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Anaerobic glycerol-3-phosphate dehydrogenase complex from hyperthermophilic archaeon *Thermococcus kodakarensis* KOD1

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Glycerol-3-phosphate (G3P) is a key intermediate of glycerol metabolism and is oxidized to dihydroxyacetone phosphate aerobically or anaerobically by appropriate G3P dehydrogenases. A hyperthermophilic archaeon *Thermococcus kodakarensis* KOD1 has a novel operon consisting of three genes encoding an anaerobic G3P dehydrogenase (G3PDH), an NADH oxidase (NOX), and a molybdopterin oxidoreductase (MOX). Typically, the G3PDH gene (*glpA*) is included in an operon with genes encoding essential subunits of the G3PDH complex, *glpB* and *glpC*. The three genes from *T. kodakarensis* were cloned and expressed in *Escherichia coli*, and their recombinant proteins, Tk-G3PDH, Tk-NOX and Tk-MOX, were characterized. The optimal temperature of Tk-G3PDH for activity was 80°C, indicating high thermal stability. Tk-G3PDH has flavin adenine dinucleotide as a prosthetic group and catalyzes oxidation of G3P with k_{cat}/K_m 1.93×10^3 $M^{-1}s^{-1}$ at 80°C, compared with 9.83×10^5 $M^{-1}s^{-1}$ for the *E. coli* G3PDH complex at 37°C. Interestingly, Tk-G3PDH can catalyze this reaction even as a monomer, whereas GlpA must form a complex with GlpB and GlpC. Tk-G3PDH also forms a putative heteropentamer with Tk-NOX and Tk-MOX (G3PDH:NOX:MOX = 2:2:1). This complex may form an electron transfer pathway to a final electron acceptor in the cell membrane, as is the case for the typical G3PDH complex GlpABC.

[**Key words:** Glycerol-3-phosphate dehydrogenase; GlpA; Archaea; Glycerol metabolism; NADH oxidase; Molybdopterin oxidoreductase; Operon; Complex]

Glycerol is a carbon and energy source for *Escherichia coli* (1) and the glycerol metabolic pathway is conserved in most organisms. As a central step of glycerol metabolism, glycerol is converted to dihydroxyacetone phosphate by phosphorylation and oxidation. Glycerol-3-phosphate (G3P), which is produced by phosphorylation of glycerol, is a key product of this metabolic pathway. G3P is the backbone of phospholipids used as membrane lipids, and also a substrate (electron donor) of the respiratory chain. In glycerol dissimilation, electrons from G3P oxidation are transported through the inner membrane to fumarate, nitrate or oxygen as a final electron accepter. In *E. coli*, this oxidation reaction is catalyzed by two different glycerol-3-phosphate dehydrogenases (G3PDH), an aerobic G3PDH encoded by *glpD*, and an anaerobic G3PDH complex consisting of three subunits encoded by the *glpABC* operon (subunits named GlpA, GlpB and GlpC). GlpA and GlpB are flavoenzymes that bind flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), respectively. GlpA is thought to be the subunit that performs G3P oxidation, and both GlpB and GlpC form a complex with GlpA to form an electron transfer pathway to the inner membrane. The bacterial-type anaerobic G3PDH (GlpABC complex) is also found in the archaeon *Halobacterium volcanii*, where the *glpABC* operon is induced by G3P and the GlpABC complex functions in respiratory oxidation of G3P (2,3).

Thermococcus kodakarensis KOD1 is a hyperthermophilic archaeon isolated from a solfatara of Kodakara Island, Kagoshima, Japan (4), which grows optimally at 85°C (5). The complete genome sequence was determined by Fukui et al. (6) and 2306 coding DNA sequences (CDSs) were annotated in the genome. Since the number of CDSs of this archaeon is less than half that of *E. coli*, *T. kodakarensis* is thought to have a minimal set of genes for survival in its favored environment. Some unique metabolic pathways, such as a modified Embden-Meyerhof pathway for glycolysis (7,8) and a pentose bisphosphate pathway (9), were found in *T. kodakarensis*.

Glycerol metabolism in archaea is interesting because glycerol is both a possible carbon source and it is connected to synthesis of phospholipids (10) and compatible solute glycerol phosphodiesters (11). Genes on *T. kodakarensis*, Tk1396, Tk-1397 and Tk1398, are encoding glycerol kinase and two glycerophosphoryl diester phosphodiesterases, those are key enzymes of glycerol 3-phosphate metabolism and forming a gene cluster (12). In our previous study, a gene Tk1396, Tk*glpK*, was cloned and the encoded protein, glycerol kinase (Tk-GK), was characterized (12). Glycerol kinase catalyzes phosphorylation of glycerol to produce G3P. According to biochemical and structural analysis of Tk-GK, it has a unique hexameric structure, while GK from *E. coli* forms a tetramer. Tk-GK is not feedback inhibited by the glycolysis intermediate fructose 1,6-bisphosphate, which is an inhibitor of *E. coli* glycerol kinase (12,13). About 3.3 kbp upstream from Tk1396, there are another genes encoding enzyme related to glycerol metabolism, Tk1391, Tk1392 and Tk1393.

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T. kodakarensis gene Tk1393 has been found to be a putative *glpA*. Although there are no orthologous genes of *glpB* and *glpC* in the *T. kodakarensis* genome, Tk1393 has two contiguous genes, Tk1391 and Tk1392, encoding a molybdopterin oxidoreductase (MOX) and an NADH oxidase (NOX), respectively. In this study, genes Tk1391, Tk1392 and Tk1393 were cloned and their encoded proteins, Tk-G3PDH, Tk-MOX and Tk-NOX, were characterized.

MATERIALS AND METHODS

Materials Reagents used for activity measurement of G3PDH were purchased from Wako Pure Chemical Corporation (Osaka, Japan) and Sigma–Aldrich (St. Louis, MO, USA). The *E. coli* host strain for gene cloning, JM109, and cloning vector pUC18 were purchased from Takara Bio Inc. (Kusatsu, Japan), and heterologous expression host strain *E. coli* BL21(DE3) and expression vector pET25b were purchased from Merck (Darmstadt, Germany). Enzymes and reagents for gene cloning were purchased from Takara Bio Inc.

