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Glyceraldehyde-3-Phosphate Dehydrogenase from the Hyperthermophilic Archaebacterium *Pyrococcus woesei*: Characterization of the Enzyme, Cloning and Sequencing of the Gene, and Expression in *Escherichia coli*

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The glyceraldehyde-3-phosphate dehydrogenase from the hyperthermophilic archaebacterium *Pyrococcus woesei* (optimal growth temperature, 100 to 103°C) was purified to homogeneity. This enzyme was strictly phosphate dependent, utilized either NAD^+ or $NADP^+$, and was insensitive to pentalenolactone like the enzyme from the methanogenic archaebacterium *Methanothermus fervidus*. The enzyme exhibited a considerable thermostability, with a 44-min half-life at 100°C. The amino acid sequence of the glyceraldehyde-3-phosphate dehydrogenase from *P. woesei* was deduced from the nucleotide sequence of the coding gene. Compared with the enzyme homologs from mesophilic archaebacteria (*Methanobacterium bryantii*, *Methanobacterium formicicum*) and an extremely thermophilic archaebacterium (*Methanothermus fervidus*), the primary structure of the *P. woesei* enzyme exhibited a strikingly high proportion of aromatic amino acid residues and a low proportion of sulfur-containing residues. The coding gene of *P. woesei* was expressed at a high level in *Escherichia coli*, thus providing an ideal basis for detailed structural and functional studies of that enzyme.

The extraordinary ability to grow above 100°C is exclusively restricted to certain members of archaebacteria. These hyperthermophilic strains with optimal growth temperatures ranging from 100 to 105°C are represented by four genera: *Pyrodictium* (41), *Pyrococcus* (19), *Pyrobaculum* (26), and *Methanopyrus* (27).

Although the hyperthermophily brings up evident questions concerning the molecular background of thermoadaptation, knowledge of the macromolecular cell constituents of these organisms is scarce. Thus, with the exception of the reports on hydrogenase and ferredoxin from *Pyrococcus furiosus* (5, 10), no description of any protein from these hyperthermophiles has yet been published.

Here we characterize some phenotypic properties of the glyceraldehyde-3-phosphate dehydrogenase from *Pyrococcus woesei* (optimal growth temperature, 100 to 103°C [47]) and report on the cloning and sequencing of the coding gene as well as on its expression in *Escherichia coli*.

To get indications about the structural adaptation to the extreme growth temperatures, we compared the sequence of the glyceraldehyde-3-phosphate dehydrogenase of *P. woesei* with the structures of the enzyme homologs from mesophilic and thermophilic archaebacteria (17).

MATERIALS AND METHODS

Bacterial strains. Cells of *P. woesei* Vul4 (DSM 3773) were grown as described previously (47). For cloning and expression of the glyceraldehyde-3-phosphate dehydrogenase gene, the *E. coli* K-12 strains JM83 [*ara* Δ(*lac-proAB*) *strA thi* φ80d*lacZ*ΔM15 (33)] and DH5α [F^- *endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1* λ^- *recA1 gyrA96 relA1* Δ(*lacZYA-argF*)*U169* φ80d*lacZ*ΔM15 (28)] were used.

Plasmids, enzymes, chemicals. The vectors for cloning and sequencing were pUC18 and M13mp18/19 (34), respectively; the expression plasmid was pJF118EH (20).

Restriction endonucleases, T4 DNA ligase, polynucleotide kinase, alkaline phosphatase, and endonuclease *Bal* 31 were purchased from either Boehringer GmbH (Mannheim, Federal Republic of Germany) or GIBCO/BRL (Eggenstein, Federal Republic of Germany). Agarose and urea (ultrapure grade) were from GIBCO/BRL, [α-^{35}S]dATP and [γ-^{32}P]dATP were from Amersham Buchler GmbH (Braunschweig, Federal Republic of Germany), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was from Boehringer, isopropyl-β-D-thiogalactopyranoside (IPTG) was from Biomol (Ilvesheim, Federal Republic of Germany), ampicillin was from Sigma Chemical Co. (Munich, Federal Republic of Germany), nitrocellulose filters were from Schleicher & Schuell (Dassel, Federal Republic of Germany), and ingredients for *E. coli* media were from Difco Laboratories (Detroit, Mich.). For isolation of *P. woesei* chromosomal DNA, an ion-exchange column (Quiagen tube 20; Diagen, Düsseldorf, Federal Republic of Germany) was used. The Sequenase sequencing kit was purchased from United States Biochemical (Cleveland, Ohio). Blue Sepharose CL-6B was from Pharmacia (Uppsala, Sweden), and Matrex Red A gel was from Amicon Corp. (Witten, Federal Republic of Germany).

The glyceraldehyde-3-phosphate dehydrogenase inhibitor pentalenolactone was a gift from D. Mecke (University of Tübingen, Tübingen, Federal Republic of Germany).

Standard enzyme assay. The activity of the glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.-) was measured photometrically (wavelength, 366 nm) at 70°C with equipment described previously (15). The standard assay (total volume, 1 ml) contained 90 mM Tris hydrochloride, 240 mM potassium arsenate, 6 mM glyceraldehyde-3-phosphate, and 9 mM NAD^+ (pH 7.3 at 70°C). To avoid heat destruction of glyceraldehyde-3-phosphate, this substrate was added to the mixture immediately before starting the enzyme reaction.

