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ORIGINAL PAPER

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Cloning, expression, and characterization of the *gsdA* gene encoding thermophilic glucose-6-phosphate dehydrogenase from *Aquifex aeolicus*

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Abstract The *gsdA* gene of the extreme thermophilic bacterium *Aquifex aeolicus*, encoding glucose-6-phosphate dehydrogenase (G6PDH), was cloned into a high-expression vector and overexpressed as a fusion protein in *Escherichia coli*. Here we report the characterization of this recombinant thermostable G6PDH. G6PDH was purified to homogeneity by heat precipitation followed by immobilized metal affinity chromatography on a nickel-chelate column. The data obtained indicate that the enzyme is a homodimer with a subunit molecular weight of 55 kDa. G6PDH followed Michaelis–Menten kinetics with a K_M of 63 μM for glucose-6-phosphate at 70°C with NADP as the cofactor. The enzyme exhibited dual coenzyme specificity, although it showed a preference in terms of k_{cat}/K_M of 20.4-fold for NADP over NAD at 40°C and 5.7-fold at 70°C. The enzyme showed optimum catalytic activity at 90°C. Modeling of the dimer interface suggested the presence of cysteine residues that may form disulfide bonds between the two subunits, thereby preserving the oligomeric integrity of the enzyme. Interestingly, addition of dithiothreitol or mercaptoethanol did not affect the activity of the enzyme. With a half-life of 24 h at 90°C and 12 h at 100°C, this is the most thermostable G6PDH described.

Key words *Aquifex aeolicus* · Glucose-6-phosphate dehydrogenase · Thermostability · Thermophilic · Enzyme · Cloning

Introduction

Thermophiles, which grow optimally above 40°C, have been isolated from high-temperature, water-containing terrestrial and marine environments. The most common biotopes are volcanically and geothermally heated hydrothermal systems, such as solfataric fields, hot springs, and submarine hot vents (Adams 1993; Stetter 1996, 1998). The *Aquificaceae* represent the most deeply branching family within the bacterial domain on the basis of phylogenetic analysis of the 16S ribosomal RNA sequences (Burggraf et al. 1992; Pitulle et al. 1994). *Aquifex aeolicus* is a chemolithoautotrophic bacterium that grows optimally at 85°C. Recently, the complete nucleotide sequence of *A. aeolicus* was determined (Deckert et al. 1998). Autotrophs such as *A. aeolicus* are generally very difficult to grow in the laboratory because they need gaseous hydrogen and carbon dioxide as substrates. In general, these microorganisms grow very slowly and often reach low cell densities because of the low concentration of dissolved gases at high temperatures (Stetter et al. 1983; Kelly and Brown 1993). This restriction makes it hard to study enzymes from these microorganisms. Expression in *Escherichia coli* facilitates the study of such proteins; however, this method is not applicable to all proteins from thermophilic microorganisms because it cannot be assumed that thermophilic enzymes fold correctly in *E. coli* (Diruggiero and Robb 1995; Laderman et al. 1993).

The genome sequence of *A. aeolicus* revealed the presence of a 1,293-bp open reading frame, which on the basis of 32% sequence identity to known G6PDHs in the GenBank nonredundant database was putatively assigned as the *gsdA* gene-encoding glucose-6-phosphate dehydrogenase (G6PDH) (E.C. 1.1.1.49) (Deckert et al. 1998). G6PDH is the first enzyme of the pentose phosphate pathway. It catalyzes the oxidation of glucose-6-phosphate (G6P) to 6-phosphoglucono-δ-lactone, yielding reducing equivalents in the form of NADH/NADPH for reductive biosynthesis and five carbon sugars for the synthesis of nucleotides. Our interest in G6PDH results from the opportunities it offers in the design of highly efficient and thermally stable biotransformation, biosensing, and enzyme-linked immunoassay systems. G6PDH from mesophilic organisms has been used in biotransformation and biosensing systems to recycle the expensive cofactors β-nicotinamide adenine dinucleotide (NAD) and β-

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nicotinamide adenine dinucleotide phosphate (NADP) (Hirschbein and Whitesides 1982; Wong and Whitesides 1981; Yao et al. 1995). In biosensing systems, G6PDH from brewers yeast coupled with hexokinase has been used in the determination of glucose (Slein 1963), and a glucose diagnostic kit based on this concept is commercially available (Sigma 17-25, 115-A). Homogenous and heterogeneous immunoassays have been developed using G6PDH from *Leuconostoc mesenteroides* (Kasahara 1992) and *Bacillus stearothermophilus* (Hosoda et al. 1992), respectively, as a label. Here, we describe the cloning and expression of the gene encoding G6PDH from the extremely thermophilic bacterium *A. aeolicus* as a fusion protein in *E. coli* and the characterization of the purified recombinant G6PDH product.

