Gluconate dehydratase from the promiscuous Entner–Doudoroff pathway in *Sulfolobus solfataricus*[☆]

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Abstract An investigation has been carried out into gluconate dehydratase from the hyperthermophilic Archaeon *Sulfolobus solfataricus*. The enzyme has been purified from cell extracts of the organism and found to be responsible for both gluconate and galactonate dehydratase activities. It was shown to be a 45 kDa monomer with a half-life of 41 min at 95 °C and it exhibited similar catalytic efficiency with both substrates. Taken alongside the recent work on glucose dehydrogenase and 2-keto-3-deoxygluconate aldolase, this report clearly demonstrates that the entire non-phosphorylative Entner–Doudoroff pathway of *S. solfataricus* is promiscuous for the metabolism of both glucose and galactose.
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1. Introduction

The hyperthermophilic Archaeon *Sulfolobus solfataricus* grows optimally at 80–85 °C and pH 2–4, utilising a wide range of carbon and energy sources, and is one of the most comprehensively researched model organisms of archaeal metabolism [1,2]. Central metabolism in *S. solfataricus* involves a non-phosphorylative variant of the Entner–Doudoroff pathway (Fig. 1) [3]. In this pathway, glucose dehydrogenase and gluconate dehydratase catalyse the oxidation of glucose to gluconate and the subsequent dehydration of gluconate to 2-keto-3-deoxygluconate (KDG). KDG aldolase then catalyses the cleavage of KDG to glyceraldehyde and pyruvate. The glyceraldehyde is oxidised to glycerate by glyceraldehyde dehydrogenase before being phosphorylated by glycerate kinase to give 2-phosphoglycerate. A second molecule of pyruvate is produced from this by the actions of enolase and pyruvate kinase. A similar pathway has also been detected in the thermoacidophilic Archaea *Sulfolobus acidocaldarius* [4], *Thermoplasma acidophilum* [5] and *Thermoproteus tenax* [6], as well as strains of *Aspergillus* fungi [7,8].

Recently, it has been discovered that glucose dehydrogenase and KDG aldolase from *S. solfataricus* have an unexpectedly high activity with galactose and 2-keto-3-deoxygalactonate (KDGal), respectively [9]. Consequently, it was proposed that the entire central metabolic pathway in this organism is promiscuous for the metabolism of both glucose and galactose. This situation contrasts with other microorganisms, where separate enzymes and pathways are present for the metabolism of the two sugars. However, a major question remained over gluconate dehydratase, which had not been investigated. The gene was originally reported to be missing from the published genomic sequence [10], although a likely candidate gene was subsequently identified [11]. There have been few examples of the biochemical characterisation of similar dehydratases and no reports of their purification and characterisation from a hyperthermophilic organism.

We now report on an investigation into gluconate dehydratase from *S. solfataricus* and demonstrate that this enzyme is also responsible for galactonate dehydratase activity. This discovery of an entire pathway that is promiscuous for the metabolism of more than one sugar may have important implications for metabolic evolution.

2. Materials and methods

2.1. Purification of gluconate dehydratase from Sulfolobus solfataricus

S. solfataricus (DSM 1616) cell paste was provided by Dr. Neil Raven (Centre for Applied Microbiological Research, Porton Down, UK). Extracts were prepared by resuspending the cell paste in 50 mM Tris/HCl (pH 8.0) containing 10 mM $MgCl_2$ and incubation at room temperature for 30 min. The extract was then sonicated by three 30-s bursts using a 150-W Ultrasonic Disintegrator (MSE Scientific Instruments). Soluble cell extract was obtained by centrifugation at $20\,000 \times g$ for 30 min. Gluconate dehydratase was purified from the extract by anion exchange chromatography using three 5 ml HiTrap Q Sepharose columns in series (Amersham Biosciences) with a 0–0.7 M NaCl gradient. Selected fractions were subjected to further purification by gel filtration using a HiLoad 16/60 Superdex 200 column (3.2 cm × 60 cm) (Amersham Biosciences) in the same buffer. Selected fractions were dialysed against 10 mM sodium phosphate buffer (pH 7.2) containing 5 mM $MgCl_2$, and further purified using a ceramic hydroxyapatite column (CHT-II cartridge, BioRad) with an elution buffer of 0.4 M sodium phosphate (pH 6.8) containing 5 mM $MgCl_2$. Selected fractions were dialysed against 50 mM Tris/HCl (pH 8.0)