Purification of glyceraldehyde-3-phosphate dehydrogenase from *P. woesei*. Cells (10 g wet weight) were thawed in 30 ml

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of 10 mM sodium phosphate buffer (pH 7.5) containing 30 mM 2-mercaptoethanol (buffer A) and disrupted by sonication (Bronson Sonifier, Danbury, Conn.). After centrifugation for 30 min at 27,000 × *g*, the glyceraldehyde-3-phosphate dehydrogenase was enriched by a two-step ammonium sulfate fractionation. The precipitate of the first fractionation (0 to 50% saturation) was removed by centrifugation (27,000 × *g*). After the ammonium sulfate concentration was increased to 70% saturation, the precipitated protein was centrifuged, dissolved in buffer A, and dialyzed against 2 liters of the same buffer. This solution was then applied to a hydroxyapatite column (3.2 by 10 cm) equilibrated with buffer A. The chromatographic separation was performed by a stepwise increase of the phosphate concentration (50 mM, 80 mM, 150 mM). Glyceraldehyde-3-phosphate dehydrogenase-containing fractions were collected and dialyzed against buffer A. The solution was then loaded on a Blue Sepharose CL-6B column (3.2 by 10 cm) equilibrated with buffer A. After the column was extensively rinsed with 30 mM potassium phosphate buffer (pH 7.3) (containing 30 mM 2-mercaptoethanol), the enzyme was eluted by increasing the potassium phosphate concentration to 80 mM. After dialysis against buffer A, a separation on ATP-Sepharose (bed volume of the column, 5 ml; equilibration with buffer A) followed. The specific elution of the enzyme from the resin was attained by the addition of 0.5 mM ATP to the buffer.

Heat stability tests. Heat stability tests were performed in glass capillaries (inner diameter, 1 to 2 mm) filled with the protein solution (protein content, 30 μg/ml; 10 mM potassium phosphate buffer [pH 7.3] containing 30 mM 2-mercaptoethanol) in an anaerobic hood. The capillaries were sealed off and incubated at the respective temperature. After incubation, the samples were cooled in an ice bath and tested for enzyme activity.

Protein sequencing. For N-terminal sequence analysis, the protein was electroblotted from a polyacrylamide-dodecyl sulfate slab gel onto a siliconized glass fiber sheet (14) and applied to a gas-phase sequencer (model 470 A; Applied Biosystems).

Preparation of chromosomal DNA from *P. woesei*. Cells (3 g) were suspended in 5 ml of buffer (10 mM Tris hydrochloride, 1 mM EDTA, pH 8.0) and lauroylsarcosine was added to a final concentration of 1% (wt/vol). After 5 min of incubation at room temperature, the lysate was extracted twice with phenol and three times with chloroform-isoamyl alcohol (24:1). The supernatant was precipitated with isopropanol (1 volume), washed, dried, and suspended in 1.5 ml of 50 mM morpholinepropanesulfonic acid–750 mM NaCl–15% ethanol (vol/vol), pH 7.0. Further purification was done by anion-exchange chromatography with a Quiagen tube 20 according to the protocol of the manufacturer (Diagen).

Cloning and sequencing of glyceraldehyde-3-phosphate dehydrogenase gene of *P. woesei*. Two oligonucleotide mixtures deduced from a part of the N-terminal amino-acid sequence of the *P. woesei* glyceraldehyde-3-phosphate dehydrogenase (V-G-I-N-G-Y-G) were synthesized by the phosphoamidite method on a DNA synthesizer (model 380 A; Applied Biosystems) (D. Oesterhelt, Max-Planck-Institute for Biochemistry, Martinsried, Federal Republic of Germany). Mixture 1 was 5′-G-T-(A, C)-G-G-(N)-A-T-(A, C, T)-A-A-(T, C)-G-G-(N)-T-A-(T, C)-G-G-3′. Mixture 2 was 5′-G-T-(G, T)-G-G-(N)-A-T-(A, C, T)-A-A-(T, C)-G-G-(N)-T-A-(T, C)-G-G-3′, where N = (A, C, G, T).

Chromosomal DNA of *P. woesei* was digested with *Eco*RI, *Pst*I, or *Xba*I. The respective fragments were separated on a 1% agarose gel, transferred to a nitrocellulose filter, and probed with the ^{32}P-end-labeled oligonucleotides (30).

A *Pst*I fragment of about 3 kilobases which gave the strongest signal after washes at the highest stringency (44°C, 30 min) was cut out from a preparative agarose gel and ligated in pUC18. *E. coli* JM83 was transformed with the recombinant plasmids, and a small gene library of about 800 clones was established.

The colony hybridization was done as described by Woods (46). Recombinant plasmids of colonies that gave positive signals were analyzed by restriction digestion, Southern blotting (40), and oligonucleotide screening.

Double-stranded recombinant plasmids were prepared as described elsewhere (16); the isolation of M13mp18/19 single-stranded DNA followed the protocol of Amersham International (*M13 Cloning and Sequencing Handbook*).

The sequencing was done by the dideoxy termination method of Sanger et al. (37), with single-stranded M13mp18/19 derivatives as the template. The method of Guo et al. (21) was used for generating overlapping subfragments with exonuclease *Bal* 31.

Expression of *P. woesei* glyceraldehyde-3-phosphate dehydrogenase gene in *E. coli*. To remove the complete upstream DNA sequence of the glyceraldehyde-3-phosphate dehydrogenase gene, the recombinant pUC plasmid containing the gene was cut with *Hph*I and the gene-containing restriction fragment was isolated.

Two oligonucleotides (26 and 25 bases long; synthesized in the department of D. Oesterhelt) were used as adaptors for an appropriate integration of the gene in the expression vector pJF118EH (Fig. 1). The respective construction changed the original start codon GTG to ATG and created a unique *Nde*I restriction site in front of the gene, thus allowing an insertion of the gene at a suitable distance from the ribosome-binding site of the vector (Fig. 1).

Transformants of *E. coli* JM83 were screened for the correct recombinant plasmid by restriction analysis and DNA sequencing. The conditions for production of the *P. woesei* glyceraldehyde-3-phosphate dehydrogenase in *E. coli* (IPTG concentration, culture conditions, etc.) were the same as reported for the heterologous expression of the *Methanothermus fervidus* glyceraldehyde-3-phosphate dehydrogenase (18) in *E. coli*.

Purification of *P. woesei* glyceraldehyde-3-phosphate dehydrogenase from *E. coli*. *E. coli* cells (2 to 3 g) expressing *P. woesei* glyceraldehyde-3-phosphate dehydrogenase were suspended in 10 ml of buffer A (10 mM potassium phosphate buffer [pH 7.5] containing 30 mM 2-mercaptoethanol) and disrupted by sonication as described for the preparation of the original enzyme. After centrifugation (30 min at 27,000 × *g*), the supernatant was heated to 90°C for 30 min and centrifuged again. The protein solution was then applied immediately to a Matrex Red A gel column (3.2 by 6 cm; equilibrated with buffer A). After the column was washed with 800 ml of buffer A containing 250 mM KCl, the enzyme was eluted with buffer A in the presence of 1 mM $NADP^+$.

