An extremely thermostable aldolase from *Sulfolobus solfataricus* with specificity for non-phosphorylated substrates

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Sulfolobus solfataricus is a hyperthermophilic archaeon growing optimally at 80–85 °C. It metabolizes glucose via a novel non-phosphorylated Entner–Doudoroff pathway, in which the reversible C₆ to C₃ aldol cleavage is catalysed by 2-keto-3-deoxygluconate aldolase (KDG-aldolase), generating pyruvate and glyceraldehyde. Given the ability of such a hyperstable enzyme to catalyse carbon–carbon-bond synthesis with non-phosphorylated metabolites, we report here the cloning and sequencing of the *S. solfataricus* gene encoding KDG-aldolase, and its expression in *Escherichia coli* to give fully active enzyme. The recombinant enzyme was purified in a simple two-step procedure, and shown to possess kinetic properties indistinguish-

able from the enzyme purified from *S. solfataricus* cells. The KDG-aldolase is a thermostable tetrameric protein with a half-life at 100 °C of 2.5 h, and is equally active with both D- and L-glyceraldehyde. It exhibits sequence similarity to the *N*-acetylneuraminate lyase superfamily of Schiff-base-dependent aldolases, dehydratases and decarboxylases, and evidence is presented for a similar catalytic mechanism for the archaeal enzyme by substrate-dependent inactivation by reduction with NaBH₄.

Key words: biotransformation, C–C bond, 2-keto-3-deoxygluconate.

INTRODUCTION

Sulfolobus solfataricus is a hyperthermophilic Archaeon that grows optimally at 80–85 °C and pH 2–4 [1]. With respect to its pathways of central metabolism, glucose is oxidized via a non-phosphorylated Entner–Doudoroff pathway (Scheme 1) [2], involving an NAD(P)-dependent dehydrogenation of glucose to gluconate that is then dehydrated to form 2-keto-3-deoxygluconate (KDG). KDG then undergoes an aldol cleavage catalysed by KDG-aldolase (EC 4.1.2.20):

2-Keto-3-deoxygluconate $\rightleftharpoons$ pyruvate + glyceraldehyde

Up to this point, there is no requirement for ATP. After the conversion of glyceraldehyde to glycerate, the action of a specific kinase produces 2-phosphoglycerate, which is further converted to generate a second molecule of pyruvate via enolase and pyruvate kinase.

This non-phosphorylated Entner–Doudoroff pathway has also been reported in the Archaeon, *Thermoplasma acidophilum* [3], and is used for glucose catabolism to a small extent in *Thermoproteus tenax* [4], but not in *Pyrococcus furiosus*, even though this organism contains the majority of the necessary enzymes (reviewed in [5]). This situation contrasts with that in bacteria, where the Entner–Doudoroff pathway, if present, involves phosphorylated metabolites.

In the current article, we report the purification of the *S. solfataricus* KDG-aldolase, which then permitted the cloning and expression of the gene in *Escherichia coli*, followed by characterization of the recombinant enzyme. The potential use of KDG-aldolase in its synthetic mode may overcome one of the most challenging tasks in synthetic chemistry, namely the

formation of the carbon–carbon bond. Furthermore, its inherent thermostability and ability to utilize non-phosphorylated substrates may offer significant advantages over currently available enzyme systems.

EXPERIMENTAL

Strains and plasmids

Cells of *S. solfataricus* (DSM 1616) were kindly provided by Dr. Neil Raven (Centre for Applied Microbiological Research, Porton Down, Wilts., U.K.). PCR products were cloned into the pGEM-T vector system (Promega, Madison, WI, U.S.A.). The expression vector pREC7/*NdeI* (provided kindly by Dr. L. C. Kurz, Washington University School of Medicine, St. Louis, MO, U.S.A.) was used for expression in the *E. coli* strain JM109 (Promega).

Purification of KDG-aldolase from *S. solfataricus*

Preparation of *S. solfataricus* cell extracts

Cell paste was resuspended in 20 mM Tris/HCl, pH 8.5, containing 1 mM PMSF and 1 mM EDTA, at approx. 0.2 g of cells/ml. Cells were broken by four 30-s bursts of sonication on ice with an MSE 150-W Ultrasonic Disintegrator. Soluble cell extract was obtained by centrifugation at 25000 *g* for 1 h at 4 °C. The insoluble pellet was discarded.

Heat step

The soluble cell extract was heated in glass test tubes (1-cm diameter) in a water bath at 90 °C for 15 min. Precipitated proteins were then removed by centrifugation at 13000 *g*, for 1 h, at 4 °C.

Abbreviations used: TBA, thiobarbituric acid; KDG, 2-keto-3-deoxygluconate (2-oxo-3-deoxygluconate); DHDPS, dihydrodipicolinate synthase; NAL, *N*-acetylneuraminate lyase; ORF, open reading frame.

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The nucleotide sequence data reported have been deposited in the GenBank sequence data base under the accession number AJ224174.

Scheme 1 The non-phosphorylated Entner–Doudoroff pathway of *S. solfataricus*

Ammonium sulphate precipitation

Pulverized ammonium sulphate was added to the heat-treated cell extract at 4 °C to a concentration of 65% (w/v), and the solution was maintained at this temperature for 15 min. Precipitated proteins were then removed by centrifugation at 11 000 g for 20 min at 4 °C, and the supernatant containing the KDG-aldolase activity was retained and further purified by gel filtration.

