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Cloning, Isolation and Characterization of the *Thermotoga Maritima* KDPG Aldolase

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Abstract—The *Thermotoga maritima* aldolase gene has been cloned into a T7 expression vector and overexpressed in *Escherichia coli*. The preparation yields 470 UL^{-1} of enzyme at a specific activity of 9.4 $U\,mg^{-1}$. During retroaldol cleavage of KDPG, the enzyme shows a k_{cat} that decreases with decreasing temperature. A more than offsetting decrease in K_m yields an enzyme that is more efficient at 40 °C than at 70 °C. The substrate specificity of the enzyme was evaluated in the synthetic direction with a range of aldehyde substrates. Although the protein shows considerable structural homology to KDPG aldolases from mesophilic sources, significant differences in substrate specificity exist. A preparative scale reaction between 2-pyridine carboxaldehyde and pyruvate provided product of the same absolute configuration as mesophilic enzymes, but with diminished stereoselectivity.

Introduction

The aldolases catalyze reversible stereoselective carbon–carbon bond formation. As one of the relatively small class of carbon–carbon bond forming enzymes, this group has been investigated extensively for utility in synthetic chemistry. In this context the aldolases are typically divided into three broad groups according to nucleophile: those that use dihydroxyacetone phosphate (DHAP),[1,2] those that use acetaldehyde,[3–6] and those that use pyruvate or phosphoenolpyruvate.[7]

2-Keto-3-deoxy-6-phosphogluconate (KDPG) and 2-keto-3-deoxy-6-phosphogalactonate (KDPGal) aldolases are pyruvate aldolases central to the Entner–Doudoroff and DeLay–Doudoroff glycolytic pathways, respectively. In this metabolic route glucose and galactose are converted to the corresponding 3-deoxy-6-phosphohexulosonate and then cleaved enzymatically to pyruvate and D-glyceraldehyde-3-phosphate (Scheme 1). We have previously reported the use of mesophilic KDPG and KDPGal aldolases as tools for stereoselective carbon–carbon bond formation.[8–10] KDPG aldolase catalyzes aldol addition of pyruvate to electrophilic aldehydes to form 4-hydroxy-2-ketobutyrates with the *S*-configuration at the newly formed stereocenter; KDPGal aldolase provides the analogous products in the opposite stereochemical series. The pyruvate aldolases provide both densely and differentially functionalized products, constructing a four-carbon backbone in which each common oxidation state of carbon is represented.

Enzymes from thermophilic organisms offer intriguing possibilities both for practical biocatalysis and for understanding structure–function relationships in protein catalysis.[11–14] Here we report the cloning, overexpression and characterization of a recombinant KDPG aldolase from the thermophilic organism *Thermotoga maritima*. The increased thermostability and altered substrate selectivity of this enzyme may provide useful properties for practical biocatalysts.

Results and Discussion

Selig et al. reported the existence of an Entner–Doudoroff glycolytic pathway in *T. maritima*.[15] Accordingly, we sought to clone and overexpress KDPG aldolase from this organism. Using the BLASTp protein

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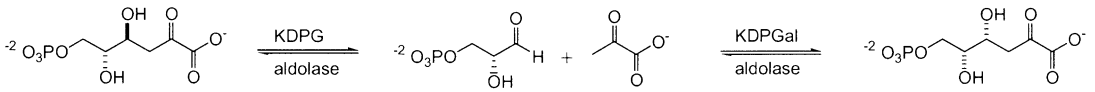


Scheme 1. In vivo reactions catalyzed by KDPG and KDPGal aldolase.