To determine the enzyme activity in crude extracts, we inhibited the glyceraldehyde-3-phosphate dehydrogenase of *E. coli* by preincubation with 100 μM pentalenolactone for 30 min.

Determination of protein and polyacrylamide-dodecyl sulfate slab gel electrophoresis were performed as described elsewhere (15).

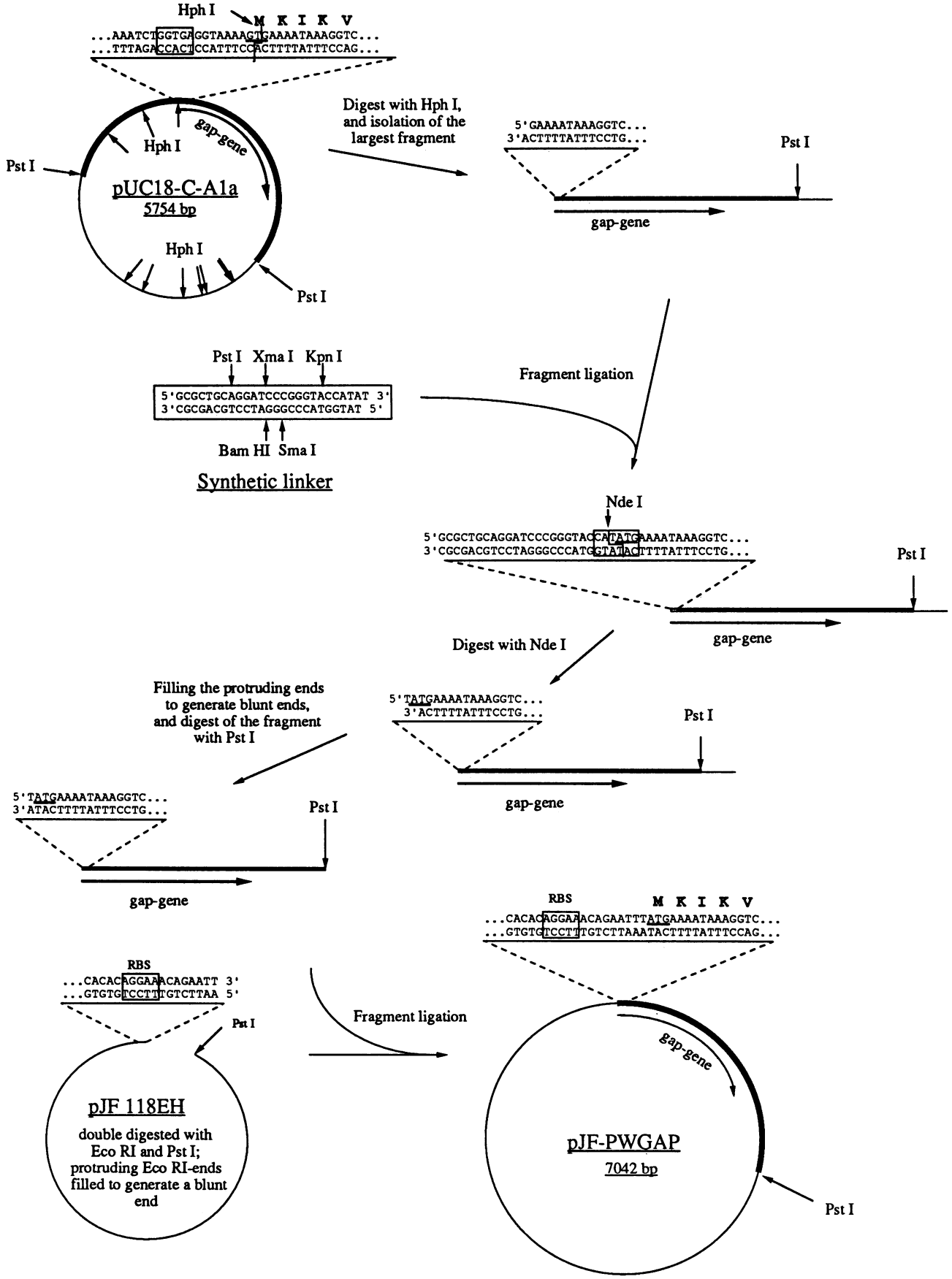

FIG. 1. Construction scheme for the recombinant plasmid pJF-PWGAP used for the heterologous expression of the *P. woesei* glyceraldehyde-3-phosphate dehydrogenase gene (gap-gene) in *E. coli*. The initiation codon is underlined. bp, Base pairs; RBS, ribosome-binding sequence.

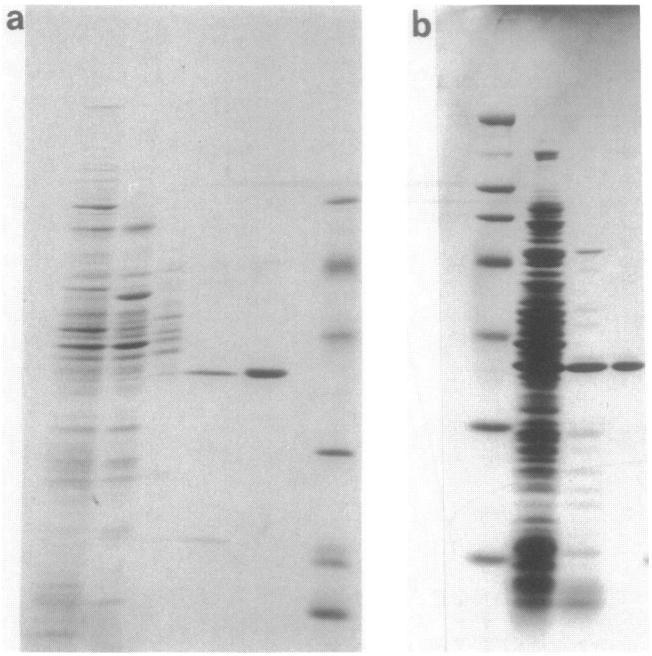

FIG. 2. Polyacrylamide-dodecyl sulfate gel electropherogram documenting the purification of the *P. woesei* glyceraldehyde-3-phosphate dehydrogenase from the original organism (a) and from transformed *E. coli* cells (b). (a) From left to right: crude extract, ammonium sulfate fractionation, hydroxyapatite chromatography, chromatography on Blue Sepharose CL-6B, chromatography on ATP Sepharose, molecular weight standards; (b) from left to right: molecular weight standards, crude extract, heat treatment, chromatography on Matrex Red A gel.