Plasmid construction The *glpA* gene from *T. kodakarensis* (Tk1393) and its contiguous genes, Tk1391 and Tk1392, were amplified from the chromosome of *T. kodakarensis* by PCR using primers shown in Table S1. Primer pairs 1391F and 1391R, 1392F and 1392R, and 1393F and 1393R, were used for amplification of Tk1391, Tk1392 and Tk1393, respectively. PCR was carried out with a GeneAmp PCR system 2400 (GE Healthcare, Chicago, IL, USA) using KOD polymerase (Toyobo, Kyoto, Japan). The amplified fragments were ligated into pET25b between *Nco*I and *Xho*I sites and the resultant plasmids were transformed into *E. coli* JM109 and BL21(DE3).

Operon analysis of contiguous genes of Tk1393 *T. kodakarensis* was cultivated in anaerobic conditions at 85°C in nutrient rich artificial sea water medium (14) containing yeast extract, tryptophan and elemental sulfur (S^0) for 12 h mRNA was extracted from the *T. kodakarensis* cells with an RNeasy Midi Kit (Qiagen, Hilden, Germany) and contaminating DNA was digested with DNase I. The DNA sequences between contiguous genes Tk1391 and Tk1392, and Tk1392 and Tk1393, were amplified from the extracted mRNA by reverse transcription-PCR with primer sets indicated in Table S1.

Heterologous protein expression and purification Putative G3PDH, MOX and NOX from *T. kodakarensis* were overexpressed in *E. coli* BL21(DE3) harboring expression plasmids pET-Tk1393, pET-Tk1391 and pET-Tk1392, respectively. Each recombinant *E. coli* strain was cultivated in 1 L of Lysogeny Broth at 37°C. Isopropyl β-D-1-thiogalactopyranoside was added to the culture to 1 mM (final concentration) when the optical density at 660 nm reached 0.6 to induce expression of heterologous protein. After 8 h of cultivation, the cells were harvested by centrifugation at 6000 × *g* for 10 min, suspended in 50 mM Tris–HCl (pH 7.5), disrupted by sonication, and centrifuged at 15,000 × *g* for 30 min. The supernatant containing recombinant proteins was incubated at 70°C for 30 min and centrifuged at 15,000 × *g* for 30 min. The supernatants were applied to a HiTrap Q HP (GE Healthcare) column and eluted by linearly increasing the NaCl concentration from 0 to 1 M in 50 mM Tris–HCl buffer, pH 7.5. The target protein was detected by SDS–PAGE and the fractions containing the target protein were dialyzed into 50 mM Tris–HCl, pH 7.5, 100 mM NaCl. The protein sample was applied to size exclusion chromatography (SEC) using HiLoad Superdex 200 pg (GE Healthcare). Elution was monitored by UV absorption and the target protein was detected by SDS–PAGE. Fractions containing the target protein were gathered as purified proteins. Purity of proteins was confirmed by SDS–PAGE followed by Coomassie Brilliant Blue staining. The protein concentration was determined from the UV absorption at 280 nm on the basis that the absorbance of a 0.1% (1 mg mL^{-1}) solution at 280 nm is 1.302 for Tk-G3PDH, 0.859 for Tk-NOX, and 1.033 for Tk-MOX.

G3P titration Purified Tk-G3PDH solution was diluted to 1 mg mL^{-1} and left in an anaerobic atmosphere for 4 h to remove oxygen from the solution. The solution was transferred to a capped quartz cell. G3P (500 mM) was added and the absorbance spectrum from 300 to 700 nm was measured every min until 15 min.

Activity assay of G3PDH and NADH oxidase G3PDH activity was determined by measuring the rate of reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan dye coupled with G3P oxidation (15). The assay was performed at 40–85°C in 100 mM glycine-NaOH buffer (pH 10), containing 150 mM NaCl, 1 mM MTT, 5 mM G3P and 0.2 mM FAD. Tk-G3PDH (2.5 μg) was added to 500 μL of assay solution to start the reaction, which was stopped by adding 500 μL of ice-cold 2-propanol after 5 min. The amount of formazan dye was measured by absorption at 565 nm (molar absorbance = 20,000 L mol^{-1} cm^{-1}). For activity assay of G3PDH complexes, 100 mM Tris–HCl (pH 7.5) buffer was used instead of 100 mM glycine-NaOH (pH 10) buffer. pH dependence of G3PDH activity was determined in the range pH 7.0 to 10.5 in appropriate buffers (Tris–HCl for pH 7.0 to 8.5, glycine-NaOH for pH 8.5 to 10.5). One unit of enzymatic activity was defined as the amount of enzyme that produced 1 μmole of G3P in 1 min.

NADH oxidase activity was determined by measuring the amount of NADH by absorption at 340 nm. Activity assay was performed at 60°C in 50 mM Tris–HCl buffer (pH 7.5) containing 0.4 mM NADH and 0.1 mM FAD.

Gel filtration analysis of putative Tk-G3PDH complexes Purified Tk-G3PDH, Tk-MOX and Tk-NOX were dialyzed into 50 mM Tris–HCl, pH 7.5, containing 100 mM NaCl and individual solutions or mixed solutions of all the possible combinations of the three proteins were prepared. The concentration of each protein was adjusted to 83 nM in the individual or mixed solutions. The protein solutions were incubated for 30 min at 37°C, filtered to remove precipitant, and 100 μL of each protein solution was applied to high-performance liquid chromatography–SEC analysis using a TSKgel G2000SW_{XL} column (Tosoh, Tokyo, Japan). Eluted proteins were detected by absorption at 280 nm and fractions were collected every 1 min. The molecular weight of proteins in solution was determined from the retention time and a standard curve prepared with Gel Filtration Standard (Bio-Rad, Hercules, CA, USA). Eluted proteins in the fractions were precipitated by adding 60 μL of trichloroacetic acid (TCA), stored on ice for 10 min, and centrifuged at 15,000 × *g* for 10 min. The precipitant was washed with 1 mL of 70% acetone, dried by vacuum, and dissolved in 20 μL of 1× sodium dodecyl sulfate (SDS) sample buffer (62.5 μM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 0.005% bromophenol blue, and 0.1 M 2-mercaptoethanol); 10 μL aliquots were applied for SDS–PAGE analysis. The amount of protein in each fraction was quantified from the gel using ImageJ software (16). Different amounts (from 0.25 to 3.0 μg) of standard Tk-G3PDH, Tk-NOX and Tk-MOX were run on the same gel as a quantification standard. The blank part on the gel image between Tk-NOX band and Tk-MOX band was cut out along with horizontal line and band of three proteins are aligned not to make gap on Fig. 1H.

