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The oxidative pentose phosphate pathway in the haloarchaeon *Haloferax volcanii* involves a novel type of glucose-6-phosphate dehydrogenase – The archaeal Zwischenferment

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ABSTRACT

The oxidative pentose phosphate pathway (OPPP), catalyzing the oxidation of glucose-6-phosphate to ribulose-5-phosphate is ubiquitous in eukarya and bacteria but has not yet been reported in archaea. In haloarchaea a putative 6-phosphogluconate dehydrogenase (6PGDH) is annotated, whereas a gene coding for glucose-6-phosphate dehydrogenase (Glc6PDH) could not be identified. Here we report the purification and characterization of a novel type of Glc6PDH in *Haloferax volcanii* that is not related to bacterial and eukaryal Glc6PDHs and the encoding gene is designated as *azf* (archaeal zwischenferment). Further, recombinant *H. volcanii* 6PGDH was characterized. Deletion mutant analyses indicate that both, Glc6PDH and 6PGDH, are functionally involved in pentose phosphate formation in vivo. This is the first report on the operation of the OPPP in the domain of archaea.

1. Introduction

The pentose phosphate pathway (PPP) that consists of an irreversible oxidative part (OPPP) and a reversible non-oxidative part is a ubiquitous pathway in bacteria and eukarya providing NADPH for reductive biosyntheses and precursors for the generation of nucleotides and aromatic amino acids [1,2]. In the OPPP glucose-6-phosphate is oxidized to ribulose-5-phosphate and CO_2 involving two $NADP^+$ dependent dehydrogenases – glucose-6-phosphate dehydrogenase (Glc6PDH) [glucose-6-phosphate + $NADP^+$ → 6-phosphogluconolactone + NADPH + H^+] and 6-phosphogluconate dehydrogenase (6PGDH) [6-phosphogluconate + $NADP^+$ → ribulose-5-phosphate + CO_2 + NADPH + H^+]. The first enzyme, Glc6PDH, was discovered by Otto Warburg in the 1930s while analyzing the oxidation of glucose-6-phosphate by O_2 in erythrocytes. This oxidation required $NADP^+$ as a novel cofactor (*Co-Ferment*) and two proteins, *gelbes Ferment* (german for "yellow enzyme") and *Zwischenferment* (german for "intermediate enzyme"), now known as glucose-6-phosphate dehydrogenase (Glc6PDH, encoded by *zwf* for zwischenferment) [3,4]. The product of the Glc6PDH reaction, 6-phosphogluconolactone, is hydrolyzed by lactonase and the product 6-phosphogluconate is further oxidized and decarboxylated to ribulose-5-phosphate (Ru5P) by $NADP^+$ dependent 6-phosphogluconate dehydrogenase (6PGDH) [2]. The OPPP is well studied in many eukarya and bacteria but it has not been demonstrated in archaea so far [5]. An alternative route for the synthesis of pentose phosphates from hexose phosphates has been reported for many archaeal groups, and has been studied in detail in *Pyrococcus horikoshii*, *Methanocaldococcus jannaschii*, *Methanosarcina barkeri* and *Thermococcus kodakaraensis* [5–10]. This alternative route involves the enzymes hexulose-6-phosphate isomerase and 3-hexulose-6-phosphate synthase of the ribulose monophosphate pathway, converting fructose-6-phosphate non-oxidatively to ribulose-5-phosphate and formaldehyde. The ribulose monophosphate pathway was first described in methylotrophic bacteria where it is involved in formaldehyde fixation [10]. Whereas hexulose-6-phosphate isomerase and 3-hexulose-6-phosphate synthase genes are widely distributed among archaeal genomes, they are absent in haloarchaea [5]. However, in contrast to all other archaea, a putative 6PGDH was annotated in haloarchaea, suggesting the operation of the OPPP in these organisms. So far, 6PGDH has not been characterized as a functional enzyme in archaea and a gene coding for classical Glc6PDH has not been found in any archaeal genome.

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In this communication, we studied enzymes of the OPPP in the halophilic archaeon *Haloferax volcanii*. An NAD^+ dependent Glc6PDH was purified from *H. volcanii* cells and the encoding gene (denoted *azf* for archaeal zwischenferment) was identified. The enzyme was characterized as a novel type Glc6PDH that belongs to the short-chain dehydrogenase/reductase (SDR) superfamily. Further, the annotated 6PGDH gene was expressed and the recombinant enzyme was characterized as NAD^+ dependent 6PGDH. Deletion mutant analyses indicated that both, Glc6PDH and 6PGDH, were essential for pentose-phosphate synthesis in *H. volcanii* in vivo. This is the first report on the operation of the OPPP in the domain of archaea involving a novel type of Glc6PDH.