Gel filtration and ion-exchange chromatography

Gel filtration was carried out on a Sephacryl S-300 HR column (5 cm × 44 cm) in 20 mM Tris/HCl buffer, pH 8.5. A HiTrap Q anion-exchange column was equilibrated in 20 mM Tris/HCl, pH 8.5. After application of the gel-filtration fractions containing KDG-aldolase activity, the column was washed with the same buffer and the bound protein was then eluted using a gradient of 0–2 M NaCl in 20 mM Tris/HCl, pH 8.5.

Preparative PAGE

Partially purified enzyme following anion-exchange chromatography was electrophoresed in three adjacent lanes of a non-denaturing polyacrylamide gel. Gel buffers were prepared as for the subsequent SDS/PAGE, but with the omission of SDS. One lane was stained with Coomassie Brilliant Blue. The remaining two lanes were cut horizontally into 1-mm fractions; slices from one of these lanes were ground in 20 µl of SDS/PAGE loading

dye and then subjected to SDS/PAGE [6]. Slices from the second lane were ground in 50 mM sodium phosphate buffer, pH 6.0, and the resulting extract was assayed for KDG-aldolase activity using the thiobarbituric acid (TBA) assay as described below.

Analytical techniques

TBA assay of KDG-aldolase activity

KDG-aldolase activity was measured using a modification of the TBA assay [7,8]. Reactions of total volume 250 µl were incubated at 70 °C in 50 mM sodium phosphate buffer, pH 6.0, with 50 mM pyruvate and 20 mM D,L-glyceraldehyde and an appropriate volume of cell extract, or purified enzyme, between 10 and 50 µl. After 10 min, 100-µl samples were removed and the reaction stopped by the addition of 10 µl of 12% (w/v) trichloroacetic acid. Precipitated proteins were removed by centrifugation, and 50 µl of the supernatant (containing the product of the KDG-aldolase, KDG) were then oxidized by the addition of 125 µl of 25 mM periodic acid/0.25 M H₂SO₄ and incubation at room temperature for 20 min. Oxidation was terminated by the addition of 250 µl of 2% (w/v) sodium arsenite in 0.5 M HCl. TBA [1 ml, 0.3% (w/v)] was then added and the chromophore developed by heating at 100 °C for 10 min. A sample of this solution was then removed and the colour intensified by adding to an equal volume of DMSO. The absorbance was read at 549 nm, where the absorbance coefficient for the chromophore was taken to be $67.8 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [7,8]. All enzyme activities were determined in duplicate, and the S.E.M. of reported activity values was determined to be ± 3.6%.

HPLC assay of KDG-aldolase activity

Samples from aldolase assay reactions (as above) were analysed by HPLC using a Bio-Rad Aminex HPX-87H Organic Analysis column (300 mm × 7.8 mm) linked to a refractive-index detector. Reaction samples (5 µl) were injected and eluted with 8 mM H₂SO₄ at a flow rate of 1 ml/min. The system was calibrated with pyruvate, glyceraldehyde and KDG.

Protein-concentration determination

Protein concentrations were determined by the method of Bradford [9] using a calibration curve constructed with BSA.

Protein microsequencing

Following preparative gel electrophoresis, the band corresponding to KDG-aldolase activity was electroblotted on to a hydrophobic PVDF membrane. The N-terminal sequence was then determined on an Applied Biosystems 470 gas-phase sequencer, coupled to an Applied Biosystems 120 phenylthiohydantoin analyser.

Gene cloning and sequencing

Degenerate PCR primers were designed based on each end of the 38-residue N-terminal amino acid sequence obtained from the purified KDG-aldolase. PCR, using *S. solfataricus* genomic DNA as template, yielded a 114-bp product that was then radiolabelled using a High Prime DNA labelling kit (Boehringer-Mannheim, Lewes, Sussex, U.K.). This was then used as a hybridization probe to screen a *S. solfataricus* genomic DNA library in lambda phage [10]. Screening was carried out as described by Sambrook et al. [11], and a secondary screen was performed on initial positives. Lambda DNA from secondary positives was purified

using the Qiagen lambda kit, and DNA sequencing was carried out using the dideoxynucleotide chain-termination method [12] on an Applied Biosystems 377 automated DNA sequencer.

Expression and purification of recombinant enzyme

Expression

The aldolase gene from the lambda library was amplified by PCR using a 5' primer that introduced a unique *NdeI* site upstream of the initiating methionine codon and a 3' primer that introduced a unique *BamHI* site downstream of the termination codon. The PCR product was digested with the appropriate restriction endonucleases and cloned into *NdeI/BamHI*-digested pREC7 expression vector; this was then used to transform *E. coli* strain JM109. Luria broth medium (1 litre) containing carbenicillin (50 µg/ml) was inoculated with 50 ml of an overnight culture of transformed JM109 cells, and the culture was then grown at 37 °C to a D_{600} of 0.6. The cells were then induced with nalidixic acid (50 µg/ml) and grown for a further 21 h at 37 °C. Cells were harvested at 2000 *g* for 10 min and the cell pellet stored at -20 °C.