Glucose

GLUCOSE DEHYDROGENASE $\rightharpoondown$ NAD(P)$^+$ $\rightharpoonup$ NAD(P)H + H$^+$

Gluconate

GLUCONATE DEHYDRATASE $\rightharpoonup$ H$_2$O

2-Keto-3-deoxygluconate

KDG ALDOLASE

Pyruvate   Glyceraldehyde

GLYCERALDEHYDE DEHYDROGENASE $\rightharpoondown$ NADP$^+$ $\rightharpoonup$ NADPH + H$^+$

Glycerate

GLYCERATE KINASE $\rightharpoondown$ ATP $\rightharpoonup$ ADP

2-Phosphoglycerate

ENOLASE $\rightharpoonup$ H$_2$O

Phosphoenolpyruvate
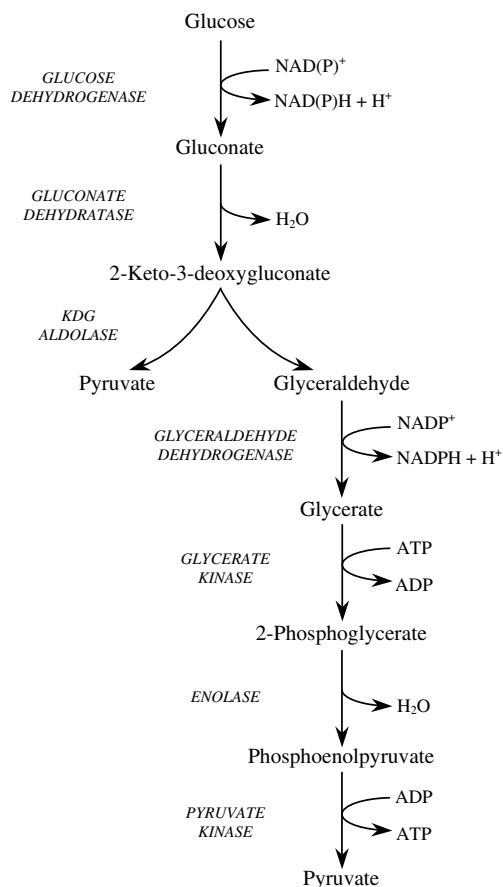
PYRUVATE KINASE $\rightharpoondown$ ADP $\rightharpoonup$ ATP

Pyruvate

Fig. 1. The non-phosphorylative Entner–Doudoroff pathway of *S. solfataricus* (reproduced from [9], with permission).
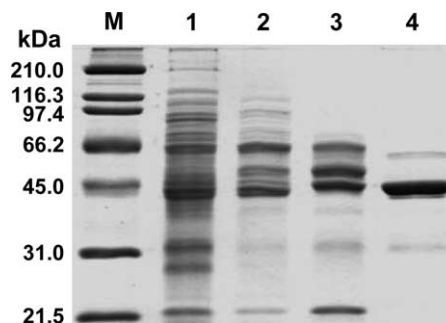

Fig. 2. SDS–PAGE gel showing samples from the purification of *S. solfataricus* gluconate dehydratase. *M*, Molecular weight markers (listed from top): Myosin, β-galactosidase, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase and trypsin inhibitor. 1 – soluble cell extract; 2 – after HiTrap Q Sepharose; 3 – after gel filtration; 4 – after hydroxyapatite.

containing 10 mM MgCl$_2$ and stored at 4 °C. Analytical gel filtration was performed with purified enzyme using a Superdex 200 10/300 GL column (1.0 cm × 30–31 cm) (Amersham BioSciences) calibrated with a molecular weight marker kit (MW-GF-200, Sigma–Aldrich).

2.2. Standard enzyme assay and protein determination

Standard assays were performed in 100 μl of 50 mM sodium phosphate buffer (pH 6.0) containing 10 mM MgCl$_2$ and 5 mM sodium gluconate. Enzyme samples were added in 1–5 μl volume and the reactions were incubated at 70 °C for 10 min before being stopped by the addition of 10 μl of 12% (w/v) trichloroacetic acid and centrifuged at 16 000 × g for 5 min. The presence of KDG in the reaction mix was quantified spectrophotometrically after reaction with thiobarbituric acid, as described previously [12]. Protein concentrations were determined by the method of Bradford [13] using a calibration curve constructed with bovine serum albumin. Selected samples from throughout the purification procedure were monitored by SDS–PAGE [14], using 10% (w/v) gels.