2. Material and methods

2.1. Strains and growth conditions

The following strains of *H. volcanii* were used and/or prepared: strain H26 (*ΔpyrE2*), *azf* and *gndA* single deletion mutants of *H. volcanii* H26 (denoted *Δazf* and *ΔgndA*), *Δazf* transformed with pTA963-his::*azf*, and *ΔgndA* transformed with pTA963-his::*gndA*. Growth experiments were performed aerobically at 42 °C in synthetic medium [11] containing 25 mM D-glucose. Growth of *H. volcanii* H26 and of *Δazf* and *ΔgndA* strains was supplemented with 50 μg/ml uracil and complemented strains with functional genes *in-trans* grew in the presence of up to 300 μM tryptophan. Additionally, *Δazf* and *ΔgndA* strains were grown in the presence of 5 mM uridine. For growth determination, the optical density at 600 nm was followed over time.

2.2. Purification of native Glc6PDH

Glc6PDH from *H. volcanii* was purified from 9,8 g wet weight cells grown on glucose. Cells were disrupted in a French pressure cell at 14000 lb/in^2 in HIC buffer (100 mM Tris–HCl, 2 M $(NH_4)_2SO_4$, pH 8.0). The obtained cell lysate was centrifuged at 100000×*g* at 4 °C and the supernatant was applied to a Phenyl Sepharose 26/10 HiLoad column (GE Healthcare), equilibrated in HIC buffer. Bound protein was eluted by a linear gradient with decreasing $(NH_4)_2SO_4$ concentrations at 1 ml/min for 400 ml. Highest Glc6PDH activity, which was eluted at about 800 mM $(NH_4)_2SO_4$ in 6 ml, was concentrated 7.5-fold by ultrafiltration (cut-off 10kDa) and applied to a HiLoad 16/60 Superdex 200 column (GE Healthcare) equilibrated with 50 mM Tris–HCl, 2 M KCl, pH 8.0. Elution was performed in an isocratic flow at 1 ml/min. Fractions with the highest activity were diluted 7-fold in HIC buffer to a final volume of 20 ml and applied to a HiTrap Butyl HP Sepharose column (GE Healthcare), equilibrated in HIC buffer. Protein was eluted for 20 ml at a flow rate of 1 ml/min with decreasing concentrations of $(NH_4)_2SO_4$. The highest Glc6PDH activity eluted at a concentration of about 550 mM $(NH_4)_2SO_4$. During the purification procedure protein concentrations were determined by the Bradford method [12] and protein purity was analyzed by SDS–PAGE. The encoding gene was identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry of the purified protein (32kDa band in SDS–PAGE) [13].

2.3. Homologous expression, purification and characterization of recombinant Glc6PDH and 6PGDH

HVO_0511 (*azf*) and HVO_1830 (*gndA*) were amplified from genomic DNA with specific primers (Supplemental Table S1). PCR products were cloned into the plasmid pTA963 and each generated plasmid (pTA963-his::*azf* and pTA963-his::*gndA*) was transformed in *H. volcanii* H1209, followed by homologous overexpression in complex medium, as described [14]. Each recombinant protein was purified, using Ni–NTA and size exclusion chromatography as described previously [15]. Enzyme activities were determined by measuring NAD(P)H formation at 340 nm in a total volume of 200 μl at 42 °C. One unit (U) of enzyme activity is defined as the conversion of 1 μmol substrate per min. Glc6PDH activity was measured in an assay, containing 0.1 M Tris pH 7.5, 2 M KCl, 2 mM NAD^+ or 20 mM $NADP^+$, 20 mM glucose-6-phosphate and up to 7 μg enzyme. The assay for the measurement of 6PGDH activity contained 0.1 M Tris–HCl pH 9.0, 1 M KCl, 3 mM NAD^+ or 10 mM $NADP^+$, 7.5 mM 6-phosphogluconate and up to 60 μg purified enzyme. For both enzymes, the salt dependence was determined with up to 3 M potassium chloride, and the optimal pH value was measured using bis-Tris (pH 5.5–7.5), Tris (pH 7.5–9) and glycylglycine (pH 9.0–11.5) buffer. Apparent K_m and V_{max} values were calculated from linear Lineweaver–Burk plots.