Purification of recombinant KDG-aldolase

Cell paste was re-suspended in 20 mM Tris/HCl, pH 8.5, containing 1 mM PMSF and 1 mM EDTA, at approx. 0.2 g of cells/ml. Triton X-100 (final concentration 0.1%, w/v) and lysozyme (100 µg/ml) were added, and the cell suspension incubated at 37 °C with occasional shaking for 1 h. Cell lysis was observed during this time. Complete lysis was achieved by five 30-s bursts of sonication on ice with an MSE 150-W Ultrasonic Disintegrator, after which the cell debris was removed by centrifugation at 11 000 *g* for 30 min at 4 °C.

The cell extract was heated in glass test tubes (1-cm diameter) in a water bath at 78 °C for 30 min, after which precipitated proteins were removed by centrifugation at 11 000 *g* for 20 min at 4 °C. The supernatant was then applied to a 2.6 cm × 1.5 cm column of Q Sepharose (Pharmacia) equilibrated with 20 mM Tris/HCl, pH 8.5. The column was washed with equilibration buffer to remove unbound protein, and then bound protein was eluted with a gradient of 0–2 M NaCl in 20 mM Tris/HCl, pH 8.5. Fractions (2 ml) were collected and analysed for catalytic activity and for purity by SDS/PAGE.

Characterization of the recombinant KDG-aldolase

Thermostability

Enzyme assay buffer, overlaid with mineral oil to minimize evaporation, was incubated at 100 °C in 0.5-ml sealed tubes. Once the correct temperature was reached, one-fiftieth volume of enzyme was added to each tube. At selected times, a tube was removed and frozen by plunging into a solid-CO₂/methanol bath. Tubes were stored at -20 °C until assayed for KDG-aldolase activity using the TBA assay described above.

Determination of relative molecular mass (M_r)

The M_r value of the KDG-aldolase was determined by centrifugal analysis on a Beckman XL-A analytical ultracentrifuge at the U.K. National Centre for Macromolecular Hydrodynamics, University of Nottingham, Nottingham, U.K. Sedimentation equilibrium experiments were carried out at 20 °C and three rotor speeds (10 000, 15 000 and 20 000 revs./min) were employed. Three solute concentrations (0.1, 0.3 and 1 mg of protein/ml)

were used, and data were captured at 236 and 280 nm, using the scanning absorption optical system. Data were analysed using the program NONLIN [13], fitting to both single species and associating system models. Sedimentation velocity experiments were carried out at 20 °C and 40 000 rev./min. Three solute concentrations (0.4, 0.6 and 0.8 mg of protein/ml) were used, and data were analysed using the program Svedberg (Amgen, Thousand Oaks, CA, U.S.A.).

The diffusion coefficient of the KDG-aldolase was determined by dynamic light scattering at 20 °C on a DynaPro 801 Dynamic Light Scattering Instrument (Protein Solutions, High Wycombe, Bucks, U.K.), using a sample at 2 mg of protein/ml [14].

Reduction with NaBH₄

A method similar to that used by Aisaka et al. ([15], and references therein) was used to investigate the possible involvement of a Schiff-base mechanism in the KDG-aldolase reaction. Enzyme was incubated at room temperature in 50 mM sodium phosphate buffer, with 100 mM NaBH₄ in the presence or absence of saturating concentrations of pyruvate or D,L-glyceraldehyde (10 and 20 mM, respectively). After 10 min the samples were dialysed twice against 2 l of 20 mM Tris/HCl, pH 8.5, and assayed for KDG-aldolase activity in the TBA assay.

RESULTS

Purification of native KDG-aldolase

The purification schedule used to obtain KDG-aldolase for N-terminal sequencing is summarized in Table 1. Briefly, as described in the Experimental section, purification from cell extracts involved a heat treatment, an ammonium sulphate fractionation, and chromatography by gel filtration and anion exchange. Samples after each step were analysed for catalytic activity, and for purity by SDS/PAGE. Approx. 15 µg of protein from the peak fraction following anion-exchange chromatography, which had a specific activity of 1.9 units/mg, was subjected to preparative PAGE. When the gel was analysed as described in the Experimental section, a major band of $M_r \approx 32 000$ was identified as the only protein that possessed KDG-aldolase activity. Protein from this band was eluted, subjected to SDS/PAGE and blotted on to PVDF membrane. The major band was excised and its N-terminal sequence was determined to be: PEIPIITPFTKDNRIDKEKLKIHAENLIRKGIDKLF.

Cloning of the KDG-aldolase gene from *S. solfataricus*

Amplification of *S. solfataricus* genomic DNA using oligonucleotide primers based on the 38-amino-acid N-terminal sequence yielded a 114-bp fragment. This was cloned into pGEM-T vector without further modification and sequenced. As expected, the sequence was found to contain an insert with a nucleotide sequence corresponding to the determined N-terminal amino acid sequence of the KDG-aldolase. The 114-bp PCR fragment was radiolabelled and used to screen an *S. solfataricus* genomic DNA library. A small number of the positive plaques obtained were used in a secondary screen. Lambda DNA from a positive plaque from the secondary screen was purified, and the sequence of the KDG-aldolase gene was obtained by direct sequencing of both strands.