2.3. Enzyme characterisation

Galactonate was prepared from its γ-lactone by incubation in 1 M NaOH for 1 h and a stock solution was then prepared directly in 50 mM sodium phosphate buffer (pH 6.0). Kinetic analysis was performed on pure enzyme using the standard assay with 0–10 mM gluconate or galactonate. Kinetic parameters were determined by the direct linear method of Eisenthal and Cornish-Bowden [15]. To determine the temperature activity optimum, the standard assay was performed at a range of temperatures from 40 to 100 °C. To investigate the thermal stability of the enzyme, small aliquots were heated at 95 °C for up to 2 h, before being transferred to ice and analysed by the standard assay. A sample of enzyme was also dialysed into 20 mM Tris/HCl (pH 8.0) containing 5 mM EGTA and standard assays were then performed without any divalent metal salt and in the presence of 10 mM MgCl$_2$, MnCl$_2$ or CoCl$_2$.

2.4. Product characterisation

To confirm the identity of the enzyme products, preparative scale reactions were performed in 50 ml of 50 mM sodium phosphate buffer (pH 6.0) with 10 mM MgCl$_2$ containing 1 g gluconate or galactonate. Purified gluconate dehydratase was added and the reactions were incubated at 50 °C for 10 h. Enzyme products, KDG and KDGal, were purified by DOWEX 1X8-formate anion exchange chromatography with a 0–0.6 M formic acid elution gradient. Samples were dried before being analysed by ^{1}H NMR spectroscopy in D$_2$O using an Avance 300 machine (Bruker).

2.5. Gene sequence identification

Pure enzyme was run on a 10% (w/v) SDS–PAGE gel before being electroblotted onto a hydrophobic PVDF membrane. An excised band was subjected to tryptic digest mass spectroscopic fingerprinting, using a TofSpec-2E machine (Micromass) calibrated using the tryptic peptides of β-galactosidase. N-terminal amino acid sequencing was performed on a separate sample using a Procise 491 machine (Applied Biosystems).

3. Results

3.1. Purification of gluconate dehydratase

Gluconate dehydratase was purified to near homogeneity from cell extracts of *S. solfataricus* as assessed by SDS–PAGE (Fig. 2). Only one peak of activity was found after each chromatographic step throughout the purification process and the specific activity was enriched 134-fold (Table 1). Galactonate dehydratase activity was traced to the same peaks and enriched by an equivalent factor at each step, suggesting that a single enzyme is responsible for both activities. The M_r of the enzyme was determined to be 45 kDa by SDS–PAGE and 48 kDa by analytical gel filtration, indicating a monomeric structure.

3.2. Enzyme kinetics

Kinetic characterisation indicated a similar catalytic efficiency of gluconate dehydratase for both gluconate and galactonate (Table 2), implying a potential physiological significance to the activity with both substrates. Moreover, assays of the enzyme in the presence of both substrates demonstrated that the activities with each substrate were not additive, suggesting that they do not result from two separate enzymes. The same enzyme therefore appears to be responsible for the dehydration of both gluconate and galactonate forming KDG and KDGal, respectively. Product identity was con-

Table 1
Summary of the purification of gluconate dehydratase from *S. solfataricus*

Enzyme sample	Total protein (mg)	Total activity[a] (U)	Specific activity (U mg^{-1})	Enrichment factor	Yield[b] (%)	Galactonate dehydratase activity (%)[c]
Cell extract	209	20.2	0.1	–	–	13.7
Anion exchange	9.65	10.3	1.1	11	51	9.1
Gel filtration	0.67	2.97	4.4	44	15	10.1
Hydroxyapatite	0.05	0.67	13.4	134	3.3	9.8

[a] Activity assays were performed at pH 6.0 and 70 °C using the standard assay.
[b] Only selected fractions were transferred from the activity peak after each chromatographic step thus reducing the recorded yield.
[c] Values are expressed as a percentage of the activity with gluconate, as determined using the standard assay.

firmed in both cases by preparative scale reactions, giving ^{1}H NMR spectra identical to those observed previously [9].