RESULTS AND DISCUSSION

Operon analysis In the *T. kodakarensis* genome (6), an open reading frame, Tk1393, was annotated as a possible *glpA* gene encoding G3PDH. The 5′- end of a contiguous gene, Tk1392, is 23 base pairs downstream from the 3′- end of Tk1393. Four base pairs of the 3′-end of Tk1392 overlap with the 5′-end of the next gene, Tk1391. Tk1394 is located 160 base pairs upstream of Tk1393. mRNAs were extracted from anaerobically cultivated *T. kodakarensis*, and cDNAs were synthesized by reverse transcription from the mRNA. In addition, genomic DNA from *T. kodakarensis* was used as the template for PCR amplification of partial sequences of adjacent genes, such as Tk1391–Tk1392 and Tk1392–Tk1393 (Fig. S1). The results indicate that Tk1391, Tk1392 and Tk1393 were transcribed as a single mRNA. Putative Shine-Dalgarno sequences, (T)GGAG(G), were found located 10 to 20 base pairs upstream from the initiation codon of each gene by searching homologous 3′end sequence of 16S rRNA, CCACU (17). Tk1391, Tk1392 and Tk1393 are located in an operon.

In the *T. kodakarensis* genome database, Tk1392 and Tk1391 are annotated as NOX and MOX encoding genes, respectively. Rashid and co-workers reported that the protein encoded by Tk1392 has FAD-dependent NADH oxidase activity (18). MOX has a 4Fe–4S cluster binding domain found in some oxidoreductases. According to the SOSUI database (http://harrier.nagahama-i-bio.ac.jp/sosui/), 17 amino acid residues from the N-terminus of MOX were predicted to form a transmembrane region. In *E. coli.*, GlpA and GlpC subunits respectively require FAD and FMN as prosthetic groups for their activity. *E. coli* GlpC has a membrane anchoring region and a 4Fe–4S cluster, indicating that this subunit has a role in transferring electrons to the inner membrane (19). The characteristics of the genes contiguous to *glpA* may be common in different organisms.

In bacteria and some halophilic archaea, the anaerobic G3PDH forms a heterotrimeric complex consisting of the essential subunits for glycerol respiration, GlpABC, and the corresponding genes form an operon. *Haloferax volcanii* is known to utilize glycerol as carbon source and it has an operon consist of *glpA, glpB* and *glpC* under the control of G3P inducible promoter (2,3). However, *T. kodakarensis* cannot grow on glycerol as a carbon source (data not shown) and Tk1393 encoding G3PDH in *T. kodakarensis* is in an operon with genes encoding NOX and MOX instead of GlpB and GlpC. Thus, Tk-

G3PDH may have different physiological role and may form a complex for glycerol 3-phosphate oxidation reaction with NOX and MOX.

Twelve representative bacteria and 30 archaeal species from 13 different orders for which complete genome sequences are available (20) were selected for analysis of *glpA* and flanking genes. The DNA sequence of Tk1393 was used for BLAST analysis against selected genome sequences; 15 homologous G3PDH genes were identified from archaeal genomes and 10 from bacterial genomes (Table 1) (21). There was, however, no G3PDH homolog in 15 archaea belonging to the Thermoproteales, Desulfurococcales, Nanoarchaea, or methanogenic archaea. Among the 25 homologous genes, 15 genes are adjacent to genes encoding enzymes or subunits related to redox reactions. Fourteen of these 15 genes form a consecutive three gene structure with related enzymes and/or subunits. Some such genes were identified as encoding G3PDH subunits GlpB and GlpC. However, *T. kodakarensis* contains a gene cluster with Tk1391 and Tk1392 encoding MOX and NOX instead of *glpB* and *glpC*, and similar gene clusters were found in other archaea belonging to the Thermococcalaes and also in two bacteria belonging to Clostridiales and Thermotogales.

Oligomeric structure analysis of Tk-G3PDH, Tk-NOX and Tk-MOX complexes Putative G3PDH, Tk-G3PDH, encoded by Tk1393, was overexpressed in *E. coli* BL21(DE3) and 6.0 mg of protein was purified from 1 L of culture. Tk-NOX and Tk-MOX were also overexpressed in *E. coli* BL21(DE3); 2.1 mg of Tk-NOX and 2.5 mg of Tk-MOX were purified per liter of culture.

Each protein was applied to SEC analysis as an individual protein, and in combinations of two or three proteins. Tk-MOX, Tk-NOX and Tk-G3PDH eluted from a TSKgel G2000SW$_{XL}$ column as major peaks with retention times of 16.3, 18.9 and 16.5 min, respectively