2.4. Generation of knockout strains

Chromosomal knockout strains were generated using the pop-in/pop-out strategy, as described previously [16]. Pop-out clones were selected in complex medium [15], containing casamino acids (1%), uracil (30 μg/ml), 5-fluoroorotic acid (50 μg/ml), adenosine, thymidine, cytidine, uridine (0.5 mM, each), and guanosine (0.2 mM). Successful deletion was verified by Southern blot analysis as described [15]. Primers used are listed in Table S2. Probes for Southern blotting were generated with the primer pairs HVO_0511frgt1_s/HVO_0511P_as (5′-CTATCACCAGTTCGCCGGCAC-3′) and HVO_1830P_s (5′-GCGGGTGCCGCCACAATCAC-3′)/HVO_1830 frgt2_as, respectively.

3. Results

The oxidative pentose phosphate pathway (OPPP) catalyzing ribulose-5-phosphate formation from glucose-6-phosphate has not been found in the archaeal domain so far. It is shown here that a functional OPPP is present in *H. volcanii* involving a novel type of Glc6PDH.

3.1. Identification of a novel glucose-6-phosphate dehydrogenase

Extracts of *H. volcanii* cells grown on glucose catalyzed the NAD^+ dependent oxidation of glucose-6-phosphate with a specific activity of 35 mU/mg. The enzyme was purified 1200-fold to apparent homogeneity by phenyl Sepharose-, gel filtration- and butyl Sepharose chromatography (Table 1). On SDS–PAGE, a single subunit with an apparent molecular mass of 32kDa was detected (Fig. 1A). By gel filtration the molecular mass of the native enzyme was determined at 55kDa, indicating a homodimeric structure. The purified enzyme catalyzed the NAD^+ dependent oxidation of glucose-6-phosphate (Glc6P) following Michelis–Menten kinetics with apparent K_m values of 5.4 mM for Glc6P, and 0.18 mM for NAD^+. The apparent V_{max} was 39 U/mg. The 32kDa protein subunit was analyzed by peptide mass fingerprinting. Matched peptides covered 58% of a protein, encoded by HVO_0511 in the genome

Table 1
Purification of glucose-6-phosphate dehydrogenase from *H. volcanii*.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Enrichment factor	Yield (%)
Cell-free extract	638	22.8	0.035	1	100
Phenyl sepharose	11.3	6.65	0.59	17	29
Gel filtration	0.16	2.06	12	343	9
Butyl sepharose	0.015	0.62	42	1200	2.7

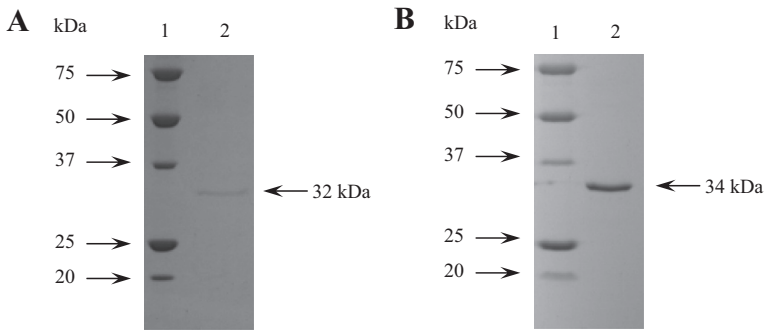

Fig. 1. SDS–PAGE of purified native (A) and His-tagged recombinant (B) Glc6PDH of *Haloferax volcanii*. (A) Lane 1: molecular mass markers; lane 2: purified enzyme after butyl Sepharose. (B) Lane 1: molecular mass markers; lane 2: purified recombinant enzyme.

of *H. volcanii*. The identified gene, previously annotated as sugar epimerase/dehydratase and now designated *azf* for archaeal zwischenferment, consists of 789 base pairs and encodes a polypeptide of 262 amino acids with a calculated molecular mass of 29kDa.

3.2. Characterization of Glc6PDH

To prove its catalytic function, *azf* was cloned into pTA963, expressed in *H. volcanii* H1209 and the recombinant enzyme was purified by Ni–NTA affinity and size exclusion chromatography. SDS–PAGE analysis revealed one subunit at 34kDa (Fig. 1B). The apparent molecular mass of recombinant Glc6PDH determined by gel filtration was 59kDa, suggesting a homodimeric structure. Glc6PDH showed the highest activity at about 3 M KCl and the pH optimum was at 8.5. The enzyme catalyzed NAD^+ dependent oxidation of Glc6P with apparent K_m values of 3.7 mM for Glc6P and 0.43 mM for NAD^+, and a V_{max} value of 212 U/mg. The enzyme also utilized $NADP^+$ as electron acceptor with apparent K_m and V_{max} values of 5.2 mM and 11 U/mg (in the presence of 20 mM Glc6P). Thus, the catalytic efficiency of the enzyme for NAD^+ ($263\ s^{-1}\ mM^{-1}$) was about 230-fold higher than for $NADP^+$ ($1.13\ s^{-1}\ mM^{-1}$), indicating NAD^+ to be the physiological electron acceptor. The enzyme was specific for Glc6P; glucose was not oxidized at significant rates.