3.3. Further characterisation

The enzyme was found to have a half-life of 41 ($\pm$2) min at 95 °C (Fig. 3(a)). This value contrasts with values of 10 min for glucose dehydrogenase (Heyer, N.I., Hough, D.W., and Danson, M.J., unpublished observations) and 7 h 48 min for KDG aldolase [12], at the same temperature. The maximum activity of the gluconate dehydratase was found to occur at a temperature above 100 °C (Fig. 3(b)) and the Arrhenius activation energy was determined to be 54 ($\pm$1) kJ mol^{-1}. Enzyme activity was reduced to 24% after dialysis in buffer containing EGTA, as assessed by standard assays performed without addition of any divalent metal salt. Activity was completely recovered in subsequent assays performed in the presence of 10 mM MgCl$_2$ and recovered to 39% and 62% in the presence of MnCl$_2$ and CoCl$_2$, respectively. This implies that Mg^{2+} has a catalytic role in this enzyme, as observed for other dehydratases [16].

3.4. Gene sequence determination

Using tryptic digest mass spectroscopic fingerprinting, the gene encoding gluconate dehydratase was identified from the published genomic sequence [10]. Fourteen peptide fragments were assigned and used to identify the gene as gi:13816633 (SSO3198) from a 49922 sequence archaeal database, with an 'expect value' of 3.1e^{-13}. A 5-residue N-terminal amino acid sequence, MRIRE, was also obtained providing further confirmation of the gene identity. The gene encodes a protein with a theoretical molecular weight of 44729 Da, which is consistent with the size of the protein established by SDS–PAGE and gel filtration. The gene appears 2 base pairs upstream of the KDG aldolase gene (gi:13816632, SSO3197), which implies that they may exist in an operon. A TATA box, TTTATA, as described by Reiter et al. [17], was found 23–28 base pairs upstream of the translation start site of the gluconate dehydratase gene, but not upstream of the KDG aldolase gene. A putative Shine–Dalgarno sequence, GGTGT, was found 7–11 base pairs upstream of the KDG aldolase gene, but was not found upstream of the gluconate dehydratase gene, consistent with the situation reported for other operons in *S. solfataricus* [18].

Table 2
Kinetic parameters determined for *S. solfataricus* gluconate dehydratase at 70 °C with gluconate and galactonate

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
Gluconate	1.57 ($\pm$0.08)	10.4 ($\pm$0.2)	6.65
Galactonate	0.81 ($\pm$0.04)	1.08 ($\pm$0.01)	1.33

4. Discussion

Gluconate dehydratase has been purified and characterised from the hyperthermophilic crenarchaeon *S. solfataricus*. It was found to possess activity, not only with gluconate, but also with its C-4 epimer, galactonate. Many dehydratases are members of the enolase superfamily, which possess a similar TIM-barrel fold but catalyse a number of different overall reactions. All members of this superfamily share a common partial mechanism involving the abstraction of the α-proton from a carboxylate anion, producing an enolic intermediate that is stabilised by a divalent cation [16]. Dehydratases then catalyse β-elimination of OH$^-$, involving a residue such as histidine, acting as a general acid catalyst [19]. The determined gene sequence of *S. solfataricus* gluconate dehydratase supports the prediction that this protein is a member of the enolase superfamily. The conserved glutamate residues 197, 223
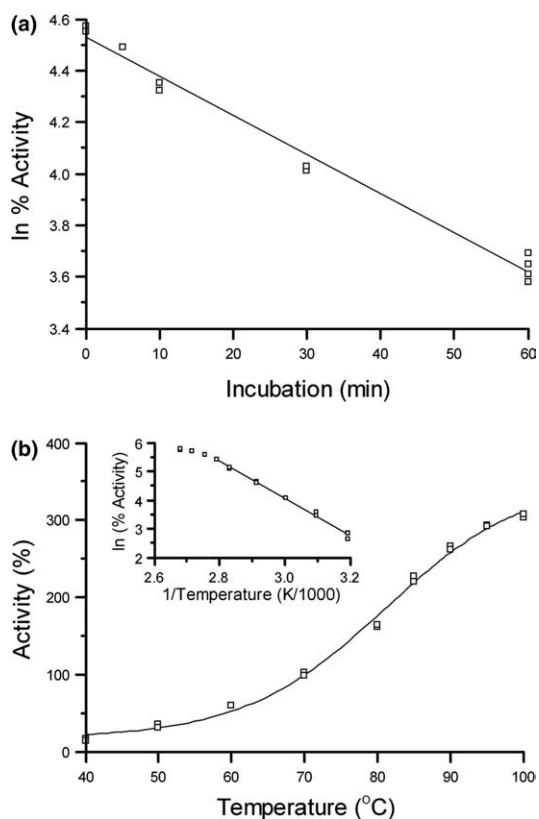