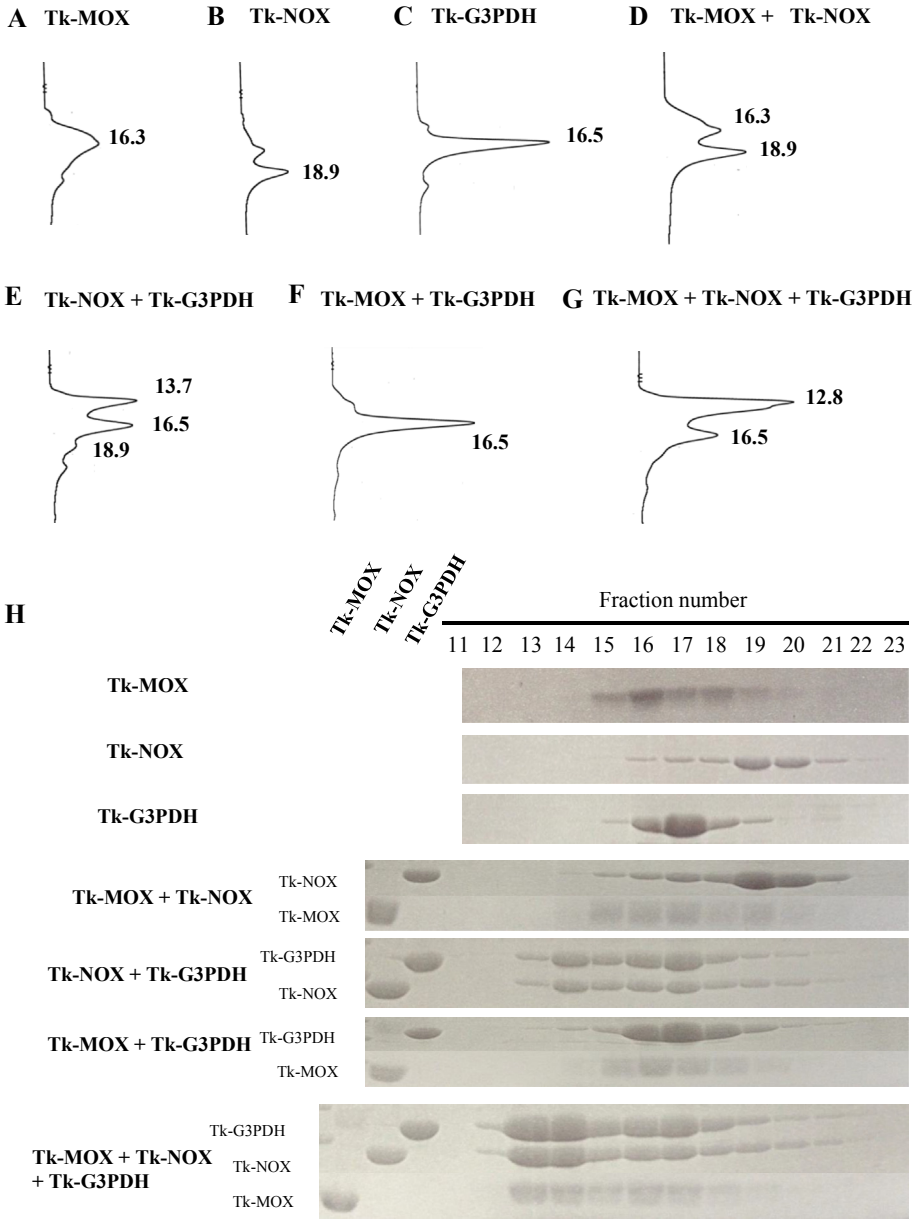
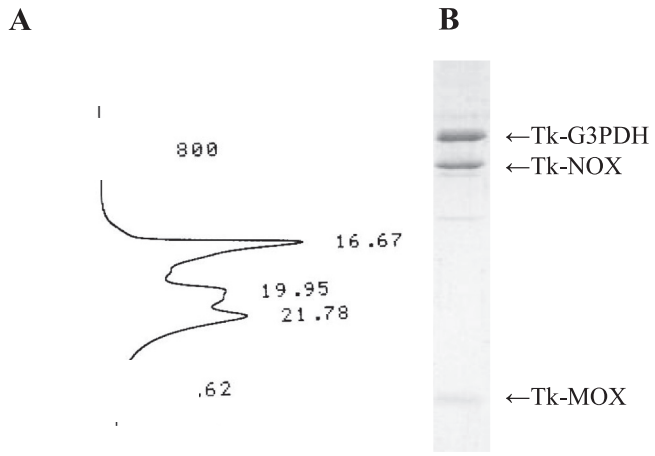


FIG. 1. Gel filtration profile of proteins and protein complexes. (A) Tk-MOX; (B) Tk-NOX; (C) Tk-G3PDH; (D) Tk-MOX and Tk-NOX; (E) Tk-NOX and Tk-G3PDH; (F) Tk-MOX and Tk-G3PDH; (G) Tk-MOX, Tk-NOX and Tk-G3PDH. The peak eluted at 13.71 min represents a Tk-G3PDH–NOX complex, and the peak eluted at 12.75 min represents a Tk-G3PDH–NOX–MOX complex. A TSKgel G2000SW$_{XL}$ column was used in this assay. (H) SDS–PAGE analysis of the proteins eluted by gel filtration in panels A–G. The fraction number indicates elution time in each size exclusion column analysis.

TABLE 1. List of genes encoding anaerobic G3PDH and rerated enzyme in archaea and bacteria.