Nucleotide sequence analysis

The complete nucleotide sequence and deduced amino acid sequence of the *S. solfataricus* KDG-aldolase gene are shown in

Table 1 Purification of KDG-aldolase from *S. solfataricus*

Step	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Fold purification
Cell homogenate	1420	34.7	0.025	100	1
Soluble cell extract	1220	32.5	0.027	93	1
90 °C heat step	1040	28.1	0.027	81	1
65% (w/v) ammonium sulphate supernatant	71	16.6	0.24	71	10
Sephacryl-300 gel filtration	7.4	4.1	0.55	12	23
HiTrap Q anion exchange	2.7	2.9	1.1	8	44

-90 -70 -50 -30 -10
 TAATAGAACAGCTAAGAGCTGAACCAATACCATAGATGTAATTGAAGAACCGGTTTGGGTCGTCAGGGAACCTGGAAGAATTATGGTGTGAGG
 10 30 50 70 90
 ATGCCAGAAATCATAACTCCAATCATAACCCCATTCCTACTAAAGATAATAGAATAGATAAGGAAAAATTAAGATACATGCGGAGAATCTCATTAGGA
 M P E I I T P I I T P F T K D N R I D K E K L K I H A E N L I R K
 110 130 150 170 190
 AGGGAATAGATAAGTTGTTGCTCAACGGTACTACTGGTCTTGGTCTTCTCGTTATCTCCAGAGGAGAAGTTAGAGAAGTTAAAGGCAGTTTATGACGT
 G I D K L F V N G T T G L G P S L S P E E K L E N L K A V Y D V
 210 230 250 270 290
 CACCAATAAGATAATATTCAAGTTGGTGGATTGAATCTAGACGATGCTATAAGATTGGCTAAATTAAGTAAAGACTTTGATATTGTCGGTATAGCC
 T N K I I F Q V G G L N L D D A I R L A K L S K D F D I V G I A
 310 330 350 370
 TCGTATGCTCCATATTATTACCCAAGAATGTCTGAGAAGCATTGGTAAAGTATTTAAGACCTTGTGTGAAGTATCTCCACACCTGTCTATTGT
 S Y A P Y Y Y P R M S E K H L V K Y F K T L C E V S P H P V Y L Y
 390 410 430 450 470
 ACAATTACCCGACGGCAACGGGAAAAGACATAGATGCAAAAGTCGCTAAAGAGATAGGCTGTTTACTGGAGTAAAGGATACTATTGAAACATAAT
 N Y P T A T G K D I D A K V A K E I G C F T G V **K** D T I E N I I
 490 510 530 550 570
 TCACACCTTAGACTACAACGCTCTAAATCCTAACATGTTAGTATATAGTGGCTCTGATATGTTAATAGCAACGGTAGCTTCTACGGGTTTATAGTGGT
 H T L D Y K R L N P N M L V Y S G S D M L I A T V A S T G L D G
 590 610 630 650 670
 AATGTTGCAGCAGGTTTCGAATTATCTTCCAGAGGTTACTGTGACAATTAAGAAATTGGCTATGGAAGGAAAAATTGATGAAGCACTTAAGTTACAAT
 N V A A G S N Y L P E V T V T I K K L A M E R K I D E A L K L Q F
 690 710 730 750 770
 TCCTTCATGACGAGGTAATAGAGGCGTCTAGAATATTTGGGAGCTTATCTTCAAATTACGTATTACCAAGTATTTCCAAGGATACGATTTAGGATA
 L H D E V I E A S R I F G S L S S N Y V L T K Y F Q G Y D L G Y
 790 810 830 850 870
 TCCTAGACCTCCAATATTTCCACTAGATGATGAAGAAGAAAGGCGACTAATTAAGAAAGTTGAGGGTATAAGGGCGAAACTTGTAGAGCTTAAATA
 P R P P I F P L D D E E E R Q L I K K V E G I R A K L V E L K I
 890 910 930 950 970
 TTGAAAGAATAGTACTATCATGTTGATGTAATAGCTTTGGGAGAGCCTTAAATCCAATTAACCTCTTTAACCCCTGGTCCGTTGAGATTGCTAA
 L K E *
 990 1010 1030 1050
 ACTATTTTAAAAACATGTAGCAGGATCTGAGTTAAATTTCTGCATTGCTGTTGTTAGGAATCATTTATCATGTGTTTAAATAGCAAGAGTAGGGAA

Figure 1 Nucleotide sequence of the KDG-aldolase gene from *S. solfataricus* and its flanking regions

The nucleotide sequence of a 1165-bp fragment is shown, with the region encoding the KDG-aldolase numbered from 1–882 (including the ATG start codon); the deduced amino acid sequence is shown below this sequence in the one-letter code. The proposed active-site lysine residue (K155) is shown boxed and in bold type. The upstream (97-bp) and downstream (186-bp) nucleotide flanking sequences of the gene are also shown.

Figure 1. The ATG initiation codon was identified by the exact match of the deduced amino acid sequence following the methionine with that determined from the purified protein. The gene encodes a polypeptide of 293 amino acids with an M_r of 32980.