Materials and methods

Materials

Restriction enzymes, sodium dodecyl sulfate (SDS), molecular weight markers, and Luria-Bertani (LB) medium were purchased from Gibco-BRL (Gaithersburg, MD, USA). Sequencing primers were synthesized by Operon Technologies (Alameda, CA, USA). Prepacked Sephacryl 200 HR gel-filtration column, the HisTrap kit, and the SDS-PAGE (polyacrylamide gel electrophoresis) PhastSystem were from Amersham Pharmacia Biotech (Piscataway, NJ, USA). NAD, NADP, D-glucose-6-phosphate (G6P), dithiothreitol (DTT), 2-mercaptoethanol, and the molecular weight kit for gel filtration were from Sigma (St. Louis, MO, USA). Tris(hydroxymethyl)aminomethane (Tris) was from Research Organics (Cleveland, OH, USA). All chemicals were of reagent grade or better. Deionized water (Milli-Q water purification system; Millipore, Bedford, MA, USA) was used in the preparation of all solutions.

Bacterial strains and plasmids

Genomic DNA of *A. aeolicus* was generously provided by Robert Huber (Lehstuhl für Mikrobiologie, Universität Regensburg, Regensburg, Germany). Plasmid pBAD-TOPO was purchased from Invitrogen (Carlsbad, CA, USA). The *E. coli* expression strain, LMG194 [F^- Δ*lac*X74 *gal* E *thi rps*L Δ*pho*A (*Pvu* II) Δ*ara*714 *leu*::Tn*10*], was grown in LB medium in a rotary shaker at 37°C. Ampicillin was added to LB medium in a final concentration of 100 μg/ml. L(+) arabinose was added at a final concentration of 0.02% (w/v) for the induction of gene expression.

Cloning and sequencing of the *gsdA* gene

Based on the *gsdA* sequence, primers were designed to amplify the gene by PCR on a GeneAmp PCR system 2400 (Perkin-Elmer, Norwalk, CT, USA). The two primers (with *Pst*I and *Sma*I restriction sites in boldface) designed were as follows: 5′-**CTG CAG** ATG AGT TCG GGA GAC ACA, sense; and 5′-**CCC GGG** AAG GCA GGA AAT GAA ATC, antisense. In addition to the template and the primers, the 50-μl reaction mixture contained 0.5 mM dNTPs, *Taq* DNA polymerase buffer, and 2.5 units of *Taq* DNA polymerase (Life Technologies, Gaithersburg, MD, USA). The reaction mixture was subjected to 25 cycles of amplification (45 s at 94°C, 60 s at 48°C, and 150 s at 72°C). The freshly prepared PCR product with the expected size was ligated into the pBAD-TOPO vector, which also contained DNA sequences for the V5 epitope and a polyhistidine tail. The *gsdA* gene was ligated so that the resulting fusion protein had the V5 epitope and poly-His tail at the C-terminus. The recombinant plasmid was transformed into *E. coli* LMG194 using standard procedures. Transformants were selected on LB agar plates containing ampicillin at 100 μg/ml. Colonies were removed from the plates, grown in overnight minicultures, and plasmids were isolated. The plasmid insert was sequenced by the dideoxynucleotide chain termination method on an ABI200 (model 377) automated sequencer at the Macromolecular Structure Analysis Facility at the University of Kentucky.