Domain	Order	Species	Gene name of G3PDH (identity %)	Contiguous genes encoding G3PDH related proteins (identity %) (protein name)	
Archea	Thermococcales	*Thermococcus kodakarensis* KOD1	Tk1393 (G3P dehydrogenase)	Tk1392 (NADH oxidase)	Tk1391 (Molybdopterin oxidoreductase, 4Fe−4S cluster binding subunit)
		Thermococcus gammatolerans	TGAM1791 (90.7)	TGAM1792 (89.4) (FAD-dependent pyridine nucleotide-disulphide oxidoreductase)	TGAM1793 (94.4)
			TGAM1023 (36.8)	TGAM1024 (40.6) (FAD-dependent pyridine nucleotide-disulphide oxidoreductase)	TGAM1025 (35.5)
		Pyrococcus abyssi	PAB0183 (80.5)	PAB 184 (72.5)	PAB183 (76.4)
		Pyrococcus furiosus	PF2005 (82.7)	PF2006 (76.3)	PF2007 (75.0)
		Pyrococcus yayanosii	PYCH18330 (84.5)	PYCH1840 (75.4)	PYCH1850 (77.8)
	Archaeoglobales	*Archaeoglobus fulgidus*	AF1328 (32.8)	–	–
	Halobacteriales	*Haloarcula marismortui*	rrnAC1955 (21.7)	rrnAC1954 (10.8) (oxidoreductase)	–
		Halobacterium salinum	OE3763F (22.0)	OE3764F (10.2) (G3PDH subunitB)	OE3765F (2.9) (G3PDH subunitC)
	Haloferacales	*Haloferax volcanii*	HVO1538 (23.8)	HVO1539 (14.2) (G3PDH subunitB)	HVO1540 (4.0) (G3PDH subunitC)
	Methanobacteriales	*Methanothermobacter thermautotrophicus*	MTH368 (14.5)	–	–
	Thermoplasmatales	*Picrophilus torridus*	PTO1486 (17.6)	–	–
		Thermoplasma acidophilum	Ta0633 (19.9)	–	–
		Thermoplasma volcanium	TVG0875243 (20.7)	–	–
	Sulfolobales	*Sulfolobus acidocaldarius*	Saci2032 (24.3)	–	–
		Sulfolobus solfataricus	SSO2526 (22.1)	–	–
Bacteria	Chloroflexales	*Chloroflexus aurantiacus*	Caur3291 (19.0)	Caur3290 (7.1) (G3PDH subunitB)	Caur3289 (3.0) (G3PDH subunitC)
	Chloroflexales	*Roseiflexus castenholzii*	Rcas4111 (20.9)	Rcas4112 (12.2) (G3PDH subunitB)	Rcas4113 (3.6) (G3PDH subunitC)
	Clostridiales	*Clostridium perfringens* str. 13	CPE2551 (36.8)	CPE2550 (39.7)	CPE2549 (36.2)
	Enterobacterales	*Escherichia coli*	JW2235 (23.0)	JW2236 (7.0) (G3PDH subunitB)	JW2237 (4.1) (G3PDH subunitC)
	Halanaerobiales	*Halothermothrix orenii*	Hore10550 (14.1)	–	–
	Lactobacillales	*Streptococcus pneumoniae*	SP2091 (13.7)	–	–
	Synechococcales	*Synechocystis* sp. PCC 6803	slr1755 (13.2)	–	–
	Thermotogales	*Thermotoga maritima*	Tm1432 (44.5)	Tm1433 (42.3)	Tm1434 (33.3)
	Thermotogales	*Thermotoga petrophila*	Tpet1362 (44.5)	Tpet1361 (41.7)	Tpet1360 (32.1)
	Thermotogales	*Fervidobacterium nodosum*	fnod0697 (43.6)	fnod0698 (40.5)	fnod0699 (40.6)

Taxonomic order of original host is indicated in first three columns and name and identity % of genes homologous to Tk1393, Tk1392 and Tk1391 are listed. The annotated protein name of each gene which is different from *Thermococcus kodakarensis* is also indicated in parenthesis.



C

	Tk-MOX	Tk-NOX	Tk-G3PDH
Amount (μg)	0.170	0.963	1.108
M.W. (Da)	12337	45247	55004
pmol	0.01378	0.02129	0.02014
Ratio	**1**	**2**	**2**

FIG. 2. Determination of the oligomerization state of protein complex. (A) Gel filtration profile of the mixture of Tk-G3PDH, Tk-NOX and Tk-MOX. A TSKgel G3000SW$_{XL}$ column was used in this assay. (B) Fractions 16–20, which contain the complex, resolved by SDS–PAGE. (C) The amount and molar ratio of Tk-G3PDH, Tk-NOX and Tk-MOX in the complex calculated from the calibration curves.

(Fig. 1). Considering the theoretical molecular weights of the proteins, this result indicates that Tk-NOX and Tk-G3PDH are monomeric, while Tk-NOX was reported as dimer in a previous report (22). And Tk-MOX exists as an oligomer of uncertain degree. The mixture of Tk-NOX and Tk-G3PDH gave an additional SEC peak at 13.7 min as well as peaks identical to those observed for the Tk-NOX and Tk-G3PDH monomers. The other two protein mixtures containing Tk-MOX (i.e., Tk-MOX + Tk-NOX and Tk-MOX + Tk-G3PDH) gave only elution peaks identical to those for the respective individual proteins. This indicates that, in these combinations, Tk-NOX and Tk-G3PDH form a complex in solution, while Tk-MOX does not. However, in the mixture of all three proteins, an additional peak appeared at 12.8 min, indicating that Tk-MOX bound to the complex of Tk-NOX and Tk-G3PDH (Fig. 1).

Each eluted peak was fractionated and applied to SDS–PAGE (Fig. 1H). In the mixture of Tk-NOX and Tk-G3PDH, both proteins were present in fractions collected from 13 to 20 min. In the three-protein mixture, all proteins were eluted in the peak centered on 12.8 min, indicating formation of a complex of all three proteins. This result indicates that Tk-MOX can form a complex only when both Tk-G3PDH and Tk-NOX are present. The formation of a Tk-G3PDH and Tk-NOX complex may precede formation of the three-protein complex.

To estimate the molar ratio of the three proteins in the complex, the mixture was applied to TSKgel G3000SW$_{XL}$ chromatography (instead of TSKgel G2000SW$_{XL}$) for better resolution (Fig. 2A). The peak identical to the complex, fractions from 16 to 19 min, was collected and the aliquot was precipitated with TCA. Precipitated proteins were dissolved with SDS–PAGE sample buffer and then applied to SDS–PAGE (Fig. 2B). As a quantification standard, various amounts (from 0.25 to 3.0 μg) of each of Tk-G3PDH, Tk-NOX and Tk-MOX were run on the same gel. The band patterns of the gel were scanned and band intensities of each protein were measured using Image J software. Quantification standard curves were determined