RESULTS AND DISCUSSION

Enzyme purification, molecular mass of subunits, and enzymatic properties. The glyceraldehyde-3-phosphate from *P. woesei* was purified to homogeneity as demonstrated by the single band in the electropherogram (Fig. 2a). The efficiency of the purification procedure is documented in Table 1. The molecular mass of the subunits was determined by polyacrylamide-dodecyl sulfate gel electrophoresis to be 36.5 kilodaltons, resembling that of the glyceraldehyde-3-phosphate dehydrogenases from other sources (22). Fourteen amino acid residues from the N terminus of the protein could be sequenced: M-K-I-K-V-G-I-N-G-Y-G-T-I-G.

TABLE 1. Purification of *P. woesei* glyceraldehyde-3-phosphate dehydrogenase from 10 g of *P. woesei* cells or from 10 g of transformed *E. coli* cells

Purification step	Protein (mg)	Total activity (U)	Sp act (U/mg)	Purification (fold)	Recovery (%)
P. woesei					
Crude extract	1,040	213	0.2		100
Ammonium sulfate fractionation	745	156	0.21	1.05	73
Hydroxyapatite chromatography	50	156	3.1	15.8	73
Blue Sepharose CL-6B	2.3	130	56	280	61
ATP-Sepharose	1.0	70	70	350	33
E. coli					
Crude extract	442	2,170	4.8		100
Heat treatment	96	2,120	22.1	4.6	98
Matrex Red A gel	16	1,200	75	15.5	56

TABLE 2. Kinetic properties of *P. woesei* glyceraldehyde-3-phosphate dehydrogenase from original organism and from transformed *E. coli* cells

Variable substrate[a]	K_m (mM) of enzyme from:		V_{max} (U/mg) of enzyme from:	
	P. woesei	*E. coli*	*P. woesei*	*E. coli*
Arsenate	120	120	9.2	11.5
Phosphate	40	40	5.7	6.5
NAD^+	1.0	1.0	18.0	17.0
$NADP^+$	0.01	0.01	11.0	11.5

[a] Concentrations of the fixed substrates were as follows: arsenate, 300 mM; 3-phosphoglyceraldehyde, 5 mM; $NADP^+$, 1 mM. Assay temperature was 45°C.

Like the glyceraldehyde-3-phosphate dehydrogenase from the methanogenic archaebacterium *Methanothermus fervidus* (15), the *P. woesei* enzyme exhibits a phosphate-dependent activity, acts on NAD^+ and $NADP^+$ (however, showing a 100-fold higher affinity to the phosphorylated cosubstrate [Table 2]), and is insensitive to the antibiotic pentalenolactone (range of concentration tested, 1 to 100 μM), a potent inhibitor of the eubacterial and eucaryotic glyceraldehyde-3-phosphate dehydrogenases (23).

Thermostability. The thermostability of the enzyme was determined by observing the velocity of the irreversible inactivation at various temperatures. With a half-life of 44 min at 100°C (Table 3), the enzyme from *P. woesei* shows a considerable thermostability. Nevertheless, considering the extreme growth temperature of the organism (upper limit of growth, 104°C), the intrinsic stability of the enzyme seems to be insufficient; thus, in vivo extrinsic stabilizing factors must be assumed.

Several salts were able to stabilize the *P. woesei* enzyme (Table 4). K^+ seems more efficient than Na^+; with respect to the anions, the stabilizing effect on the *P. woesei* enzyme increases in the order chloride, sulfate, phosphate, citrate. Indications that K^+ ions improve the thermostability of the cytoplasmic enzymes of *P. woesei* in vivo may be deduced from studies on the intracellular ion milieu of *P. woesei*, yielding potassium concentrations in the range of 500 to 600 mM (A. Haas and R. Hensel, unpublished data). However, the counterion balancing the positive charge of the potassium ions in *P. woesei* remains to be identified.

Cloning and sequencing of glyceraldehyde-3-phosphate gene: characteristics of coding and noncoding regions. Several clones carrying an identical 3.1-kilobase *Pst*I insert were identified by colony hybridization of an enriched gene bank

TABLE 3. Thermostability of *P. woesei* glyceraldehyde-3-phosphate dehydrogenase from the original organism and from transformed *E. coli*[a]

Incubation temp (°C)	Half-life (min) of irreversible inactivation of enzyme from:	
	P. woesei	*E. coli*
97	65	65
100	44	44
104	9	9
107	4.5	4.5

[a] Incubation buffer was 10 mM sodium phosphate buffer–30 mM 2-mercaptoethanol (pH 7.3). Enzyme concentration was 30 μg. The half-lives were deduced from respective semilog plots.

TABLE 4. Effectiveness of several salts in stabilizing *P. woesei* glyceraldehyde-3-phosphate dehydrogenase from the original organism and from transformed *E. coli*

Salt (250 mM)	Residual activity (%) after 30-min incubation at 104°C of enzyme from:	
	P. woesei	*E. coli*
Sodium chloride	22	18
Potassium chloride	14	12
Sodium sulfate	12	12
Potassium sulfate	28	28
Sodium phosphate	70	62
Potassium phosphate	72	72
Sodium citrate	68	63
Potassium citrate	100	100

of *P. woesei* DNA with two oligonucleotide mixtures derived from the N-terminal protein sequence. Restriction analysis confined the hybridizing region to a 300-base-pair *Hin*dIII subfragment. As proved by sequencing, this fragment contained the 5′ part of the glyceraldehyde-3-phosphate dehydrogenase gene. The nucleotide sequence of the 3.1-kilobase insert was completely determined. The coding region of the glyceraldehyde-3-phosphate gene was read from both strands, and parts of the flanking regions were read from only one (Fig. 3). The total nucleotide sequence of the 3.1-kilobase insert is given in Fig. 4a.