Overexpression and enzyme purification

An overnight culture of *E. coli* LMG194 harboring pG6PDH was diluted 1:100 into 200 ml of LB supplemented with 100 μg/ml ampicillin and grown to an OD_{600} value of 0.8. Expression was induced by addition of 0.02% (w/v) of L(+) arabinose for 8 h. *E. coli* cells that expressed the cloned gene were harvested by centrifugation at 4,000 *g* for 10 min. Following centrifugation, the cells were resuspended in 20 mM NaH_2PO_4/HCl, 0.5 M NaCl, 10 mM imidazole, pH 7.4 lysis buffer without lysozyme, and sonicated using a Sonic Dismembrator Model 550 (Fisher). Cell debris was removed by centrifugation (10,000 *g* for 15 min). The resulting supernatant was heated for 5 min at 80°C, and the precipitated host cell proteins were removed by centrifugation. To purify the protein, the supernatant was loaded onto a 1-ml HiTrap chelating column charged with nickel ions. Bound proteins were eluted by a stepwise gradient of imidazole-containing buffer (60–500 mM in 20 mM NaH_2PO_4/HCl, 0.5 M NaCl, pH 7.4). Active fractions eluted around 300 mM imidazole. The typical activity recovered after the purification steps was 65%, and the protein yield was 15–20 mg/l of culture. The M_r of G6PDH was estimated by gel filtration using a prepacked Sephacryl S-200 HR column with 50 mM Tris/HCl, 0.15 M NaCl, pH 7.0 as the eluent. The protein standards used were sweet potato β-amylase (M_r = 200,000), yeast alcohol dehydrogenase (M_r = 150,000), bovine serum albumin (M_r = 66,000), bovine carbonic anhydrase (M_r = 29,000), and equine cytochrome C (M_r = 12,400). The purity of the protein samples was analyzed by SDS-PAGE on a 12.5% gel using the PhastSystem. Western blot analysis was performed on the SDS-PAGE fractions by blotting onto nitrocellulose. The blots were incubated with an anti-V5 antibody conjugated to horseradish peroxidase (Invitrogen) and detected using a colorimetric assay.

G6PDH assay

G6PDH activity was measured by following the increase in absorbance at 340 nm on a HP 8453 diode array spectrophotometer, equipped with a thermostated cuvette holder. The reaction mixture was allowed to reach the desired temperature, and the reaction was then initiated by injecting the cofactor. The standard assay (total volume, 750 µl) contained 120 mM NaH_2PO_4/HCl, 0.5 M NaCl, 1.6 mM glucose-6-phosphate, 1.6 mM NADP, pH 7.0, and an appropriate amount of enzyme. The enzyme activity was determined from the initial velocity of the reaction. G6P, NAD, and NADP were confirmed as being stable at temperatures up to 85°C for at least the time period of the assay by variable temperature NMR studies. All pH values are room temperature values unless otherwise stated. For the determination of the Michaelis–Menten constants, initial rate measurements were performed by varying substrate concentrations of G6P and NAD/NADP. The concentration of the fixed substrate was always kept saturating. Data from these measurements were plotted as Eadie–Hofstee plots.

Protein concentrations were determined by the BCA Protein Assay kit (Pierce, Rockford, IL, USA) using bovine serum albumin as a standard. N-terminal amino acid sequencing of the protein was performed by immobilizing the pure enzyme on a polyvinylidene fluoride (PVDF) membrane and subjecting it to 25 cycles of Edman degradation. The phenylthiohydantoin derivatives were identified by HPLC. The sequencing analysis was performed at the Macromolecular Structure Analysis Facility at the University of Kentucky.

Determination of optimum pH and temperature

The optimal pH of the enzyme was determined at 70°C by performing the assay using phosphate buffer in the pH range 3.1–9.7 (corresponding to pH 3–10.5 at 25°C). The optimal temperature was determined by performing the standard assay at temperatures from 40° to 105°C in 120 mM NaH_2PO_4/HCl, 0.5 M NaCl, pH 7.0 (ΔpK_a/°C = –0.0028). Half-lives (time at which activity was reduced to 50% of the original) were determined at 70°, 80°, 90°, and 100°C in 120 mM Tris/HCl, 0.5 M NaCl, pH 7.0. For a given temperature, samples were taken at different time intervals, cooled in an ice bath, and residual activity was determined by the standard assay at 70°C.

Tryptic digestion and mass spectrometry

Purified G6PDH was resolved by SDS-PAGE, and the gels were Coomassie-stained. The gel bands of interest were excised with a razor blade, placed in an Eppendorf tube, and destained by washing sequentially for 30 min with each of the following solutions: (1) 200 mM ammonium bicarbonate, (2) 50% methanol/10% acetic acid, and (3) 40% v/v ethanol. The three washing steps were repeated until the gel bands were clear. In-gel tryptic digestion of the bands was performed by adding a solution of 20 mM ammonium bicarbonate containing 0.03 µg/µl sequencing-grade trypsin and incubated at 37°C for 4 h. Mass spectrometric analysis was carried out on a MALDI-FTMS instrument at the University of Kentucky Mass Spectrometry Facility.