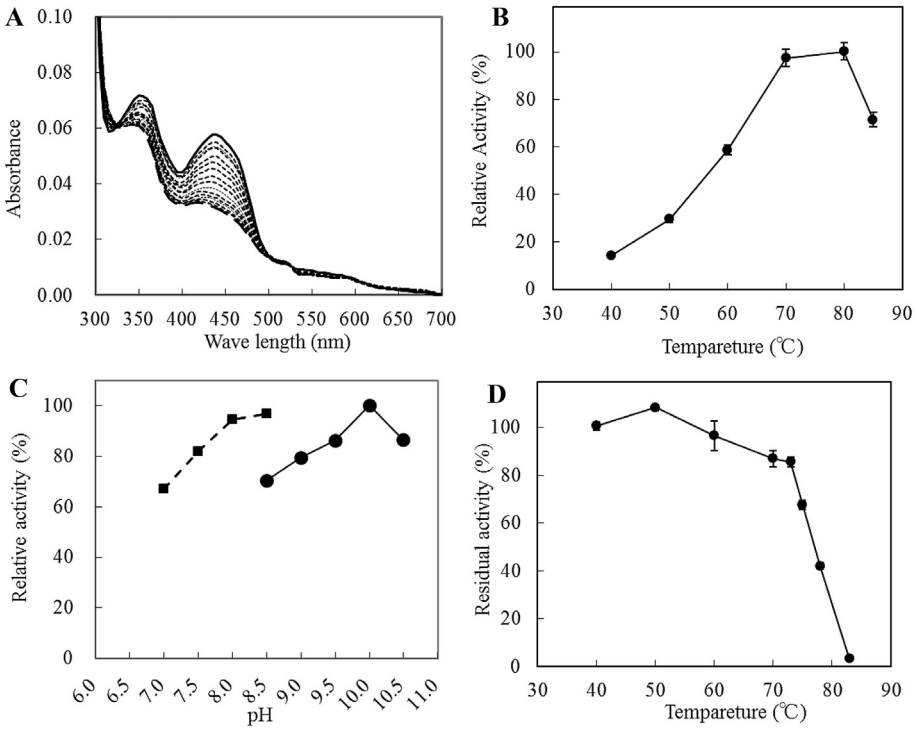


FIG. 3. G3PDH activity of Tk-G3PDH. (A) Time dependent change of UV absorption spectrum of FAD coordinated to Tk-G3PDH after G3P addition. The solid line indicates UV absorption before adding G3P, dotted line indicates the spectrum measured every 1 min until 14 min after adding G3P and the broken line indicates the spectrum of 15 min after G3P addition. (B) Temperature dependence of Tk-G3PDH activity. (C) pH dependence of Tk-G3PDH activity. (D) Residual activity of Tk-G3PDH after thermal denaturation.

TABLE 2. Kinetic parameters of G3PDHs.

	V_{max} (U·mg^{-1})	K_m (M)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Tk-G3PDH	5.63	2.68×10^{-3}	5.16	1.93×10^{3}
Ec-GlpAC[a]	34.4	3.39×10^{-4}	33.3	9.83×10^{5}

[a] Data from Schryvers et al. (18).

from the band intensities of the individual proteins on the gel (Fig. S2). The amounts of Tk-MOX, Tk-NOX and Tk-G3PDH in the complex were determined to be 0.170, 0.963 and 1.108 mg, respectively, and the estimated numbers of moles each protein were 0.01378, 0.02129 and 0.02014. The molecular weights were 12 kDa for Tk-MOX, 45 kDa for Tk-NOX, 55 kDa for Tk-G3PDH, and 205 kDa for their complex. Thus, the molar ratio of the proteins was determined to be Tk-MOX:Tk-NOX:Tk-G3PDH = 1:2:2 (Fig. 2C).

Enzymatic activity of Tk-G3PDH The purified Tk-G3PDH solution showed absorption from 300 nm to 700 nm originating from FAD. The UV absorption decreased with time after addition of 2.5 mM G3P as the substrate was oxidized in anaerobic conditions (Fig. 3A) and electrons were transferred to FAD. The G3PDH activity was determined from 40°C to 85°C and Tk-G3PDH showed the highest activity at 80°C (Fig. 3B). pH dependence of G3PDH activity was determined in the range pH 7.0 to 10.5 in appropriate buffers (Tris—HCl for pH 7.0 to 8.5, and glycine-NaOH for pH 8.5 to 10.5). The activity of Tk-G3PDH increased as the pH increased up to 10.0 (Fig. 3C). Tk-G3PDH (1.0 mg mL^{-1}) was incubated in 100 mM glycine-NaOH buffer from 40°C to 83°C for 15 min. Tk-G3PDH retained >85% residual activity at <73°C, while irreversible denaturation occurred at ≥75°C (Fig. 3D).

The kinetic parameters of Tk-G3PDH were determined at substrate concentrations from 0.125 to 8 mM at 70°C and using Lineweaver—Burk plots. V_{max}, K_m and k_{cat} of Tk-G3PDH were estimated as 5.63 U mg^{-1}, 2.68 mM and 5.16 s^{-1}, respectively. The catalytic reaction efficiency (k_{cat}/K_m) of Tk-G3PDH at 70°C was 1.93×10^3 M^{-1} s^{-1}, which was lower than that of *E. coli* GlpA (Ec-GlpA) at 37°C (Table 2). The Ec-GlpA, GlpB and GlpC complex may have evolved as a specific G3P oxidation complex, while the Tk-G3PDH forms a complex with Tk-NOX which oxidizing NADH. The physiological advantage of this complex is still unclear.