search algorithm,[16] we identified a distantly related homologue (32% identity and 50% similarity over 203 amino acids) to *E. coli* KDPG aldolase in the genomic sequence of *T. maritima*.[17,18] The homologous fragment was amplified using the polymerase chain reaction (PCR) and cloned into a pET 28-b(+) expression plasmid behind an inducible T7 RNA polymerase promoter to induce expression following treatment with isopropyl β-D-thiogalactopyranoside (IPTG).[19] Constructs were prepared encoding the putative *T. maritima* KDPG aldolase in both native form and with a hexahistidine-tag engineered onto the C-terminus of the protein to facilitate purification. A band corresponding to the predicted molecular weight for *T. maritima* KDPG aldolase (23,229 Da) was identified in induced cell-free extracts from *E. coli* BL21(DE3) transformed with the plasmid encoding this gene. The identity of the protein was confirmed by assay against authentic KDPG using a lactate dehydrogenase-coupled assay. After induction, the construct produces 470 units of enzyme per liter of cell culture (one unit of enzyme catalyzes cleavage of 1 μmol of 2-keto-3-deoxy-6-phosphogluconate to pyruvate and D-glyceraldehyde 3-phosphate per min at pH 7.5, 70 °C).

T. maritima KDPG aldolase was purified via nickel affinity chromatography. This method also removes endogenous *E. coli* KDPG aldolase. SDS–PAGE analysis of purified *Thermotoga* KDPG aldolase suggests a protein purity of 90%; enzymatic assay of this preparation shows a specific activity of 9.4 U mg^{-1} at 70 °C for catalysis of the retro aldol cleavage of KDPG. This specific activity is somewhat reduced compared to the values of 50–250 U mg^{-1} determined for KDPG aldolases from other sources.[8] Under conditions of saturating substrate the enzyme is maximally activite at 80 °C (Fig. 1), consistent with both the optimum growth temperature of *T. maritima* (80 °C) and the observed maximal activity of most enzymes isolated from this species (80–105 °C).[11] The enzyme displays significant activity over a broad temperature range, retaining roughly 15% of its activity at room temperature.

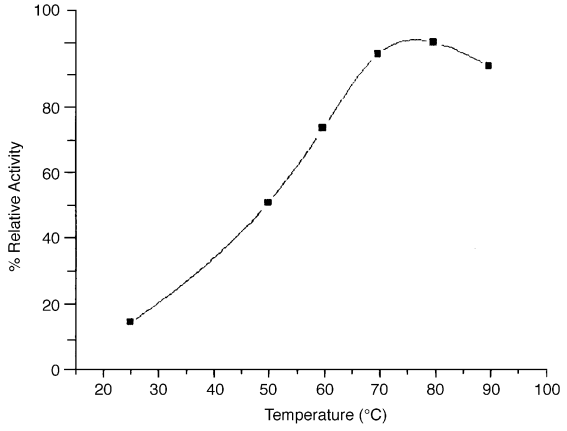


Figure 1. Temperature profile of *T. maritima* KDPG aldolase catalyzing the retroaldol cleavage of saturating KDPG assayed using the standard coupled assay with L-lactic dehydrogenase.

Kinetic parameters for the retro-aldol cleavage of KDPG were determined at two temperatures (Table 1). Over a 30 °C increase in temperature, a 1.9-fold increase in k_{cat} is accompanied by a 4-fold increase in K_m to diminish k_{cat}/K_m by a factor of two. While a drop in catalytic efficiency at higher temperatures is not an unusual feature of thermophilic enzymes, it is hardly ubiquitous.[13,20] Such temperature dependences might arise from conformational changes that interfere with substrate binding or from thermal sensitivity of the substrate. Given the mechanism of Schiff-base aldolases it is unlikely that the Michealis constant reflects a simple dissociation constant and the origin of the increase in K_m is unclear. We are currently conducting binding studies designed to establish the molecular basis of KDPG recognition by the *Thermotoga* aldolase.