Results

Cloning, expression, and purification of G6PDH from *Aquifex aeolicus*

The genome sequence of *A. aeolicus* revealed the presence of a 1,293-bp open reading frame, which was putatively assigned as the *gsdA* gene encoding G6PDH (Deckert et al. 1998). Upstream of the gene, the consensus promoter sequence $TTTA^{A}/_{T}A$ was found. The *gsdA* gene was amplified by the PCR method, and the PCR product was ligated directly into pBAD-TOPO under the control of the P_{BAD} promoter of the *araBAD* operon. The expression vector was designed to incorporate the V5 epitope along with a polyhistidine tag at the C-terminus of G6PDH. The resulting plasmid, pG6PDH, was sequenced, and there was no difference in the nucleotide sequence found in comparison to the 1,293-bp open reading frame that was putatively assigned as coding G6PDH by Deckert et al. (1998). The plasmid was transformed into *E. coli* LMG194, grown until the OD_{600} reached 0.8, and induced by adding L(+) arabinose. The G6PDH fusion protein could be purified to homogeneity by a two-step purification procedure consisting of heating for 5 min at 80°C to denature the majority of the *E. coli* proteins, followed by affinity chromatography on a HiTrap chelating column. The protein was estimated to be at least 95% pure from SDS-PAGE analysis and was used in all subsequent studies.

Sequence analysis of G6PDH from *Aquifex aeolicus*

The pure enzyme was immobilized on a PVDF membrane and subjected to amino-terminal sequencing. The sequencing studies resulted in an unambiguous amino-terminal sequence of MSSGDTELRK. This sequence corresponds to the first ten amino acids coded by the *gsdA* gene. The deduced amino acid sequence of the *A. aeolicus gsdA* gene was compared with sequences deposited in the GenBank nonredundant database using the BLAST program. The sequence showed a 32% identity to other known G6PDHs, including the *L. mesenteroides* G6PDH. Multiple alignments of *A. aeolicus* G6PDH, with other known G6PDH sequences from different eukaryotes and prokaryotes, were analyzed with the algorithm PILEUP (Feng and Doolittle 1987). The *A. aeolicus* G6PDH shows a strong sequence similarity, especially to the active site amino acid residues Asp177, His178, and His240 of *L. mesenteroides* G6PDH (Cosgrove et al. 1998, 2000) (Fig. 1). The substrate-binding pocket, which contained the eight-amino-acid sequence RIDHYLGK, is conserved in the *A. aeolicus* G6PDH (Rowland et al. 1994). Presently, there is one other