3.3. Characterization of 6PGDH

In *H. volcanii* HVO_1830 is annotated as *gndA* gene encoding putative 6PGDH, consisting of 299 amino acids with a calculated molecular mass of 32kDa. The *gndA* gene was cloned into pTA963, followed by homologous overexpression of the his-tagged protein in *H. volcanii* H1209 and purification by Ni–NTA affinity and size exclusion chromatography. Analysis on SDS–PAGE revealed a single protein band at 37kDa (Fig. 2) and by gel filtration a molecular mass of 143kDa was determined indicating the enzyme to be a homotetramer. The highest activity of the enzyme was determined at 1 M KCl and at a pH of 10.5. The enzyme catalyzed the NAD^+ dependent oxidation of 6-phosphogluconate (6PG) with apparent K_m values of 0.021 mM for 6PG and

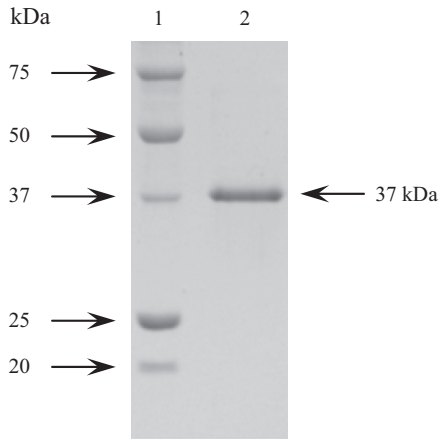

Fig. 2. SDS–PAGE of purified, His-tagged 6PGDH of *Haloferax volcanii*. Lane 1, molecular mass markers; lane 2, purified recombinant enzyme.

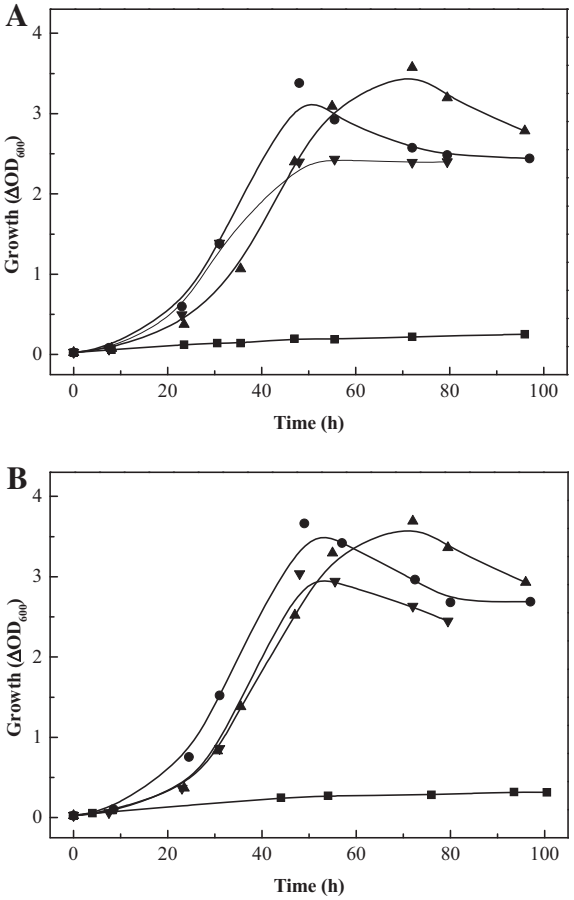

Fig. 3. Growth analyses of deletion mutants of genes involved in pentose phosphate synthesis in *H. volcanii*. Growth of *H. volcanii* strains *Δazf* (A) and *ΔgndA* (B) on 25 mM glucose (■) compared to the wild type (●) and complementation strains with functional genes *in-trans* (▼). Further, growth of *Δazf* (A) and *ΔgndA* (B) on 25 mM glucose in the presence of 5 mM uridine is shown (▲). For growth experiments, precultures grown in complex medium containing 1% casamino acids and nucleosides were used. Growth was measured by determining the optical density at 600 nm (ΔOD_{600}).