The glyceraldehyde-3-phosphate dehydrogenase gene encompasses 1,002 nucleotides, starting at position 1297 (Fig. 4) with the rare initiation codon GTG. The coding region is preceded by a putative ribosome-binding site (GAGGT) which shows strong homology to the complementary sequence of the 3′ terminus of 16 S rRNA of the closely related archaebacterium *Thermococcus celer* (1). The G+C content of the glyceraldehyde-3-phosphate dehydrogenase gene (40.3%) is slightly higher than the value for the total genome (37.5% [47]). As expected for genes of an organism with a low G+C content, A and T are the preferred bases in the third position of the codons. Remarkably, the dinucleotide CG is strongly discriminated against in the coding region. As a consequence, none of the arginine residues in the *P. woesei* glyceraldehyde-3-phosphate dehydrogenase is encoded by the triplets starting with CG.

Immediately downstream from the TAA stop codon, a pyrimidine-rich region of 28 nucleotides including the sequence TTTTTT is found, resembling the putative termination signals of the five transcripts of the *Sulfolobus* viruslike particle SSV-1 (36).

In the upstream and downstream regions of the glyceraldehyde-3-phosphate dehydrogenase gene, three complete open reading frames (ORFs) (ORF a, ORF b, ORF y) and two truncated ORFs (ORF x, ORF z) could be identified (Fig. 4b). The deduced amino acid sequence of ORF y shows high similarity (31% identical residues) to the ribosomal protein L35a from *Rattus norvegicus* (42) and to the partial sequence of ribosomal protein L32 from *Xenopus laevis* (4). None of them, however, indicates similarity to any other glycolytic enzyme from eubacteria or eucaryotes. Obviously, the glyceraldehyde-3-phosphate dehydrogenase gene of *P. woesei* is not integrated in a glycolytic or gluconeogenetic operon as recently described for several eubacteria (3, 8, 9, 12).

Expression of *P. woesei* glyceraldehyde-3-phosphate gene in *E. coli* and comparison of enzyme produced in *E. coli* with original enzyme. For expression of the *P. woesei* glyceraldehyde-3-phosphate dehydrogenase in *E. coli*, the respective gene was inserted in the expression vector pJF118EH. To provide favorable conditions for expression, the original start codon of the gene GTG was changed to ATG and the distance between the vector ribosome-binding site and the start codon was adjusted to 9 base pairs (Fig. 1).

IPTG-induced *E. coli* cells transformed with the recombinant plasmid pJF-PWGAP produced about 3 mg of protein per g of cells (Table 1); thus, the expression is about 10-fold higher than for the *Methanothermus fervidus* glyceraldehyde-3-phosphate dehydrogenase (18).

The heat stability of the *P. woesei* enzyme allowed a simple two-step purification (Table 1). Heat incubation and consecutive affinity chromatography yielded a homogeneous enzyme preparation (Fig. 2b).

The heterologously expressed enzyme was compared with the original enzyme regarding various kinetic and stability

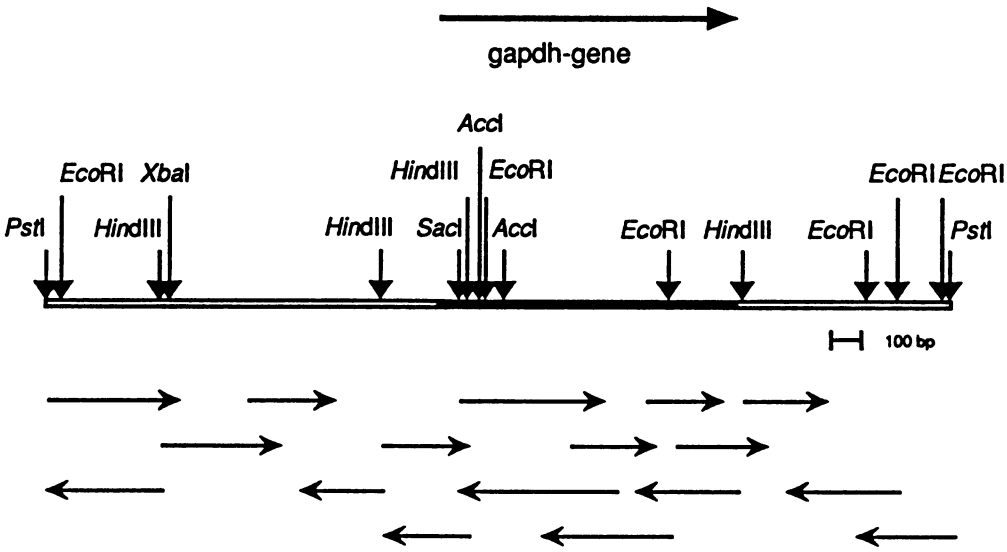

FIG. 3. Restriction map and sequencing strategy of the cloned *Pst*I fragment carrying the glyceraldehyde-3-phosphate dehydrogenase gene (gapdh-gene) of *P. woesei*. Arrows indicate the individual sequence runs. bp, Base pairs.

a)

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CTGCAGGAGTCTCTAAGGCAAACCTAATTCCCCGAATTCCAGTTGCAGAAAGTGCATCCAAAACAATCTTAGGTTTTAGAACATTCAATAGCAAAACAACTACATCCCTATTTGGAGCCATTCGAGGATTATAAAAAACA 140
GACGTCCTCAGAGATTCCGTTTGGATTAAGGGGCTTAAGGTCAACGTCTTTCACGTAGGTTTTGTTAGAATCCAAAATCTTGTAAGTTATCGTTTTGTTGATGTAGGGATAAACCTCGGTAAGCTCCTAATATTTTTTGT
...A P T E L A F R I G R I G T A S L A D L V I K P K L V N L L L V V V D R N P A M R P N Y F V