Characterization of putative complexes Three pairs of proteins (i.e., Tk-NOX + Tk-MOX, Tk-NOX + Tk-G3PDH, and Tk-MOX + Tk-G3PDH) and a complex of all three proteins were prepared from SEC fractions, and the G3PDH and NADH oxidase activities of each mixture were determined at 70°C and compared to those of the individual proteins. Tk-G3PDH complexes with NOX and/or MOX showed higher GAPDH activities than that of Tk-G3PDH alone (Fig. 4A). It is surprising that Tk-G3PDH activity was increased when mixed with Tk-MOX. 4Fe—4S cluster in Tk-MOX may work as electron acceptor while it cannot form stable complex with Tk-G3PDH. The Tk-NOX complex with Tk-G3PDH and/or MOX showed almost the same NADH oxidase activity as that of Tk-NOX alone (Fig. 4B). The residual activities of Tk-G3PDH and its complexes were determined after 15-min thermal inactivation at 80°C. Tk-G3PDH activity was 29% of that before heat inactivation, while the rate of heat inactivation of the

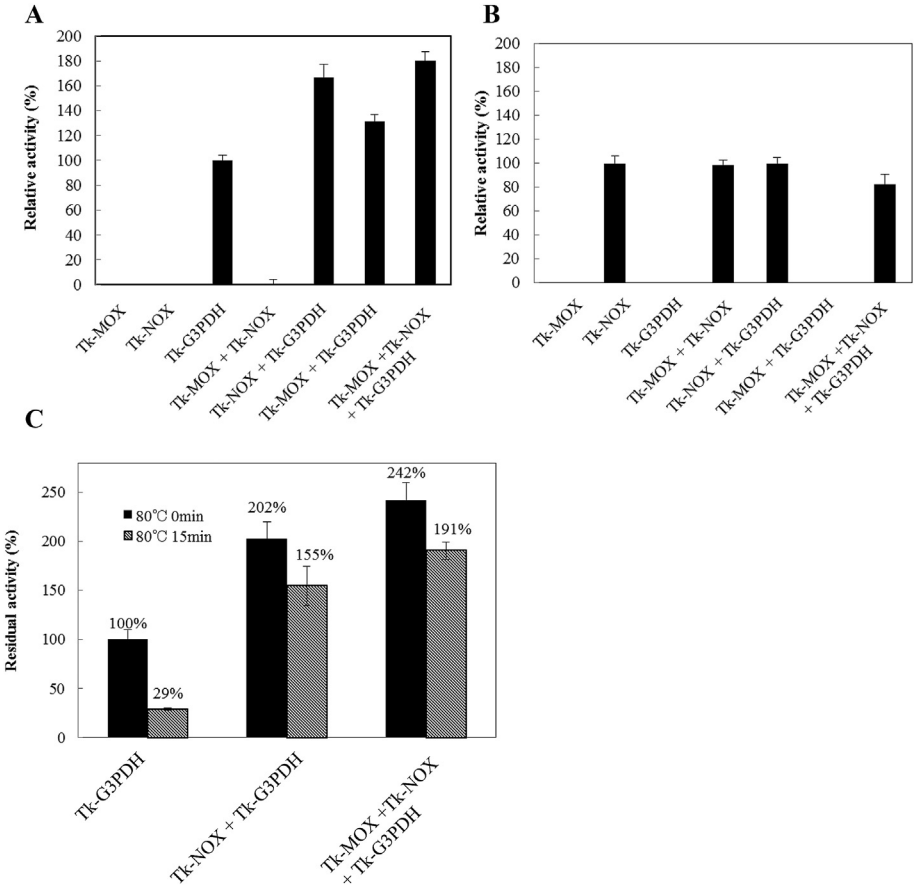


FIG. 4. Enzymatic activity of Tk-G3PDH, Tk-NOX, Tk-MOX and their complexes. (A) G3PDH activity of individual proteins and their combinations. The value is normalized by the activity of Tk-G3PDH. (B) NADH oxidase activity normalized to Tk-NOX activity. (C) Residual activity of Tk-G3PDH after heat inactivation at 80°C. The black bar indicates Tk-G3PDH activity before heat inactivation and the gray bar indicates after the heat inactivation. The activity of Tk-G3PDH before heat inactivation is defined as 100%.

complexes was around 76–79% of the activity before heat inactivation (Fig. 4C). There is a possibility that the conformational change of G3PDH depends on the binding of NOX or MOX makes rearrangement of geometry of amino acid residues related to electron transferring. This can be caused the enhancement of G3PDH activity however it should be confirmed in further research.

Yang et al. (23,24) found that the NOX from *Thermotoga maritima* contributed to protection of cells from oxidative stress by producing NAD^+. There are seven NAD(P)H oxidase genes in the *T. kodakarensis* genome. Kobori et al. (25) characterized one of the NAD(P)H oxidase, TK1481, for which was found to unique to *T. kodakarensis*. Their study suggest that the NADH oxidase from TK1481 participate in reduction of sulfur and oxygen and it may participate in the oxygen sensitivity of *T. kodakarensis* (25). Harnvoravongchai et al. (26) performed detailed phylogenetic analysis of the seven homologues along with 63 sequences of potential NADH oxidase homologues from 15 well-studied hyperthermophiles. Among the seven homologues TK1392 was close to alpha subunit of H_2O_2 forming NADH oxidase from *T. maritima* (26). However, Tk-1393 is the only gene encoding G3PDH, the key enzyme of glycerol metabolism.

In this study, a novel redox complex consisting of G3PDH, NADH oxidase and MOX was isolated from *T. kodakarensis*. The complex was different from the typical bacterial anaerobic G3PDH complex, GlpABC. The operon structure of Tk-1391, Tk-1392 and Tk1393 is also found in several anaerobic archaea and bacteria which are not phylogenetically close to *T. kodakarensis*. GlpABC may form a complex that evolved to specifically oxidize G3P and efficiently transfer the resultant electrons to the inner membrane. However, Tk-G3PDH may form a complex with NADH oxidases that share the electron transfer pathway to the inner membrane.