0.033 mM for NAD^+ and a V_{max} value of 10 U/mg. The apparent K_m and V_{max} values for $NADP^+$ (in the presence of 5 mM 6PG) were determined at 2.94 mM and 0.42 U/mg, respectively. Thus, the catalytic efficiency (k_{cat}/K_m) of the enzyme for NAD^+ (174 s^{-1} mM^{-1}) was about 2000-fold higher than for $NADP^+$ (0.082 s^{-1} mM^{-1}), defining the enzyme as NAD^+ specific 6PGDH.

3.4. Characterization of azf and gndA deletion mutants

To prove the functional involvement of both, Glc6PDH and 6PGDH, in pentose-phosphate formation, deletion strains of *azf* and *gndA* were generated. Successful deletions were verified by Southern blot analyses (Fig. S1) and growth of the deletion mutants on glucose was analyzed. Compared to the wild type, the *Δazf* and *ΔgndA* strains did not grow, but growth was fully recovered by *in-trans* complementation with *azf* and *gndA*, respectively (Fig. 3). Growth of the deletion mutants could also be recovered by the addition of uridine to the medium (Fig. 3), suggesting that *H. volcanii* can circumvent the metabolic block for pentose phosphate formation via the OPPP by converting uridine to ribose-5-phosphate, catalyzed by uridine phosphorylase and phosphopentomutase as proposed for *T. kodakaraensis* [8]. Together, the analyses of knockout mutants indicate that the novel Glc6PDH and annotated 6PGDH are essential for the biosynthesis of pentose phosphates from glucose-6-phosphate during growth of *H. volcanii* on glucose as growth substrate.