GGAGAATCATAAATAGTTTTTGCTTTGGGAACTAAAACTTTAGCTTTCCCCTCATGAACTTCAAATAATTCCATAGACATCCCATTAGACTATTTCCACATAATCTCCTAAGGCCTGTCCTGGAAGTCCTTTTTCAAACC 280
CCTCTTAGTATTTATCAAAAACGAAACCCTTGATTTTGAAATCGAAAGGGAGTACTTGAAGTTTATTAAGGTATCTGTAGGGTAATCTGATAAAGGTGTATTAGAGGATTCCGGACAGGACCTTCAGGAAAAAGTTTGG
P S D Y I T K A K P V L V K A K G E H V E F L E M (S M)  * V I E V Y D G L A Q G P L G K E F
                                                  < orf z

TGGCCCTTACTGCCCCCTTTGTCCCATGAACTCTAACTATTTGCCCTTCAAAATCTTTCCACTTGGACTCTTCCAGAGAACAAGTCTCCCTATGAGTTTTGAAGCTTCTTCCCTACTGTTGACGTCTAGAGGCTTAATT 420
ACCGGGAATGACGGGGGAAACAGGGTACTTGAGATTGATAAAACGGGAAGTTTAGAAAGGTGAACCTGAGAAGGTCTCTTGTTCAGAGGGATACTCAAAACTTCGAAGAAGGGATGACAACTGCAGATCTCCGAATTAA
R A R V A G K T G H V R V I K G K L I K G S P S K W L V L R G I L K S A E E R S N V D L P K I

ATCATTACATTGTTGTGTTGGTTCTCCTTACTCCTTCTGTAACTTAGAACGACTCCCTTTATGCTCATTCTCGGACCCCAGCCTAATTTTATTATGTTGTTTTTTAAAGATTATTCCCAAGCAAAAAACATTTATAGGTG 560
TAGTAATGTAACAACACAACCAAGAGGAATGAGGAAGACATTGAATCTTGCTGAGGGAAATACGAGTAAGAGCCTGGGGTCGGATTAAAATAATACAACAAAAAATTTCTAATAAGGGTTCGTTTTTTGTAAATATCCAC
I M V N N H Q N E K S R R Y S L V V G K I S M
          orf a >                    < orf y
          (M K D S) M F L Y E F D L C R Y S K D L T A L S L G L L I G A S L T V R W V S L S M
ACCATTCACTCATGCAAACATGAAAGATTCTATGTTCTTGTATGAATTTGATCTATGTCGATACTCGAAGGATCTCACAGCATTGTCCTTGGGGCTGCTCATAGGCGCCTCTCTTACAGTGCGCTGGGTTTCTTTATCAA 700
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S S S R D L L H Y L V L P L I L V V I L H E G L H A L T A K L S G A K T S L G V L T K Y G I
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I L A V Y V G I N T P L P V K K I R Y I T I A P I I I S I V A F F F S W V T Y S P F W A I L Y
ATTTTAGCCGTTTATGTTGGCATTAACACTCCTCTTCCAGTTAAAAAAATAAGATACATCACTATAGCTCCAATTATTATATCCATTGTGGCGTTCTTCTTTTCCTGGGTTACATATTCTCCCTTCTGGGCCATATTGTA 980
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I F N T T G I V G D L I V F L V L S K M P S D A I V V D E G T I M K S N A E F P E P Y P S W F
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S K L I I G L A V L V F L Y I L T N I R I E F E V V G T L P N Q T M P V N S H F E *
TCTCTAAGCTTATCATTGGGCTTGCAGTATTGGTATTCCTCTATATTCTCACAAACATTAGAATCGAATTTGAAGTTGTGGGTACTCTTCCTAATCAGACAATGCCAGTAAATAGTCACTTTGAGTGAAAACAACCCTTA 1260
AGAGATTCGAATAGTAACCCGAACGTCATAACCATAAGGAGATATAAGAGTGTTTGTAATCTTAGCTTAAACTTCAACACCCATGAGAAGGATTAGTCTGTTACGGTCATTTATCAGTGAAACTCACTTTTGTTGGGAAT
                                  gapdh-gene >
                                  M K I K V G I N G Y G T I G K R V A Y A V T K Q D D M E L I G V T K T
AAATTTCATTTTATAATCTAAATCTGGTGAGGTAAAGTGAAAATAAAGGTCGGAATTAATGGATATGGGACAATTGGAAAGAGGGTAGCCTACGCCGTAACTAAGCAAGATGACATGGAGCTCATAGGAGTTACAAAAAC 1400
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K P D F E A Y R A K E L G I P V Y A A S E E F L P R F E K A G F E V E G T L N D L L E K V D I
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R G P I N A I K P S V T I P S H H G P D V Q T V I P I N I E T S A F V V P T T I M H V H S I M
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V E L K K P L T R E D V I D I F E N T T R V L L F E K E K G F E S T A Q L I E F A R D L H R
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E W N N L Y E I A V W K E S I N V K G N R L F Y I Q A V H Q E S D V I P E N I D A I R A M F E
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                                                                                                                                    orf b >
I A E K W E S I K K T N K S L G I L K *                                                                                              (M E N N N I) M S
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Q V K E I I E A A I K E L E D D G F E P D I I L A G P I F I R Y L P E D V R L K V Y E I E E L
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G S D A I I A D S K Y L G Q I K K A A K R I S I D P L L Q E K E W E K I I E E I P T Q E *
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                                                                                                                                    * E L I P

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G I R K K L I Y G L G G G I I I G S V A Q V I D N Y F E A V A G G P G Y L V Y A V S V Y G L V

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M V I G G L V T G L L I R G Y N T T G K S F W G V I V G E T G K I V L T F L A W S P Y E I V D

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A L A S G V G G A F G G V L P G F L V A V L M I M I D G F N L Y G Q S A P I P I R V L M T T

