition of at least two inactivation processes. Since the inactivation curve did not change

Table II

Structure of he hybrid GAPDHs and their specific activity and thermostability compared to the properties of the parental enzymes

Name	Structure[a]	Phenotypic properties	
		Spec. act. at 70°C (U/mg)	Thermostability relative to stability of the parental *Mt. fervidus* GAPDH[b] ΔT (°C)
Parental *Mt. fervidus* GAPDH		55	0.0
Rec 42	294 42 (10)	54	−9.0
Rec 94	242 94 (25)	47	−10.0
Rec 160	176 160 (47)	47	−10.5
Revrec	176 118(37) 42	73	−4.6
Parental *Mb. bryantii* GAPDH		n.d.	−12.3

[a]Numbers indicate the length (number of residues) of the exchanged peptide, numbers in brackets give the real residue changes in the recombinant structure

[b]For calculation of the relative thermostabilities see legend to Fig. 2

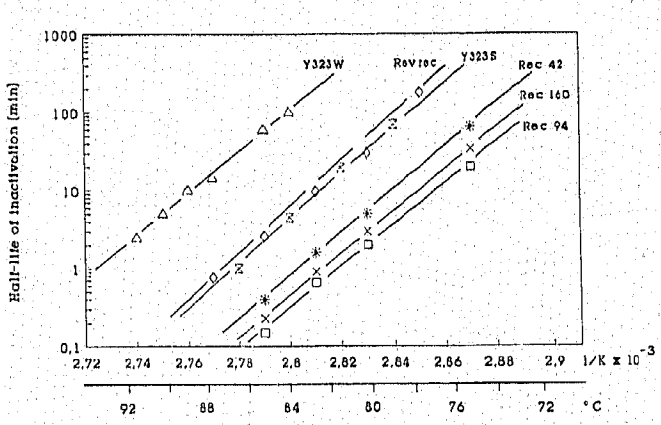


Fig. 3. Temperature dependence of irreversible inactivation of the hybrid and mutant GAPDHs.

by varying the protein concentration between 30 and 150 μg/ml, dissociation processes can be excluded as rate limiting steps under the conditions used.

For comparison of the thermostabilities we determined the temperature with the half-life of 10 min deduced from the first fast inactivation reaction (Fig. 2). From this, the GAPDH from *Pc. woesei* is found to be by 20°C and 32°C more stable than the enzymes from *Mt. fervidus* and from *Mb. bryantii,* respectively.

3.2. *Construction of chimeric genes between the* gap *genes of* Mt. fervidus and Mb. bryantii *and thermostability of the gene products*

From the 3′-terminus of the *Mt. fervidus gap* gene fragments of different length (126 bp, 282 bp, 477 bp) were substituted by equivalent parts of the *Mb. bryantii gap* gene. The chimeric genes *(rec 42, rec 94, rec 160)* were expressed in *E. coli* and the respective GAPDH hybrids (Rec 42, Rec 94, Rec 160) were purified and analyzed with respect to specific activity and thermostability.

All three enzymes possess specific activities which are comparable to that of the wild-type enzyme from *Mt. fervidus* (Table II). However, the thermostabilities of the hybrid enzymes differ significantly from that of the wild-type enzyme from *Mt. fervidus:* the exchange of the short 42 amino acid fragment in the *Mt. fervidus* GAPDH (Rec 42) – corresponding to a real substitution of only 10 residues – results in a decrease of stability by 9°C, whereas the exchange of larger fragments (Rec 94, Rec 160) yields only an additional decrease of thermostability by 1.3°C (Fig. 3, Table II).

The obvious importance of the short C-terminal fragment for the conformational stability was supported by substitution of the corresponding C-terminal part of the hybrid Rec 160 by the 42 amino acid fragment of the *Mt. fervidus* GAPDH (Revrec). As shown in Fig. 3 and Table II, this reverse change of the C-terminal fragment yielded an over-average effect: the substitution of only one fourth of the mesophilic part in Rec 160 by the thermophilic sequence (Revrec) resulted in a 50% restoration of the thermostability.

3.3. *Site-directed mutagenesis of the GAPDH from* Mt. fervidus

To define the interactions, by which the short C-terminal fragment stabilizes the native conformation of the GAPDH from *Mt. fervidus,* single residues in that region were exchanged and the effect of the replacement on thermostability was tested. For the exchange we focused on position 323 where the respective residues in the different GAPDHs show an increase in hydrophobicity from mesophilic to thermophilic structures: at that position the enzymes from the closely related mesophilic methanogens *Mb. bryantii* and *Mb. formicicum* possess Ser, the more thermostable enzyme from *Mt. fervidus* Tyr and the enzyme from *Pc. woesei* with the highest thermostability Trp (Fig. 4).

As can be deduced from Fig. 3, the change to Ser (mutant GAPDH $Y_{323}S$) resulted in an decrease of thermostability by 4.5°C, the change to Trp (mutant GAPDH $Y_{323}W$) in an increase of thermostability by 1.3°C as compared to the wild-type enzyme.

For detailed structural interpretation of the observed data the knowledge of the three-dimensional structure of the *Mt. fervidus* and *Mb. bryantii* enzyme would be necessary, which, however, is not yet available. Therefore, we interpret our data on the basis of the known structures of eubacterial and eukaryotic GAPDHs [17–19]. As demonstrated by the respective spatial models of these enzymes, the C-terminal 20–25 residues form an amphipathic α-helix (helix α_3) contacting the β_E-strand of the extended parallel β-sheet of the N-terminal, nucleotide-binding domain. Exclusively

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  I     V       I  M    DNPS   E      G  .   Mb. formicicum
I I     V       I  M    D PS   Q      G  .   Mb. bryantii
QAVHQESDIVPENVDAVRAILEMEEDKYKSINKTNKAMNILQ   Mt. fervidus
        VI      I  MF IA . W   K    SLG  K   Pc. woesei

                323
```

Fig. 4. Amino acid sequence of the 42 residues comprising C-terminal fragment of the GAPDH from *Mt. fervidus* [3] compared to the homologous sequences of the enzymes from the mesophiles *Mb. bryantii* and *Mb. formicicum* [3] as well as from the hyperthermophile *Pc. woesei* [4]. Dots denote gaps introduced for optimal alignment. Residues at position 323, where the exchanges were made, are in bold type.

these contacts clamp the C-terminal, so-called catalytic domain and the N-terminal, nucleotide-binding domain together. Assuming a respective arrangement in the archaebacterial GAPDH [3], the influence of the performed exchanges on thermostability can be explained by the involvement of the residue at position 323 in interdomain contacts governing the rigidity and thus the stability of the protein conformation.

Obviously, hydrophobic interactions play a crucial role in these interdomain contacts. The participation of aromatic residues in these contacts in the thermophilic archaebacterial GAPDHs supports our suggestion that especially these residues are of importance for the conformational stability of enzymes from organisms living at extremely high temperatures [4].

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