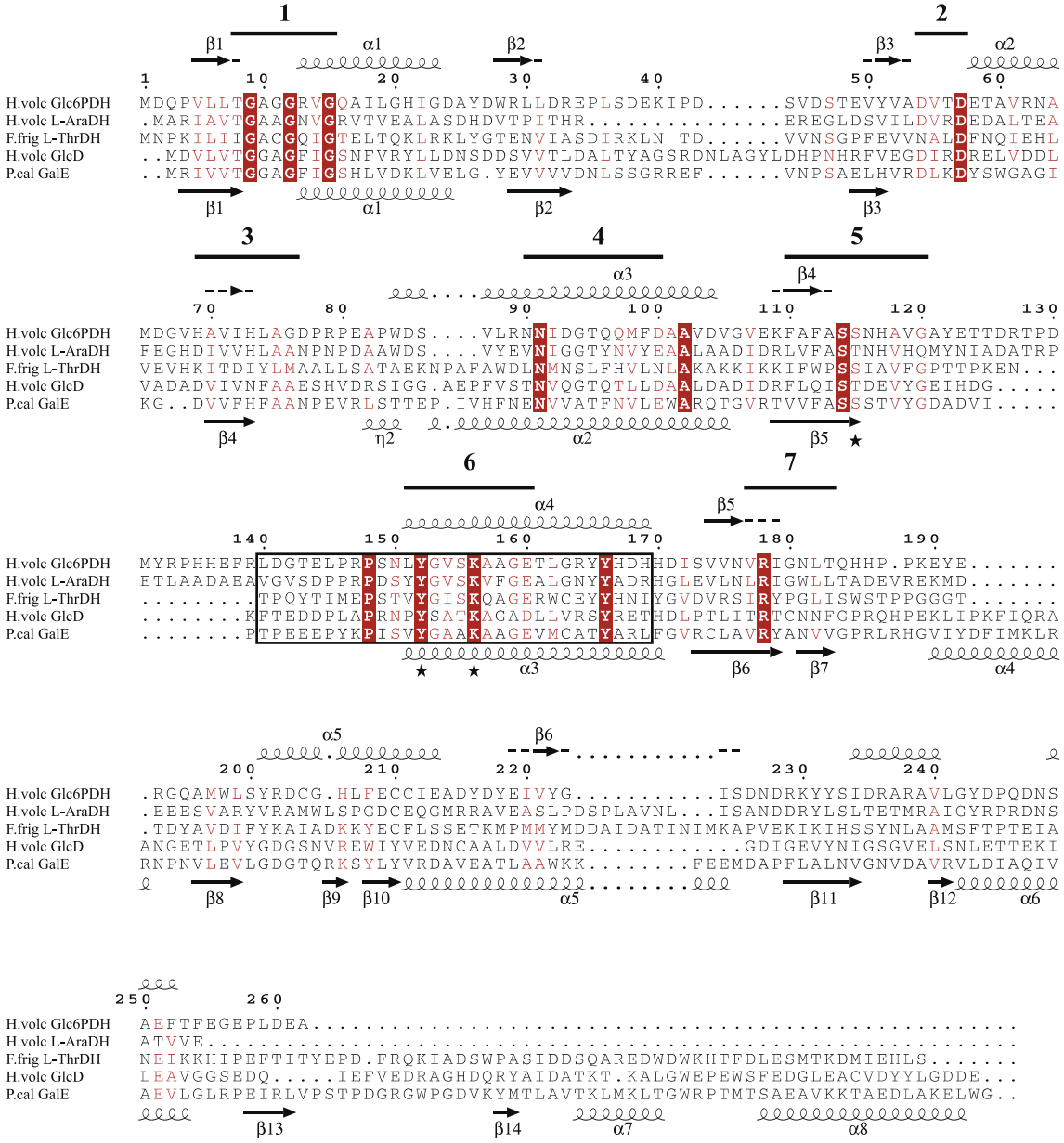

Fig. 4. Multiple amino acid sequence alignment of Glc6PDH from *H. volcanii* with selected archaeal and bacterial homologs of the SDRe family. The secondary structure in the lower line from *P. calidifontis* GalE was derived from crystal structure data [24]. In the upper line, the predicted secondary structure of *H. volcanii* Glc6PDH is shown (PSIPRED, [35]). The ADH_SHORT consensus pattern (PROSITE: PDOC00060), indicative for the SDR superfamily, is highlighted by a box. Seven sequence motifs that are characteristic for members of the SDRe family [19] are numbered and indicated by bars, and the conserved catalytic triad (S/T-Y-K) is marked by asterisks. Amino acid sequences from *H. volcanii* (H. volc Glc6PDH, H. volc L-AraDH and H. volc GlcD, ADE03728, YP_003533112 and ADE03524), *Flavobacterium frigidimaris* (Q8KZM4) and *Pyrobaculum calidifontis* (3KO8) were used for the alignment. The figure was generated with ESPript [36].

4. Discussion

In the present communication we report that *H. volcanii* utilizes the OPPP for the formation of pentose phosphates from glucose 6-phosphate involving a novel type of Glc6PDH and annotated 6PGDH. This is the first report on the operation of an OPPP in the archaeal domain.

4.1. Glucose-6-phosphate dehydrogenase from H. volcanii belongs to the SDRe family and represents the archaeal Zwischenferment

Glc6PDH was purified and characterized as a homodimeric enzyme of 32kDa subunits, catalyzing the NAD^+ specific oxidation of Glc6P. The encoding gene was identified as HVO_0511 (designated as *azf*, archaeal zwischenferment). Conserved domain search classifies the novel *H. volcanii* Glc6PDH as a member of the extended short-chain dehydrogenase/reductase (SDRe) family [17]. The SDRe family belongs to the SDR superfamily that comprises functionally diverse enzymes such as NAD(P)(H) dependent oxidoreductases with a broad substrate spectrum. SDR proteins show a low overall sequence identity of 20–30%, but share a common α/β fold and contain the ADH_SHORT consensus pattern (PROSITE:PDOC00060), indicative for the SDR superfamily. Further, SDR enzymes have a common catalytic mechanism, involving a highly conserved triad of Ser/Thr-Tyr-Lys residues [18]. Based on conserved sequence motifs the majority of SDR proteins can be classified as "classical" and "extended" type [19] and at least four minor types can be defined [20]. An amino acid sequence alignment of *H. volcanii* Glc6PDH with the characterized SDRe members L-arabinose dehydrogenase (L-AraDH) from *H. volcanii* [21], L-threonine dehydrogenase (L-ThrDH) from *Flavobacterium frigidimaris* [22], dTDP-glucose-4,6-dehydratase (GlcD) from *H. volcanii* [23] and UDP-galactose-4-epimerase (GalE) from *Pyrobaculum calidifontis* [24] is shown in Fig. 4. All sequences, including *H. volcanii* Glc6PDH, contain – with few deviations – the seven sequence motifs characteristic for the SDRe family [19], the catalytic conserved Ser/Thr-Tyr-Lys triad and the ADH_SHORT consensus motif. Also, the predicted secondary structural elements of *H. volcanii* Glc6PDH are in accordance with the structure of GalE from *P. calidifontis* [24], indicating a high degree of structural similarity to other SDR proteins. Together, the data indicate that *H. volcanii* is a member of the SDRe family.