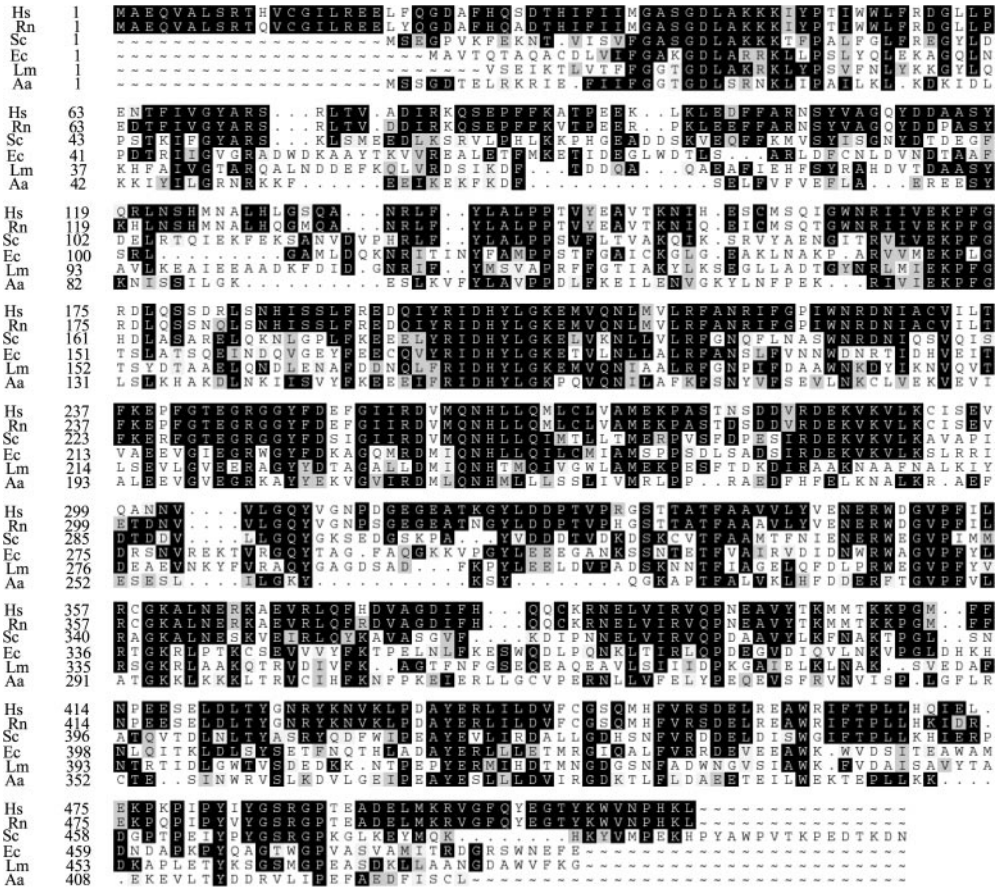

Fig. 1. Alignment of *Aquifex aeolicus* glucose-6-phosphate dehydrogenase (G6PDH) with other known G6PDHs. Sequences were deduced from Aa, *A. aeolicus gsdA*; Rn, *Rattus norvegicus* (NCBI accession number P05370); Ec, *Escherichia coli* (NCBI accession number P22992); Sc, *Saccharomyces cerevisiae* (NCBI accession number NP014158); Ln, *Leuconostoc mesenteroides* (NCBI accession number P11411), Hs, *Homo sapiens* (NCBI accession number NP000393). Different sections of the alignment are shown; the residue numbers for the individual sequences are given at the beginning of each line. The identical and homologous amino acid residues are indicated as *black* and *shaded* backgrounds, respectively

report of a G6PDH from the thermophilic bacterium *B. stearothermophilus* (Okuno et al. 1985); however, its amino acid sequence has not been determined and hence was not used for comparison.

Physical properties of G6PDH

SDS-PAGE analysis of the purified protein (reducing conditions) revealed the presence of a band of approximately 55 kDa, corresponding to the calculated molecular mass of the *gsdA* gene product fused to the V5 and polyhistidine peptides (Fig. 2, lane 4). Western blot analysis confirmed the presence of the V5 epitope in the ~55-kDa band (data not shown). However, SDS-PAGE analysis under nonreducing conditions revealed the presence of two bands corresponding to molecular weights of ~55 kDa and ~120 kDa. To determine whether the 120-kDa band was the result of undissociated *A. aeolicus* G6PDH dimer or a host (*E. coli*) protein, in-gel tryptic digestion of the two bands was performed. Mass spectrometric (MS) analysis of the trypsin-digested peptides showed a similar peak pattern for both bands. The peak pattern was also verified using the computer software GPMAW32, which calculates the theoretical molecular weights of tryptic peptides (Wass 1999). Assuming complete digestion of the protein, all the major calculated molecular weights by GPMAW32 were observed in both mass spectra. On the basis of the MS and SDS-PAGE analysis, we concluded that this enzyme is a homodimer. The native protein was further confirmed to be a dimer by performing gel filtration chromatography and comparing the retention volume of the protein to those of protein standards.

Optimal catalytic conditions and thermostability

The recombinant enzyme had a very broad pH range within which it demonstrated high catalytic activity, with the optimal pH being 6.6 ± 0.2 at 70°C (Fig. 3). Addition of EDTA (2 mM) did not affect G6PDH activity, indicating that divalent cations were not required. It should be noted that metal ions are also not essential for the activity of *L. mesenteroides* G6PDH (Cosgrove et al. 1998). Addition of DTT (100 mM) and 2-mercaptoethanol (10 mM) to the buffers did not result in any difference in activity. Addition of 0.1% (w/v) SDS inactivated G6PDH completely. The optimum temperature of the purified enzyme in 120 mM NaH_2PO_4/HCl, 0.5 M NaCl, pH 7.0 buffer was 90°C (Fig. 4); this is near the physiological optimum growth temperature (85°C) of the organism (Deckert et al. 1998). An Arrhenius plot of the data (inset, Fig. 4) was linear over the 40°–90°C range, indicating