The substrate specificity of *T. maritima* KDPG aldolase was explored in the synthetic direction with respect to both the electrophilic and nucleophilic substrate components (Table 2). As is the case for other KDPG aldolases, the *Thermotoga* enzyme shows a usefully broad

Table 1. Kinetic constants for the cleavage of KDPG catalyzed by *T. maritima* KDPG aldolase

Temperature (°C)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})
40	2.0±0.1	0.22±0.06	9.0
70	3.9±0.4	0.9±0.4	4.5

Table 2. Substrate specificities of *T. maritima* and *E. coli* KDPG aldolases

Nucleophile	Electrophile	*T. maritima* % relative activity[a]	*E. coli* % relative activity[a]
Pyruvate	D-Glyceraldehyde	100	100
	L-Glyceraldehyde	+	–
	D-Erythrose	+++	+
	L-Erythrose	+	–
	D-Threose	+	+
	L-Threose	+	+
	2-Pyridine Carboxaldehyde	+	++++
	Glycolaldehyde	++	+
	Valeraldehyde	–	–
	2-Furaldhyde	–	–
	Benzaldehyde	–	–
2-Ketobutyrate	D-Glyceraldehyde	+	++++
3-Fluoropyruvate	D-Glyceraldehyde	–	+

[a] – < 1%; + 1–25%; ++ 25–50%; +++ 50–100%; ++++ > 100%.

substrate specificity, accepting a range of electrophilic substrates with polar functionality at C2 or C3. Consistent with the *E. coli* enzyme, D-glyceraldehyde is preferred over the L-isomer, and D-erythrose, a substrate with the R-configuration at C2, is preferred over its enantiomer. D- and L-threose are accepted with roughly equal facility. In stark contrast to all mesophilic aldolases examined to date, 2-pyridine carboxaldehyde is only a modest substrate.[8] Simple aliphatic aldehydes and benzaldehyde are not substrates. The enzyme shows a greatly reduced tolerance for variation in the nucleophilic component, although weak activity is observed with 2-ketobutyrate.

Although the crystal structure of the *Thermotoga* enzyme has yet to be solved, inferences regarding protein–substrate interactions are possible based on recently reported structures of the *E. coli* KDPG aldolase (Fig. 2).[21,22] The hydrophobic residues that line the *E. coli* active site are conserved or homologous in the *Thermotoga* enzyme; valine 20 and isoleucine 135 are both conserved while isoleucine 92 and phenylalanines at positions 132 and 134 are replaced by a valine and two leucines, respectively, in the *Thermotoga* enzyme. The hydrophilic residues are even more stringently conserved between the two enzymes, and threonines 47, 73, and 161 are present in both proteins. Another difference between the two enzymes is the R49T modification from the *E. coli* to the *Thermotoga* enzyme. This arginine, which lies at the terminus of the putative substrate binding site, presumably interacts with a charged moiety of the native substrate—either phosphate or carboxylate—and its modification in the *Thermatoga* enzyme may explain the lower specific activity of this enzyme relative to the *E. coli* protein. Site-directed mutagenesis studies are currently underway to provide a better understanding of the roles of these residues in substrate recognition and turnover. Directed evolution studies may also broaden our understanding of these structure–function relationships.[23]

A preparative scale synthesis of 4-hydroxy-2-keto-4-(2′-pyridyl)butyrate was undertaken to definitively establish product identity and establish the stereospecificity of the enzyme (Scheme 2). The initial condensation product was converted to (2,2-dithioethyl-4-(2′-pyridyl)-4-butyro-γ-lactone for characterization; ^{13}C and ^{1}H NMR spectra were identical to those previously reported.[9] Consistent with mesophilic enzymes, the *Thermotoga* aldolase catalyzes enantioselective addition to the *si*-face