Database searches and sequence alignments were performed using Basic Local Alignment Search Tool (BLAST) [16]. The highest scores were observed with dihydrodipicolinate synthase (DHDPS; EC 4.2.1.52) from *Haemophilus influenzae* (30% identity), *Bacillus subtilis* (29%) and *E. coli* (28%), and the *N*-acetylneuraminase lyase (NAL; EC 4.1.3.3) of *H. influenzae* (28%) and *E. coli* (26%). Significant identities were also seen with the *Rhizobium meliloti* MosA protein (27%) and the probable 5-dehydro-4-deoxyglucarate dehydratase from *B. subtilis* (26%). Throughout these enzymes, an active-site lysine is found that forms a Schiff-base intermediate with the pyruvate substrate [17,18]. Despite its low overall sequence identity with

these proteins, the *S. solfataricus* KDG-aldolase also has this conserved lysine residue (K155; Figure 2). Further evidence that KDG-aldolase is a member of the NAL superfamily is provided by the fact that it possesses three of the other five residues identified by Lawrence et al. [19] as strictly conserved in all members of the superfamily. Two of these (G45 and Y137) correspond to residues involved in stabilizing the intermediate enzyme–substrate complex, and the other (G201) occurs at the N-terminus of a β -strand.

Expression and purification of the *S. solfataricus* KDG-aldolase gene in *E. coli*

Recombinant KDG-aldolase was expressed using pREC7-transformed JM109 cells grown to logarithmic phase in Luria broth and induced with nalidixic acid for 21 h at 37 °C. The specific

			*
Aldolase	<i>S. solfataricus</i>	(148)	IGCFTGVKDTIENIIHT
NAL	<i>E. coli</i>	(158)	LPGVGALKQTSGLDYQM
	<i>H. influenzae</i>	(157)	NPKVLGVKFTAGDFYLL
DHDPS	<i>E. coli</i>	(154)	VKNIIIGIKEATGNLTRV
	<i>H. influenzae</i>	(160)	IENIVGIKEATRDVSR I
	<i>B. subtilis</i>	(156)	IPNVVAIKEASGDLEAI

Figure 2 Comparison of the amino acid sequence of *S. solfataricus* KDG-aldolase with members of the NAL superfamily

NAL from *E. coli* (P06995) and *H. influenzae* (P44539), and DHDPS from *E. coli* (P05640), *H. influenzae* (P43797) and *B. subtilis* (Q04796) were aligned with *S. solfataricus* KDG-aldolase. Numbers in parentheses refer to the relevant entry in the SWISSPROT database; the alignment was produced using CLUSTAL [32]. The conserved active-site lysine residue is marked with an asterisk.

Table 2 Kinetic parameters of KDG-aldolase

Data were analysed using the direct linear plot [33]; values in parentheses are standard errors.

	K_m (mM)	V_{max} (units/mg)
Wild-type KDG-aldolase (semi-purified)		
D,L-Glyceraldehyde	3.6 (± 0.2)	8.4 (± 0.2)
Pyruvate	0.9 (± 0.2)	6.3 (± 0.2)
Recombinant KDG-aldolase (purified)		
D,L-Glyceraldehyde	5.2 (± 0.1)	17.1 (± 0.4)
D-Glyceraldehyde	3.9 (± 0.3)	18.0 (± 1.0)
L-Glyceraldehyde	7.1 (± 0.6)	18.0 (± 1.9)
Pyruvate	1.0 (± 0.1)	15.7 (± 0.3)

activity of the enzyme was 0.5 unit/mg of protein, a value 18-fold higher than endogenous activity in *S. solfataricus* cells. A heat step of 78 °C for 30 min followed by anion-exchange

chromatography yielded enzyme with a specific activity of 9 units/mg of protein, which was shown to be homogeneous by SDS/PAGE.

Characterization of recombinant KDG-aldolase

Thermoactivity and thermostability

KDG-aldolase activity was measured in the direction of KDG synthesis using a modification of the TBA assay. The standard assay temperature in all studies reported in this paper was 70 °C, at which temperature the linearity of the assay with time was demonstrated for both native and recombinant enzymes, as was the measurement of initial rates. Using this assay, the recombinant protein was found to possess similar catalytic properties to the native enzyme, both proteins displaying Michaelis–Menten kinetics (Table 2). No activity could be detected with glyceraldehyde 3-phosphate as substrate in place of glyceraldehyde or with the pyruvate analogues β -hydroxypyruvate and α -keto-butyrate. It has been reported that β -hydroxypyruvate is an inhibitor of *E. coli* NAL [19], and we have found that this compound is also an inhibitor of *S. solfataricus* KDG-aldolase ($K_i = 0.3$ mM).

Using the purified recombinant enzyme, the KDG-aldolase was shown to have equal catalytic activity towards D- and L-glyceraldehyde, although there are minor differences in their respective K_m values. For assays carried out on the racemic mixture, D-glyceraldehyde will act as a competitive inhibitor of the L-glyceraldehyde, with a K_i equal to its K_m , and vice versa. In this situation, the rate equation describing the velocity (v) observed is:

$$v = \frac{(V_{max}^D \cdot S^D / K_m^D) + (V_{max}^L \cdot S^L / K_m^L)}{1 + S^D / K_m^D + S^L / K_m^L}$$

where the concentrations (S) and kinetic constants (K_m and V_{max}) of the D- and L-glyceraldehydes are indicated by their respective superscripts ([20], p. 141). Given identical V_{max} values for the two

Figure 3 Temperature-dependence of KDG-aldolase activity

Purified recombinant KDG-aldolase was assayed as described in the Experimental section at temperatures from 20 to 100 °C. Activities are expressed as a percentage of the activity at 70 °C, the routine assay temperature. An Arrhenius plot of these data is shown in the inset.