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References

1. **Ingledew, W. and Poole, R. K.:** Respiratory chains of *Escherichia coli*, Microbiol. Rev., **48**, 222–271 (1984).
2. **Sherwood, K. E., Cano, D. J., and Maupin-Furlow, J. A.:** Glycerol-mediated repression of glucose metabolism and glycerol kinase as the sole route of glycerol catabolism in the haloarchaeon *Haloferax volcanii*, J. Bacteriol., **191**, 4307–4315 (2009).
3. **Rawls, K. S., Martin, J. H., and Maupin-Furlow, J. A.:** Activity and transcriptional regulation of bacterial protein-like glycerol-3-phosphate dehydrogenase of the haloarchaea in *Haloferax volcanii*, J. Bacteriol., **193**, 4469–4476 (2011).
4. **Morikawa, M., Izawa, Y., Rashid, N., Hoaki, T., and Imanaka, T.:** Purification and characterization of a thermostable thiol protease from a newly isolated hyperthermophilic *Pyrococcus* sp., Appl. Environ. Microbiol., **60**, 4559–4566 (1994).
5. **Atomi, H., Fukui, T., Kanai, T., Morikawa, M., and Imanaka, T.:** Description of *Thermococcus kodakaraensis* sp. nov., a well studied hyperthermophilic archaeon previously reported as *Pyrococcus* sp. KOD1, Archaea, **1**, 263–267 (2004).
6. **Fukui, T., Atomi, H., Kanai, T., Matsumi, R., Fujiwara, S., and Imanaka, T.:** Complete genome sequence of the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1 and comparison with *Pyrococcus* genomes, Genome Res., **15**, 352–363 (2005).
7. **Imanaka, H., Yamatsu, A., Fukui, T., Atomi, H., and Imanaka, T.:** Phosphoenolpyruvate synthase plays an essential role for glycolysis in the modified Embden-Meyerhof pathway in *Thermococcus kodakarensis*, Mol. Microbiol., **61**, 898–909 (2006).
8. **Matsubara, K., Yokooji, Y., Atomi, H., and Imanaka, T.:** Biochemical and genetic characterization of the three metabolic routes in *Thermococcus kodakarensis* linking glyceraldehyde 3-phosphate and 3-phosphoglycerate, Mol. Microbiol., **81**, 1300–1312 (2011).
9. **Aono, R., Sato, T., Imanaka, T., and Atomi, H.:** A pentose bisphosphate pathway for nucleoside degradation in Archaea, Nat. Chem. Biol., **11**, 355–360 (2015).
10. **Koga, Y.:** From promiscuity to the lipid divide: on the evolution of distinct membranes in archaea and bacteria, J. Mol. Evol., **78**, 234–242 (2014).
11. **Rodrigues, M. V., Borges, N., and Santos, H.:** Glycerol phosphate cytidylyltransferase stereospecificity is key to understanding the distinct stereochemical compositions of glycerophosphoinositol in Bacteria and Archaea, Appl. Environ. Microbiol., **83**, e02462-16 (2017).
12. **Koga, Y., Morikawa, M., Haruki, M., Nakamura, H., Imanaka, T., and Kanaya, S.:** Thermostable glycerol kinase from a hyperthermophilic archaeon: gene cloning and characterization of the recombinant enzyme, Protein Eng., **11**, 1219–1227 (1998).
13. **Koga, Y., Katsumi, R., You, D.-J., Matsumura, H., Takano, K., and Kanaya, S.:** Crystal structure of highly thermostable glycerol kinase from a hyperthermophilic archaeon in a dimeric form, FEBS J., **275**, 2632–2643 (2008).
14. **Sato, T., Fukui, T., and Atomi, H.:** Targeted gene disruption by homologous recombination in the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1, J. Bacteriol., **185**, 210–220 (2003).
15. **Kistler, W. S. and Lin, E. C.:** Purification and properties of the flavine-stimulated anaerobic L- -glycerophosphate dehydrogenase of *Escherichia coli*, J. Bacteriol., **112**, 539–547 (1972).
16. **Schneider, C. A., Rasband, W. S., and Eliceiri, K. W.:** NIH Image to ImageJ: 25 years of image analysis, Nat. Methods, **9**, 671–675 (2012).
17. **Pallejà, A., García-Vallvé, S., and Romeu, A.:** Adaptation of the short intergenic spacers between co-directional genes to the Shine-Dalgarno motif among prokaryote genomes, BMC Genomics, **10**, 537 (2009).
18. **Rashid, N., Hameed, S., and Siddiqui, M. A.:** Gene cloning and characterization of NADH oxidase from *Thermococcus kodakarensis*, Afr. J. Biotechnol., **10**, 17916–17924 (2011).
19. **Varga, M. E. R. and Weiner, J. H.:** Physiological role of GlpB of anaerobic glycerol-3-phosphate dehydrogenase of *Escherichia coli*, Biochem. Cell Biol., **73**, 147–153 (1995).
20. **Gribaldo, S. and Brochier-Armanet, C.:** The origin and evolution of Archaea: a state of the art, Phil. Trans. Roy. Soc. Lond. B Biol. Sci., **361**, 1007–1022 (2006).
21. **Schryvers, A. and Weiner, J. H.:** The anaerobic sn-glycerol-3-phosphate dehydrogenase of *Escherichia coli*. Purification and characterization, J. Biol. Chem., **256**, 9959–9965 (1981).
22. **Rashid, N., Akmal, S., and Akhtar, M.:** Gene cloning and characterization of TK1392, an NADH oxidase from *Thermococcus kodakaraensis* with a distinct C-terminal domain, Turkish J. Biochem., **36**, 107–115 (2011).
23. **Yang, X. and Ma, K.:** Purification and characterization of an NADH oxidase from extremely thermophilic anaerobic bacterium *Thermotoga hypogea*, Arch. Microbiol., **183**, 331–337 (2005).
24. **Yang, X. and Ma, K.:** Characterization of an exceedingly active NADH oxidase from the anaerobic hyperthermophilic bacterium *Thermotoga maritima*, J. Bacteriol., **189**, 3312–3317 (2007).
25. **Kobori, H., Ogino, M., Orita, I., Nakamura, S., Imanaka, T., and Fukui, T.:** Characterization of NADH oxidase/NADPH polysulfide oxidoreductase and its unexpected participation in oxygen sensitivity in an anaerobic hyperthermophilic archaeon, J. Bacteriol., **192**, 5192–5202 (2010).
26. **Harnvoravongchai, P., Kobori, H., Orita, I., Nakamura, S., Imanaka, T., and Fukui, T.:** Characterization and gene deletion analysis of four homologues of group 3 pyridine nucleotide disulfide oxidoreductases from *Thermococcus kodakarensis*, Extremophiles, **18**, 603–616 (2014).