CCGTTACTGCAG 3092
GGCAATGACGTC
V T V A..
    < orf x
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b)

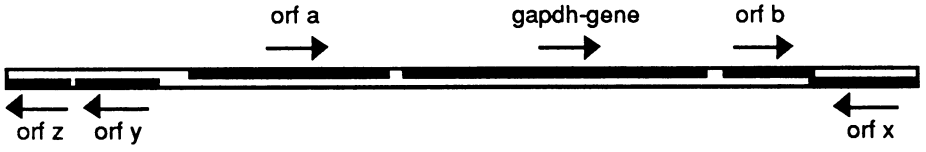

FIG. 4. Nucleotide sequence of the cloned *Pst*I fragment carrying the glyceraldehyde-3-phosphate dehydrogenase gene (gapdh-gene) and location of the reading frames. (a) Nucleotide sequence of the glyceraldehyde-3-phosphate dehydrogenase gene (gapdh-gene) and its flanking regions. The derived amino acid sequences are given in one-letter code above or below the respective nucleotide sequence. Hypothetical ORFs and their reading direction are indicated. Putative ribosome-binding sites are underlined. Residues in brackets refer to an alternative start of translation (ORF a, b, z). (b) Schematic presentation of the relative position and reading direction of the glyceraldehyde-3-phosphate dehydrogenase gene (gapdh-gene) and the neighboring hypothetical ORFs.

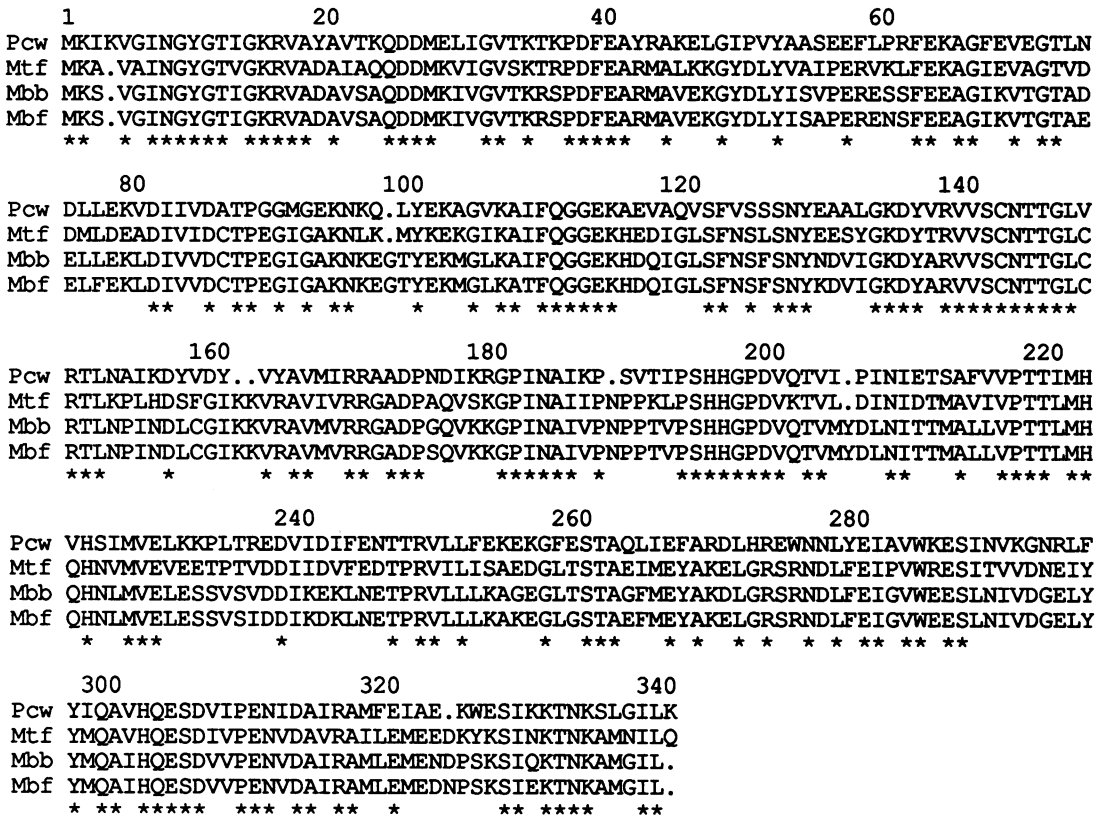

FIG. 5. Sequence alignment of the archaebacterial glyceraldehyde-3-phosphate dehydrogenases from *P. woesei* (Pcw), *Methanothermus fervidus* (Mtf), *Methanobacterium bryantii* (Mbb), and *Methanobacterium formicicum* (Mbf). Gaps are marked by dots. Asterisks indicate residues conserved through all four sequences.

properties. As demonstrated in Tables 2 to 4, both enzyme preparations appear phenotypically identical.

Primary structure of *P. woesei* glyceraldehyde-3-phosphate dehydrogenase and comparison with protein sequences of enzyme homologs from mesophilic and thermophilic archaebacteria. The derived amino acid sequence of the *P. woesei* glyceraldehyde-3-phosphate dehydrogenase (Fig. 4) comprises 334 amino acids, resulting in a subunit molecular mass of 37.5 kilodaltons, which corresponds well to the apparent molecular mass of the protein as determined by polyacrylamide-dodecyl sulfate gel electrophoresis (36.5 kilodaltons).

A sequence alignment of the glyceraldehyde-3-phosphate dehydrogenases from *P. woesei* and from the methanogenic archaebacteria *Methanothermus fervidus*, *Methanobacterium bryantii*, and *Methanobacterium formicicum* (17) is given in Fig. 5. The average sequence similarity of the *P. woesei* glyceraldehyde-3-phosphate dehydrogenase to the enzymes of the methanogens has been calculated to be 50% (identity) or 62% (similarity score based on the Dayhoff mutational matrix [13]).