Figure 4 Thermal inactivation of KDG-aldolase from *S. solfataricus*

Purified recombinant KDG-aldolase was incubated at 100 °C for up to 12 h, as described in the Experimental section. After rapid cooling, residual enzymic activity was measured at each time point in the normal aldolase assay.

substrates, the K_m that would be determined from assays with the racemic mixture can be calculated from the rate equation to be:

$$K_m = \frac{2K_m^D \cdot K_m^L}{K_m^D + K_m^L}$$

From the values given in Table 2 for the individual substrates, it was therefore predicted that the value of K_m for the racemic mixture would be 5.0 mM, as indeed was observed.

KDG-aldolase activity was confirmed using the HPLC assay. Incubation of pyruvate and glyceraldehyde in the presence of purified enzyme resulted in utilization of both substrates in equimolar quantities and the concomitant appearance of a product peak with a retention time identical to the KDG marker.

KDG-aldolase is active over a wide range of temperatures (Figure 3), with activity approximately doubling with every 10 °C rise in assay temperature between 30 and 80 °C. An Arrhenius plot of these data is linear over this temperature range (Figure 3, inset), but a break is observed at 80 °C. However, as indicated from the thermostability data described below, the lower-than-expected activity above 80 °C is not due to inactivation of the enzyme (only a 3 % loss of activity is expected at 100 °C over the 10-min period of the assay), and we have shown that there is no significant destruction of the substrates at these temperatures. As expected, therefore, the recombinant enzyme is extremely thermostable under normal assay conditions. This was also found to be the case in the absence of substrates, the enzyme having a half-life of 2.5 h at 100 °C (Figure 4) and 7.8 h at 95 °C, as measured by the rate of irreversible thermal inactivation.

Determination of M_r

Fitting of nine data sets from the equilibrium centrifugation analysis at three different rotor speeds and covering a protein concentration range of 0.1–1.0 mg/ml demonstrated that the recombinant KDG-aldolase exists as a single species of M_r ($\pm$ S.E.) 133 000 ($\pm$ 14 000). From a sequence polypeptide M_r of 32 980, it is concluded that the enzyme comprises four identical subunits. This was confirmed by sedimentation velocity analysis, which showed a single species of $s_{20,w}^0 = 7.0(\pm 0.3)$ S. The diffusion coefficient ($D_{20,w}^0$) of the KDG-aldolase was determined by dynamic laser-light scattering to be $5.4(\pm 0.1) \times 10^{-11}$ m²/s, giving, via the Svedberg equation, an M_r of 126 000 ($\pm$ 6000).

Table 3 Effect of NaBH₄ reduction on catalytic activity of KDG-aldolase

KDG-aldolase was incubated with NaBH₄ in the presence and absence of the substrates pyruvate and glyceraldehyde, as described in the Experimental section.

Incubation conditions	Activity remaining (%)
Enzyme	100
Enzyme + NaBH ₄	78
Enzyme + NaBH ₄ + pyruvate	31
Enzyme + NaBH ₄ + D,L-glyceraldehyde	90

Reduction with NaBH₄

The involvement of a Schiff-base mechanism in the KDG-aldolase reaction was examined by treating the enzyme with sodium borohydride in the presence or absence of substrates pyruvate and D,L-glyceraldehyde in a similar method to that of Aisaka et al. ([15], and references therein). The results shown in Table 3 indicate the participation of a Schiff-base mechanism in catalysis; 69 % inactivation of the enzyme was observed in the presence of NaBH₄ and pyruvate, and complete inactivation was observed when the reaction time was increased to 20 min.

Effect of metal ions

Aldolases can be categorized as type-I enzymes, which form a Schiff base with the donor substrate and an active-site lysine residue, and type-II enzymes, which require a metal-ion cofactor. Using the standard assay, KDG-aldolase activity was unaffected by the presence of 0.1 mM ZnCl₂ and there was no decrease in activity in the presence of 50 mM EDTA. This is in contrast with the thermostable type-II aldolase from *Methanococcus janaschii*, which was inactivated in the presence of EDTA and re-activated by addition of Zn²⁺ ions [21].

DISCUSSION

Aldolases in biotransformations

In the field of organic synthesis, complex structures are often difficult to prepare by conventional chemical means. Consequently, the use of enzymes to effect specific synthesis under mild reaction conditions, without the need for protecting groups and with chiral control, is gaining in favour. Stereoselective carbon-carbon-bond synthesis is a demanding synthetic challenge in this area, and aldolases, which mediate this reaction through the aldol condensation of an aldehyde acceptor with a ketone donor, are therefore of particular interest [22–24].

Over 30 different aldolases have been identified, and these can be divided into two types. Type-I aldolases, which are found primarily in animals and higher plants, form a Schiff-base intermediate and do not require a metal cofactor, whereas type-II aldolases, found predominantly in micro-organisms, use Zn²⁺ as a cofactor. Fructose-1,6-bisphosphate aldolase, a type-I aldolase from rabbit muscle, has been studied extensively. In common with many other aldolases, mostly type-II enzymes of microbial origin, this enzyme uses dihydroxyacetone phosphate as ketone donor. These aldolases have all been studied to assess their potential as biotransformation catalysts [25].