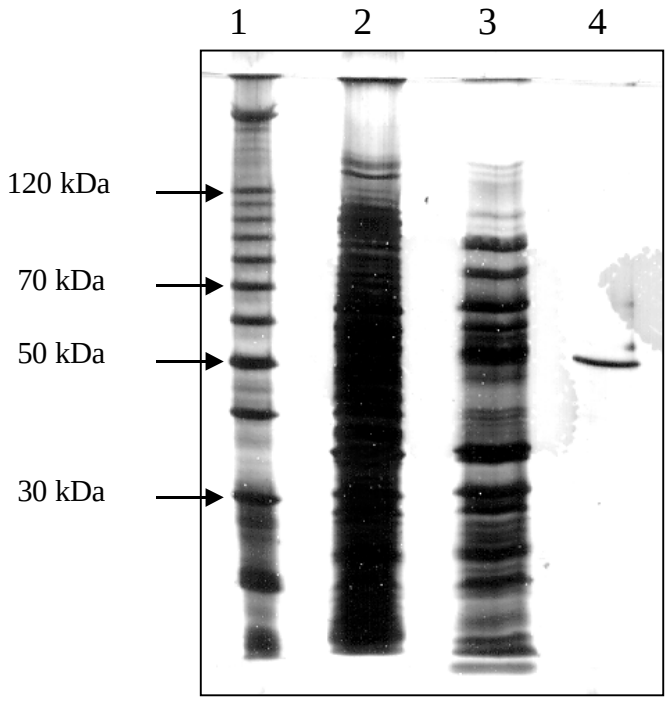

Fig. 2. SDS gel electropherogram of fractions from purification steps of G6PDH from *A. aeolicus*. Separation was performed on a 12.5% SDS gel and silver-stained. *Lane 1*, protein ladder; *lane 2*, crude extract; *lane 3*, crude extract after incubation for 5 min at 80°C; *lane 4*, purified fraction after affinity chromatography

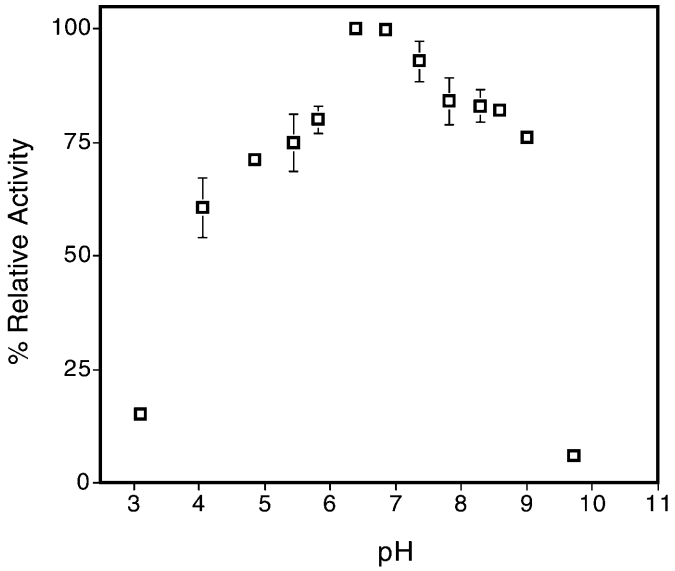

Fig. 3. Influence of pH on the activity of the purified G6PDH from *A. aeolicus*. Enzyme activity was measured in phosphate buffer (*x*-axis pH values are at 70°C). Each assay was performed with 20 µg protein/ml. *Error bars*, standard deviation (n = 3)

a single rate-limiting step with an activation energy of 58.9 kJ/mol. Tris buffer was not suited for the determination of the temperature optimum because of the strong temperature dependence of its pK_a (ΔpK_a/°C = –0.031).

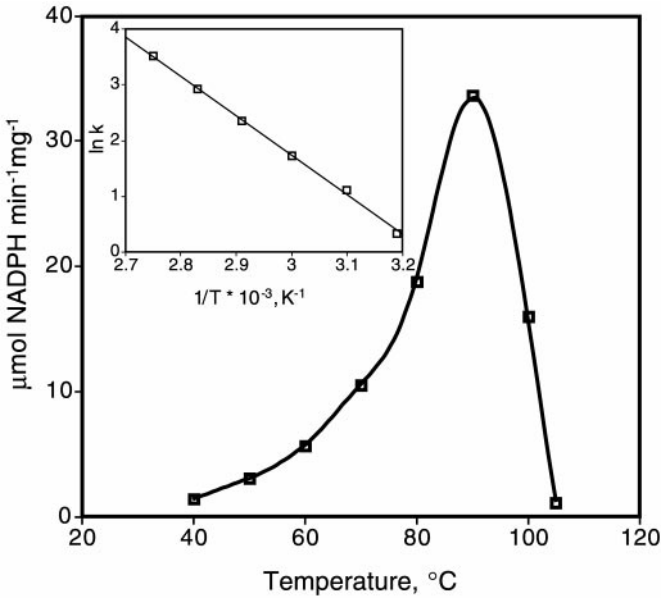

Fig. 4. Optimum temperature for G6PDH activity from *A. aeolicus*. Activity was determined in 120 mM NaH_2PO_4/HCl, 0.5 M NaCl, pH 7.0, and expressed as µmol NADPH min^{-1} mg^{-1}. *Inset*: Arrhenius plot of the data in the 40°–90°C range. Enzyme concentration, 20 µg/ml