The phylogenetic relationship of Glc6PDH from *H. volcanii* and haloarchaeal homologs, and other selected characterized SDRe members and their corresponding homologs, are shown in Fig. 5. The SDRe proteins cluster in distinct phylogenetic groups which are in accordance with their specific catalytic function. E.g. separate clusters are formed by L-AraDH involved in L-arabinose degradation, L-ThrDH involved in threonine degradation, GDP-D-mannose-4,6-dehydratase (D-ManD) and GDP-L-fucose synthase (L-FucS), both involved in L-fucose synthesis, GlcD involved in dTDP-rhamnose biosynthesis, and GalE involved in galactose catabolism. Glc6PDH from *H. volcanii* and haloarchaeal homologs form a novel distinct cluster that is closely related to the L-AraDH cluster

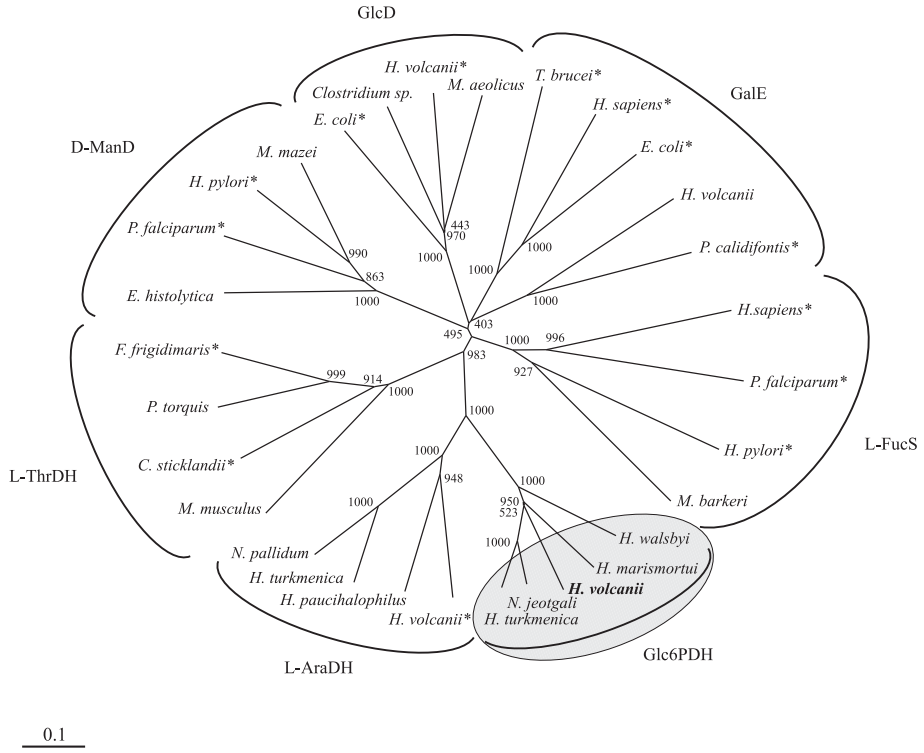

Fig. 5. Phylogenetic relationships of SDRe proteins, including haloarchaeal glucose-6-phosphate dehydrogenase (Glc6PDH), L-arabinose dehydrogenase (L-AraDH), L-threonine dehydrogenase (L-ThrDH), GDP-D-mannose-4,6-dehydratase (D-ManD), dTDP-glucose-4,6-dehydratase (GlcD), UDP-galactose-4-epimerase (GalE) and GDP-L-fucose synthase (L-FucS). The numbers at the nodes are bootstrapping values according to neighbor-joining. *H. volcanii* Glc6PDH is highlighted by bold letters and other characterized enzymes are marked by asterisks. Accession numbers and references: Glc6PDH: *Haloquadratum walsbyi* (WP_021049862), *Haloarcula marismortui* YP_136835, *Haloferax volcanii* ADE03728, *Natronococcus jeotgali* WP_008421663, *Haloterrigena turkmenica* WP_012941655; L-AraDH: *Haloferax volcanii* YP_003533112 [21], *Haladaptatus paucihalophilus* WP_007982414, *Haloterrigena turkmenica* WP_012943574, *Natrinema pallidum* WP_006184508; L-ThrDH: *Mus musculus* Q8K3F7, *Clostridium sticklandii* WP_013361834 [37], *Psychroflexus torquis* WP_015024260, *Flavobacterium frigidimaris* Q8KZM4 [22]; D-ManD: *Entamoeba histolytica* EMH75851, *Plasmodium falciparum* ETW61934 [38], *Helicobacter pylori* NP_206845 [39], *Methanosarcina mazei* NP_632683; GlcD: *Escherichia coli* EDV64919 [40], *Clostridium* sp. WP_022441977, *Haloferax volcanii* ADE03524 [23], *Methanococcus aeolicus* WP_011973099; GalE: *Trypanosoma brucei* 2CNB [41], *Homo sapiens* GALE_HUMAN [42], *Escherichia coli* EDV64919 [43], *Haloferax volcanii* ADE04757, *Pyrobaculum calidifontis* 3KO8 [24]; L-FucS: Human AAC50786 [44], *Plasmodium falciparum* XP_001347422 [38], *Helicobacter pylori* ACX98429 [39], *Methanosarcina barkeri* WP_011305081.