Clearly, in biotransformation reactions there would be a considerable advantage in using an aldolase that does not require

phosphorylated substrates. Such enzymes are known, one group of which uses pyruvate as the donor substrate. The best characterized is neuraminic acid lyase/aldolase (NAL), a type-I aldolase that catalyses the condensation of pyruvate with *N*-acetyl-D-mannosamine to form *N*-acetylneuraminic acid (sialic acid). Although the enzyme has a catabolic function *in vivo*, synthesis of the aldol product can be achieved by using an excess of pyruvate [26]. NAL is highly specific for pyruvate as donor substrate, but will utilize a wide range of compounds as acceptors, including hexoses, pentoses and tetroses of both D- and L-configuration. In addition to specificity studies [27], the application of NAL in an efficient process for production of neuraminic acid, with potential for scale-up, has been described [28].

In addition to its substrate specificity, a second important feature for any enzyme used in a biotechnological process is its stability, which not only limits the conditions under which it can be used but may determine to a large extent the economics of the particular process. In this regard, extremozymes, which are enzymes from organisms growing in extreme environments (extremophiles), may be particularly useful (reviewed in [29]). That is, extremozymes possess stabilities commensurate with the growth conditions of the parent organism, and extremophiles are therefore a rich source of novel and hyperstable enzymes.

KDG-aldolase from *S. solfataricus*

In the context of these requirements, we have chosen to characterize the enzyme KDG-aldolase from the hyperthermophilic Archaeon, *S. solfataricus*. This aldolase uses non-phosphorylated substrates, pyruvate and glyceraldehyde, and was predicted to be highly thermostable, since *S. solfataricus* grows optimally at 80–85 °C. Moreover, the product of the reaction, 2-keto-3-deoxygluconate, contains four different oxidation states of carbon in four contiguous carbons, and therefore provides a framework for a number of sugar transformations in aqueous solution without the need for protecting groups [22].

To this end, we purified the *S. solfataricus* KDG-aldolase and, from the determined N-terminal sequence, were able to generate by PCR amplification an oligonucleotide probe that then allowed us to clone and sequence the gene. Expression in *E. coli* permitted the easy purification of active recombinant enzyme that was then shown to be a tetrameric protein with a high thermal stability.

Kinetically, the enzyme was indistinguishable from the native KDG-aldolase purified from *S. solfataricus* cells. One interesting feature with respect to the enzyme's thermostability and thermoactivity is that the enzyme shows the expected increase in catalytic activity with temperature up to 80 °C, the growth temperature of the parent organism, but then the activity is considerably less than expected at 90 and 100 °C. Over the time period of the assay, there is no significant thermal inactivation of the enzyme at these temperatures, and therefore it is possible that the enzyme undergoes a reversible dissociation or unfolding affecting catalytic activity. This effect has also been proposed for *Thermoanaerobacter* 3-phosphoglycerate kinase [30], and emphasizes the point that thermostability alone does not guarantee thermoactivity.

Relationship to other aldolases

Analysis of the translated amino acid sequence has shown the *S. solfataricus* KDG-aldolase to be a putative member of the NAL enzyme superfamily of Schiff-base-dependent aldolases, dehydratases and decarboxylases [18], showing 28 and 29% identity respectively with NAL and DHDPS. Both NAL and DHDPS catalyse reactions involving C–C bond formation with

pyruvate as the ketone donor, utilizing a protonated lysine to form a Schiff-base intermediate with the keto-group of the substrate. This lysine is conserved in all members of the superfamily and has been identified in the three-dimensional structure of both NAL and DHDPS. A lysine in the sequence of KDG-aldolase (K155; Figure 2) aligns well with this conserved lysine residue, suggesting that the *Sulfolobus* enzyme employs a similar reaction mechanism. As in the case of the other type-I aldolases, this has been confirmed by the sodium borohydride-induced inactivation of the enzyme in the presence of pyruvate. Furthermore, KDG-aldolase activity is not affected by the presence of EDTA or Zn²⁺, confirming that it is not a member of the type-II (metal-ion-dependent) family. Finally, the enzyme was shown to be a tetramer, a further characteristic of the type-I aldolases that is distinct from the type-II enzymes, which are generally dimeric.

The *S. solfataricus* KDG-aldolase shows no significant sequence similarity to microbial 2-keto-3-deoxyphosphogluconate aldolases, which catalyse the aldol condensation of pyruvate and glyceraldehyde 3-phosphate, and the enzyme shows no activity with glyceraldehyde 3-phosphate as substrate. The three-dimensional structure of 2-keto-3-deoxyphosphogluconate aldolase from *Pseudomonas putida* has been determined [31]. It also has an α/β barrel structure, but in this case the enzyme is a trimer. The reaction mechanism again proceeds via a Schiff-base intermediate, involving an active-site lysine residue, and appears to resemble that of mammalian fructose-bisphosphate aldolase.

Gene sequence

The protein-coding sequence of the KDG-aldolase gene was identified by comparison of the translated base sequence with the determined N-terminal amino acid sequence, where an exact match was found. However, we could not find a recognizable Shine–Dalgarno sequence upstream of the start codon, nor a possible promoter site in the –25 to –50-bp region. Moreover, there is no good potential transcriptional stop signal (poly-dT tract) downstream of the KDG-aldolase open reading frame (ORF). The absence of these regions is suggestive of the aldolase gene being part of an operon structure, and translation of the flanking sequences is consistent with this possibility. The upstream sequence (97 bp) translates into a potential ORF that terminates 2 bp before the aldolase ATG start codon; however, no matches to this partial sequence were found in the databases. A second potential ORF (180 bp) begins 9 bp downstream of the aldolase gene, and this partial sequence, when aligned with the protein databases, shows the highest identity score with a *Bacillus* fructokinase (41%). The metabolic significance of the flanking genes and the possible operon structure will only become apparent when further and more extensive sequencing is carried out; clearly, this is beyond the scope of the current paper.