To analyze the effect of temperature on enzyme denaturation, aliquots of enzyme were subjected to 15-min incubation from 50° to 100°C, followed by cooling of the enzyme and monitoring G6PDH activity at 50°C (Fig. 5). The enzyme retained 98% activity at 65°C. A similar experiment performed on G6PDH from *B. stearothermophilus* showed 60% retention of activity at 65°C (Okuno et al. 1985). The half-life of the *A. aeolicus* G6PDH was determined at 70°, 80°, 90°, and 100°C in 120 mM Tris/HCl, 0.5 M NaCl, pH 7.0. The enzyme showed remarkable resistance to heat inactivation, with a half-life of 38 h at 80°C (Table 1). The enzyme showed lower resistance to heat inactivation ($t_{1/2}$ = 4 h at 80°C) when incubated in 120 mM NaH_2PO_4/HCl, 0.5 M NaCl, pH 7.0 instead of Tris-HCl. The increase in thermostability in the presence of Tris has also been observed previously for β-glucosidase from *Pyrococcus furiosus* and was attributed to Tris-mimicking cytoplasmic components that may be involved in thermostability (Kengen et al. 1993).

Kinetic properties of recombinant G6PDH

The rate dependence on the substrate concentration followed Michaelis–Menten kinetics. The kinetic constants K_M and V_{max} were obtained at 40° and 70°C from Eadie–Hofstee representation of the data obtained (Table 2). *A. aeolicus* G6PDH showed a dual coenzyme specificity with a preference in k_{cat}/K_M of 20.4 fold for NADP over NAD at 40°C and 5.7 fold at 70°C. The K_M values of *A. aeolicus* G6PDH showed a 3- and 4-fold increase when the temperature was raised from 40° to 70°C for the NAD- and NADP-linked reactions, respectively.

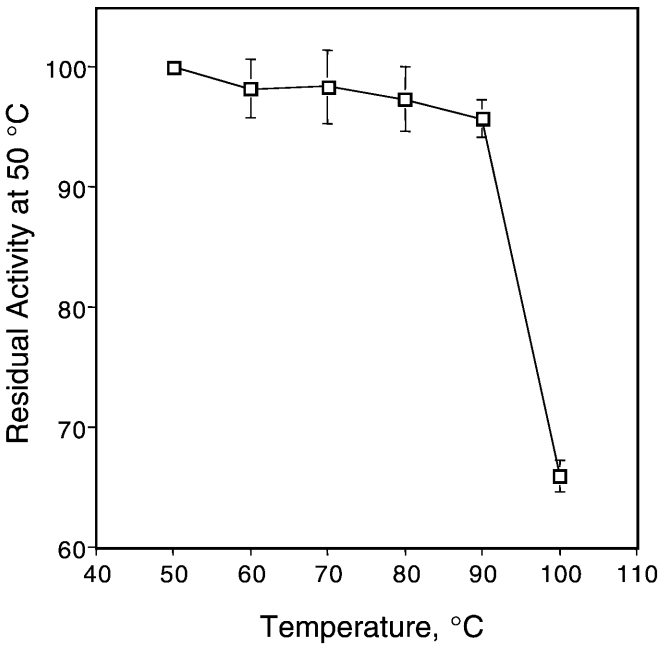

Fig. 5. Effect of temperature on the activity of G6PDH from *A. aeolicus*. Activity was determined at 50°C in 120 mM NaH_2PO_4/HCl, 0.5 M NaCl, pH 7.0 after a 15-min incubation at the desired temperature. *Error bars*, standard deviation (n = 3)

Table 1. Thermostability of *Aquifex aeolicus* glucose-6-phosphate dehydrogenase (G6PDH)[a]

Incubation temperature (°C)	Half-life (h) of G6PDH
70	45
80	38
90	24
100	12

Enzyme concentration was 20 µg/ml
Half-lives were deduced from respective semilog plots
[a] Incubation buffer was 120 mM Tris/HCl, 0.5 M NaCl, pH 7.0

Discussion

In this article, we describe the cloning and expression of the *gsdA* gene, which encodes a thermostable glucose-6-phosphate dehydrogenase from *A. aeolicus*, as a fusion protein in *E. coli*. The optimal temperature of the *A. aeolicus* G6PDH is 90°C. The enzyme has a half-life of 38 h at 80°C, making it the most thermostable G6PDH ever reported. The *A. aeolicus* G6PDH sequence alignment showed a strong similarity to the active site amino acid residues of *L. mesenteroides* G6PDH (Cosgrove et al. 1998). Of particular significance was the substrate-binding pocket, which contained the eight-amino-acid sequence, RIDHYLGK, that is also conserved in most of the G6PDHs sequenced to date (Rowland et al. 1994). A model for the catalytic mechanism was proposed for the *L. mesenteroides* G6PDH in which His240 acts as the general base that abstracts the proton from the C1-hydroxyl group of G6P, and the carboxylate