Fig. 3. Thermal inactivation (a) and thermal activity (b) profiles of the gluconate dehydratase from *S. solfataricus*. Thermal activity data are expressed as a percentage of the activity in the standard assay at 70 °C. The inset in graph (b) shows an Arrhenius transform of the data.

and 249 have been identified as the predicted ligands for Mg^{2+}, and His 199, Asp 272 and His 299 have been identified as the general acid/base catalysts involved in the reaction mechanism (J.A. Gerlt, University of Illinois, Urbana, USA, personal communication). The protein has 30% amino acid sequence identity to the structurally characterised galactonate dehydratase from *Escherichia coli* and the critical residues are conserved apart from Asp 183, which is replaced by Glu 197 [19]. An alignment of the *S. solfataricus* protein sequence alongside the *E. coli* sequence and several putative archaeal orthologues is included as supplementary information. The C-4 hydroxyl of gluconate or galactonate is not directly involved in the reaction mechanism and the enzyme is likely to accommodate both substrates by alternative active site residue interactions at this position. The $(\beta/\alpha)_8$ barrel structure provides an ideal framework for divergent evolution of an enzyme towards altered substrate specificity or reaction mechanism, as the various functional residues are positioned on separate β-strands, permitting their independent variation [20,21]. This observation may have particular significance in the case of *S. solfataricus* gluconate dehydratase, given the potential importance of enzyme catalytic promiscuity [22] and metabolic pathway promiscuity [9] in microbial evolution.

It has now been demonstrated that all the enzymes of the non-phosphorylative Entner–Doudoroff pathway can function in the metabolism of both glucose and galactose. Firstly, glucose dehydrogenase oxidises glucose and galactose forming gluconate and galactonate, respectively. Gluconate dehydratase then dehydrates both gluconate and galactonate, forming KDG and KDGal, which are both cleaved by KDG aldolase to yield pyruvate and glyceraldehyde, allowing the lower part of the pathway to proceed for both sugars. Although the dehydratase was found to have a lower activity with galactonate than gluconate, its catalytic promiscuity is likely to have physiological significance, particularly given the promiscuity of glucose dehydrogenase and KDG aldolase [9], and the constitutive expression of all three enzymes in the organism [3].

The discovery of a single central metabolic pathway for the metabolism of both glucose and galactose in *S. solfataricus* contrasts with the situation observed in other organisms. The Entner–Doudoroff pathway in its various forms and physiological modes is widely distributed among Bacteria and Archaea, and found in several eukaryotes [23]. In *Aspergillus* sp., central metabolism proceeds via the non-phosphorylative variant of the Entner–Doudoroff pathway; however, in this case separate enzymes exist for the metabolism of the two sugars [7,8,24]. A large number of both Gram-negative and Gram-positive Bacteria use the classical Entner–Doudoroff pathway for the metabolism of glucose [23,25,26]. In this pathway glucose is phosphorylated to glucose-6-phosphate, which is oxidised to 6-phosphogluconate. 6-Phosphogluconate dehydratase then catalyses the dehydration of this compound to 2-keto-3-deoxy-6-phosphogluconate (KDPG), which is cleaved by KDPG aldolase to give glyceraldehyde-3-phosphate and pyruvate. In these organisms, the Delay–Doudoroff pathway often exists as an equivalent pathway for the metabolism of galactose, involving separate, inducible enzymes [27].

S. solfataricus can grow on either glucose or galactose as the sole carbon source [1], and a single transporter has been shown to be responsible for the uptake of both sugars [28]. Furthermore, a single enzyme is responsible for both the β-glucosidase and β-galactosidase activities in the organism [29,30]. Given the discovery of a 'promiscuous' metabolic pathway, reported herein, it seems that at no point during uptake or catabolism does the organism distinguish between the two sugars. This may be indicative of a primitive evolutionary state in this hyperthermophilic organism or may simply be an adaptation to its hostile environment, allowing it to scavenge efficiently for energy substrates.

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