Concluding remarks

The significance of the *S. solfataricus* KDG-aldolase in biotransformations and organic syntheses lies in its thermostability and its catalytic activity with non-phosphorylated substrates. Future applications may require the manipulation of the enzyme structure to recognize other substrates, and to this end we have crystallized the KDG-aldolase and collected diffraction data to 2.2 Å (E. Hendry, C. L. Buchanan, D. W. Hough, M. J. Danson and G. L. Taylor, unpublished work). Structural analysis is in progress.

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REFERENCES

- Grogan, D. W. (1989) *J. Bacteriol.* **171**, 6710–6719
- De Rosa, M., Gambacorta, A., Nicolaus, B., Giardina, P., Poerio, E. and Buonocore, V. (1984) *Biochem. J.* **224**, 407–414
- Budgen, N. and Danson, M. J. (1986) *FEBS Lett.* **196**, 207–210
- Siebers, B. and Hensel, R. (1993) *FEMS Microbiol. Lett.* **111**, 1–8
- Selig, M., Xavier, K. B., Santos, H. and Schonheit, P. (1997) *Arch. Microbiol.* **167**, 217–232
- Laemmli, U.K. (1970) *Nature (London)* **227**, 680–685
- Skoza, L. and Mohos, S. (1976) *Biochem. J.* **159**, 457–462
- Gottschalk, G. and Bender, R. (1982) *Methods Enzymol.* **90**, 283–287
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 284–255
- Connaris, H., West, S. M., Hough, D. W. and Danson, M. J. (1998) *Extremophiles* **2**, 61–66
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor Press, Cold Spring Harbor
- Sanger, F., Nicklem, S. and Coulson, A. R. (1977) *Proc. Natl Acad. Sci. U.S.A.* **74**, 5463–5467
- Johnson, M. L., Correia, J. J., Yphantis, D. A. and Halvorsen, H. R. (1981) *Biophys. J.* **36**, 575–588
- Claes, P., Dunford, M., Kenney, A. and Vardy, P. (1992) In *Laser Light Scattering in Biochemistry* (Harding, S.E., Sattelle, D. B. and Bloomfield, V. A., eds.), pp. 66–76, Royal Society of Chemistry, Cambridge
- Aisaka, K., Igarashi, A., Yamaguchi, K. and Uwajima, T. (1991) *Biochem. J.* **276**, 541–546
- Atschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990) *J. Mol. Biol.* **215**, 403–410
- Izard, T., Lawrence, M. C., Maltby, R. L., Lilley, G. G. and Coleman, P. M. (1994) *Structure* **2**, 361–369
- Babbitt, P. C. and Gerlt, J. A. (1997) *J. Biol. Chem.* **272**, 30591–30594
- Lawrence, M. C., Barbosa, J. A. R. G., Smith, B. J., Hall, N. E., Pilling, P. A., Ooi, H. C. and Marcuccio, S. M. (1997) *J. Mol. Biol.* **226**, 381–399
- Wharton, C. W. and Eisinger R. (1981) *Molecular Enzymology*, Blackie and Son, Glasgow
- Choi, I.-G., Cho, S. K. and Yu, Y. G. (1998) *J. Biochem. Mol. Biol.* **31**, 130–134
- Shelton, M. C., Cotterill, I. C., Novak, S. T. A., Poonwala, R. M., Sudarshan, S. and Toone, E. J. (1996) *J. Am. Chem. Soc.* **118**, 2117–2125
- Takayama, S., McGarvey, G. J. and Wong, C.-H. (1997) *Ann. Rev. Microbiol.* **51**, 285–310
- Fessner, W.-D. (1998) *Curr. Opin. Chem. Biol.* **2**, 85–97
- Wong, C.-H., Halcomb, R. L., Ichikawa, Y. and Kajimoto, T. (1995) *Angew. Chem. Int. Ed. Engl.* **34**, 412–432
- Uchida, Y., Tsukada, Y. and Sugimori, T. (1984) *J. Biochem. (Tokyo)* **96**, 507–522
- Fitz, W., Schwark, J.-R. and Wong, C.-H. (1995) *J. Org. Chem.* **60**, 3663–3670
- Mahmoudian, M., Noble, D., Drake, C. S., Middleton, R. F., Montgomery, D. S., Piercey, J. E., Ramlakhan, D., Todd, M. and Dawson, M. J. (1997) *Enz. Microb. Technol.* **20**, 393–400
- Hough, D. W. and Danson, M. J. (1999) *Curr. Opin. Chem. Biol.* **3**, 39–46
- Thomas, T. M. and Scopes, R. K. (1998) *Biochem. J.* **330**, 1087–1095
- Mavridis, I. M., Hatada, M. H., Tulinsky, A. and Lebioda, L. (1982) *J. Mol. Biol.* **162**, 419–444
- Higgins, D. G. and Sharp, P. M. (1988) *Gene* **73**, 237–244
- Eisinger, R. and Cornish-Bowden, A. (1974) *Biochem. J.* **139**, 715–720

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