Table 2. Kinetic properties of *A. aeolicus* G6PDH (kinetic constants ± SD; n = 3)

Temperature	$K_{M,\ G6P}$ (µM)	K_M (µM)	Specific activity[a] (µmol min^{-1} mg^{-1})	k_{cat}/K_M[b] (μM^{-1})
NADP-linked reaction				
40°C	15 ± 4	9 ± 1	0.97 ± 0.03	5.11 ± 0.61
70°C	63 ± 15	161 ± 40	18.9 ± 2.2	5.55 ± 1.53
NAD-linked reaction				
40°C	58 ± 15	230 ± 32	1.24 ± 0.06	0.25 ± 0.03
70°C	180 ± 23	2,096 ± 390	42.7 ± 4.0	0.96 ± 0.20

G6P, glucose-6-phosphate
[a] µmol NAD(P)H min^{-1} mg^{-1} at the respective temperatures
[b] Per mole of enzyme subunit

group of Asp177 stabilizes the positive charge that forms on His240 in the transition state. His178 was shown to bind to the phosphate moiety of G6P (Cosgrove et al. 1998). Because all the amino acids discussed here are conserved in 28 other known G6PDH enzymes sequenced to date, including the one from *A. aeolicus*, it is likely that the same mechanism is taking place in the *A. aeolicus* G6PDH.

Aquifex aeolicus G6PDH was shown to be a homodimer with a subunit molecular weight of 55 kDa. The three-dimensional structure of the *A. aeolicus* G6PDH monomer was generated, using the *L. mesenteroides* G6PDH as the template, by submitting the amino acid sequence of *A. aeolicus* G6PDH to the SWISS-MODEL Automated Protein Modeling Server (Geneva, Switzerland) (Guex and Peitsch 1997; Peitsch 1995, 1996). The resulting monomer structure revealed possible intersubunit disulfide bonds between cysteines184 and 352 of the two subunits. These disulfide bonds may help preserve the structural integrity of the protein, thereby contributing to its thermostability. Interestingly, crystal structures of other G6PDHs from mesophilic sources, such as human and *L. mesenteroides*, did not show the presence of intersubunit disulfide bonds (Rowland et al. 1994; Au et al. 2000). It should be noted that to fully dissociate the two subunits it was necessary to have a reducing agent, DTT, in addition to SDS (Fig. 2).

The *A. aeolicus* G6PDH showed some similarity in the kinetic constants to its counterparts from *L. mesenteroides* and *B. stearothermophilus* (Lee and Levy 1992; Okuno et al. 1985; Olive and Levy 1967). G6PDHs from all three microorganisms exhibit dual specificity toward the nicotinamide-adenine dinucleotide coenzymes and show a preference for NADP over NAD as the cofactor. The *A. aeolicus* G6PDH showed a preference in k_{cat}/K_M of 5.7-fold at 70°C. The *B. stearothermophilus* and the *L. mesenteroides* G6PDH showed a 100- and 20-fold lower K_M for NADP over NAD, respectively. One aspect of metabolism that was found to be very interesting was that the dual coenzyme specificity of *L. mesenteroides* comes from the fact that this organism has an incomplete glycolytic pathway in which it uses either NADP or NAD depending on the demands for catabolic or anabolic metabolism (Rowland et al. 1994). Somewhat consistent to that, the genome of *A. aeolicus* did not show the presence of genes encoding fructose-1,6-bisphosphatase (FBPase), an essential enzyme of the gluconeogenetic

pathway (Deckert et al. 1998). An exhaustive literature search for glycolytic and gluconeogenetic enzymes in *B. stearothermophilus* also did not show the presence of FBPase. The absence of this enzyme still needs to be verified after the *B. stearothermophilus* genome sequence has been completed (sequencing of the genome is currently underway at the Advanced Center for Genome Technology, University of Oklahoma). The related microorganism *Bacillus subtilis* showed the presence of a FBPase; however, the genome encoded a G6PDH that did not exhibit dual coenzyme specificity (Fujita et al. 1998; Tanahashi et al. 1976; Ujita and Kimura 1975).

In conclusion, the gene encoding G6PDH from *A. aeolicus* was successfully cloned and expressed in *E. coli*. The recombinant enzyme exhibited dual coenzyme specificity and excellent thermostability, which makes it a robust catalyst suitable for use in biotransformation, biosensing, and enzyme-linked immunoassay systems.

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