from Haloarchaea. The close phylogenetic proximity suggests that both dehydrogenase clusters evolved and functionally diversified from a common SDRe ancestor.

Glc6PDH from *H. volcanii* is the first identified Glc6PDH in the archaeal domain. The enzyme differs from the classical Glc6PDH involved in OPPP in bacteria and eukarya in various aspects. The haloarchaeal Glc6PDH is an NAD^+ specific, 60kDa homodimeric SDRe protein, whereas the classical bacterial/eukaryal Glc6PDHs are $NADP^+$ dependent 50–60kDa proteins of different oligomeric states that all belong to the glucose-6-phosphate dehydrogenase superfamily (TIGR00871: zwf). It should be noted that *Mycobacterium* and *Nocardia* species contain, in addition to classical $NADP^+$ dependent Glc6PDH, a deazaflavin F_{420} dependent Glc6PDH that is not part of the OPPP [25]. This enzyme is not related to classical pyridine nucleotide dependent enzymes and to archaeal Glc6PDH from *H. volcanii* [26].

4.2. 6-Phosphogluconate dehydrogenase from H. volcanii is related to NAD^+ dependent bacterial 6PGDH

6PGDH from *H. volcanii* was characterized as homotetramer of 37kDa subunits, oxidizing 6-phosphogluconate with a high specificity for NAD^+ as electron acceptor. The enzyme shows a high degree of sequence identity (33–42%) to NAD^+ dependent 6PGDHs from bacteria, e.g. *Bradyrhizobium* sp. [27], *Gluconobacter oxydans* [28] and *Leuconostoc lactis* [29], that also constitute homotetrameric proteins of ~36kDa subunits. A significant lower sequence identity ($\leqslant$20%) to *H. volcanii* 6PGDH was found to $NADP^+$ dependent 6PGDHs from bacteria and eukarya, e.g. *Saccharomyces cerevisiae* [30], *Bacillus subtilis* [31], *Lactococcus lactis* [32] and *Escherichia coli* [33]. Classical $NADP^+$ dependent 6PGDHs constitute homodimeric proteins of about 50kDa subunits. A phylogram of *H. volcanii* 6PGDH and 6PGDHs from bacteria and eukarya indicate that NAD^+ and $NADP^+$ dependent enzymes form distinct clusters (Fig. S2). The *H. volcanii* enzyme clusters within the NAD^+ dependent enzymes, which is in accordance with its properties being an NAD^+ specific homotetrameric protein of 37kDa subunits. Thus, the haloarchaeal 6PGDH is likely derived from bacterial NAD^+ dependent homologs via a lateral gene transfer.

In summary, we report here that *H. volcanii* utilizes the OPPP for the synthesis for pentose phosphates from glucose-6-phosphate involving a novel type of Glc6PDH and bacterial type of 6PGDH. The first Glc6PDH in archaea belongs to the SDRe family and is not related to the classical Glc6PDH of the OPPP in bacteria and eukarya and thus is the result of a convergent enzyme evolution within the haloarchaea. 85 years after the discovery of the classical Glc6PDH, the *Zwischenferment* by Otto Warburg, we designate the first Glc6PDH in archaea as archaeal Zwischenferment, encoded by *azf*. In contrast, 6PGDH of *H. volcanii* is related to NAD^+ dependent bacterial 6PGDHs and has likely been acquired by a lateral gene transfer event.

Close homologs of both, Glc6PDH and 6PGDH, (>35% sequence identity) are present in almost all sequenced haloarchaea, but were not found in other archaeal groups, suggesting a functional OPPP to be restricted to haloarchaea. So far, a 6-phosphogluconolactonase in *H. volcanii* has not been analyzed. The OPPP in *Haloarchaea*, in contrast to the OPPP in bacteria and eukarya involves two NAD^+ dependent dehydrogenases. Thus, the organisms have to generate NADPH for reductive biosyntheses by different reactions, e.g. by the $NADP^+$ dependent isocitrate dehydrogenase [34].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.03.026.

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