From the sequence comparison of the four glyceraldehyde-3-phosphate dehydrogenases from mesophilic and thermophilic archaebacteria, trends in the structural adaptation of enzyme proteins to growth temperatures from 37 to 100°C can be recognized. Although we do not know in which direction the adaptation occurred during evolution (from cold to hot or from hot to cold; at least in the archaebacteria some hints favor the latter alternative), we discuss the observed changes in the conventional cold-to-hot direction.

The adaptation to higher temperatures is paralleled by an increase of average hydrophobicity and a respective decrease of average chain flexibility in the archaebacterial glyceraldehyde-3-phosphate dehydrogenase (Table 5). Similar tendencies have also been deduced from comparisons between eubacterial mesophilic and thermophilic glyceraldehyde-3-phosphate dehydrogenases (Table 5) or other eubacterial and eucaryotic mesophilic and thermophilic enzyme pairs (31, 32, 45). Thus, with strengthening of the hydrophobic interactions and increasing chain rigidity, the same tendencies in the structural thermoadaptation are visible in archaebacterial and eubacterial-eucaryotic enzyme proteins.

TABLE 5. Hydrophobicity and chain flexibility of glyceraldehyde-3-phosphate dehydrogenases from mesophilic and thermophilic archaebacteria and eubacteria

Organism (reference)	Optimal growth temp of organism (°C)	Hydrophobicity[a] (kJ)	Chain flexibility[b]
Archaebacteria			
Methanobacterium bryantii (17)	37	4.39	1.007
Methanothermus fervidus (16)	83	4.72	0.996
Pyrococcus woesei	100	5.00	0.991
Eubacteria			
Bacillus subtilis (44)	37	3.93	0.994
Bacillus stearothermophilus (7, 39)	55	4.45	0.991
Thermus aquaticus (24, 25)	75	4.72	0.988

[a] Calculated by the method of Tanford (43).
[b] Calculated by the method of Karplus and Schulz (29).

TABLE 6. Differences in the amino acid composition and preferred residue changes in glyceraldehyde-3-phosphate dehydrogenases from mesophilic and thermophilic archaebacteria

Comparison	Difference in amino acid composition[a]		Preferred amino acid change	
	Amino acid	Change	Substitution	Net change[b]
Methanobacterium bryantii (37°C[c]) and *Methanothermus fervidus* (83°C)	Ile	+9	Leu→Ile	6
	Ala, Asp	+4	Ser→Ala	4
	Lys, Val	+2	Asn→Glu	3
	Arg, Glu, His	+1	Glu→Asp	2
	Pro, Tyr	+1	Gly→Ala	2
	Phe, Thr, Try	0	Gly→Asn	2
	Cys, Gln	−1	Gly→Glu	2
	Met	−2	Met→Ile	2
	Asn	−3		
	Ser	−5		
	Leu	−7		
	Gly	−8		
Methanothermus fervidus (83°C) and *Pyrococcus woesei* (100°C)	Phe	+6	Val→Ile	3
	Ala	+4	Met→Leu	3
	Glu, Lys	+3	Asp→Asn	3
	Asn, Gln	+1	Asp→Glu	2
	Leu, Tyr	+1	Asp→Pro	2
	Arg, Gly, Ile, Thr	0	Val→Leu	2
	His, Val	−1	Ser→Ala	2
	Cys	−2	Ser→Glu	2
	Pro, Ser	−3	Pro→Ala	2
	Met	−5	Ile→Phe	2
	Asp	−10	Leu→Phe	2
			Arg→Tyr	2

[a] Net gain (+) or loss (−) of amino acids in glyceraldehyde-3-phosphate dehydrogenase of the organism with the higher growth temperature.
[b] Change minus back change.
[c] Optimal growth temperature of the organism.

Obviously, in both systems similar cold-to-hot amino acid changes are responsible for the shifts of these parameters. Thus, as already found in eubacterial-eucaryotic proteins (6, 35, 38, 48), alanine is preferred (mainly by serine → alanine and glycine → alanine exchanges), but serine and, especially, glycine are concomitantly discriminated against in the thermophilic structures (Table 6).

Differences, however, from the eubacterial-eucaryotic systems are visible in the dramatic increase of the branched residue isoleucine in the glyceraldehyde-3-phosphate dehydrogenase of the thermophilic archaebacterium *Methanothermus fervidus* (17). Also, the ratio of arginine to lysine does not increase in the thermophilic archaebacaterial glyceraldehyde-3-phosphate dehydrogenases, and the lysine → arginine exchanges typical for the transition to the hot environment in the eubacterial-eucaryotic system (6, 31, 32, 48) do not occur in the archaebacterial enzymes at all. Additionally, threonine does not play a role as a thermophilic substituent for serine in the archaebacterial glyceraldehyde-3-phosphate dehydrogenase comparable to that of eubacterial or eucaryotic proteins (6, 31, 38, 48). Studies on further enzyme systems will show whether these differences represent general archaebacterial features of thermoadaptation.

Comparing the amino acid net changes between the glyceraldehyde-3-phosphate dehydrogenases of the extremely thermophilic *Methanothermus fervidus* (optimal growth temperature, 83°C) and the hyperthermophilic *P. woesei* (optimal growth temperature, 100 to 103°C) with those between the enzymes of the mesophilic *Methanobacterium bryantii* and the extremely thermophilic *Methanothermus fervidus* (Table 6), differences are notable, which may be due to the different stringency in the growth temperature. Thus, the transition from the extremely thermophilic to the hyperthermophilic habitat is characterized by a striking increase of the aromatic residue phenylalanine and by a respective decrease of aspartic acid residues as well as of the sulfur-containing residues methionine and cysteine; in the *P. woesei* enzyme, the cysteine content is even reduced to the catalytically essential residue.

Whereas the increase of phenylalanine seems to be beneficial for the stabilization of the protein conformation (by aromatic-aromatic interactions [11] or by generally strengthening the hydrophobic interactions), the decrease of aspartic acid residues and sulfur-containing residues, especially the cysteine residues, may rather serve the stabilization of the covalent protein structure at temperatures around 100°C (2).

The precise characterization of the thermophily-specific elements of the enzyme requires knowledge of the three-dimensional structure. Crystallization experiments with the enzyme produced in *E. coli* are in progress.

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