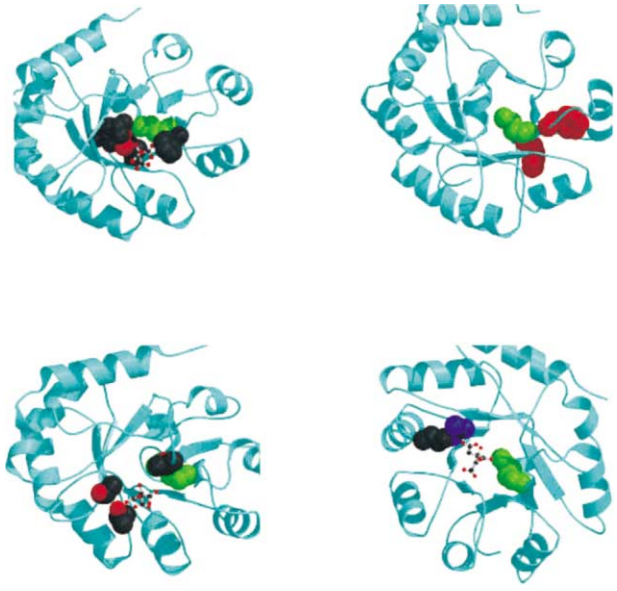
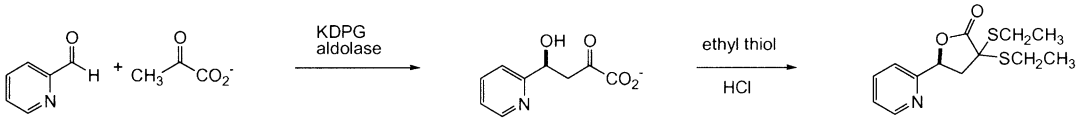


Figure 2. The binding pocket of *E. coli* KDPG aldolase. Residues are shown in relation to the position of the active site Lys133 (green). Citrate is shown in ball and stick format in panels A, C and D. Panel A (top left): position of the active site hydrophobic residues (Val20, Ile92, Phe135 (black)); panel B (top right): position of Phe132 and Phe134 (red); panel C (bottom left): position of threonines 47, 73 and 161 (black); panel D (bottom right): position of Arg49.



Scheme 2. Preparative-scale condensation of pyridine carboxaldehyde.

of the electrophile although with diminished stereoselectivity, furnishing the S-product in 50% ee.

Conclusions

We have cloned, expressed and characterized a KDPG aldolase from *T. maritima*. The enzyme shows significant differences from its mesophilic counterparts that may prove useful in the development of biocatalysts with synthetic utility. We are interpreting these differences in activity in terms of enzyme structure, and will report our results in due course.

Experimental

Materials

T. maritima genomic DNA was a generous gift from Dr. James Brown, North Carolina State University. Restriction endonucleases and T4 DNA ligase were from New England Biolabs. Oligonucleotides were purchased from Gibco BRL, *Pfu* DNA polymerase from Stratagene, and His bind nickel affinity resin from Novagen. Plasmid preparation, bacterial transformation, enzymatic manipulation of DNA and PCR DNA amplification were performed according to standard procedures.[24] XL1 Blue and BL21(DE3) strains of *E. coli* were used for cloning and overexpression, respectively.[19] UV kinetic assays were performed on a Hewlett-Packard 8453 UV–vis spectrophotometer fitted with a thermocoupled cuvette holder. ^{1}H and ^{13}C NMR spectra were recorded on a Varian 300 MHz NMR spectrometer operating at 300.071 and 75.018 MHz, respectively.

Cloning of *T. maritima* KDPG aldolase gene

The *T. maritima* KDPG aldolase gene was identified by a BLASTp search of the *E. coli eda* sequence against the complete genome of *T. maritima*.[16–18] Primers complementary to the N- and C-terminal regions of the *T. maritima* KDPG aldolase homologue were designed and the gene was amplifed by PCR (forward primer: 5′-CATGCCATGGCAATGGAAGAACTGTTCAAAAAACAC-3′; reverse primer 1: 5′-CCGCTCGAG*TCA*TTCTGTGCACCCCCTGATC-3′; reverse primer 2: 5′-CCGCTCGAGTTCTGTGCACCCCCTGATC-3′). The forward primer contains a *Nco* I restriction enzyme site (underlined) that changes the second amino acid from lysine in wild-type to alanine in the cloned sequence. The reverse primers contain a *Xho* I site for subcloning the fragment into the multiple cloning region of the pET28b vector. Reverse primer 1 contains the stop codon in the native gene (italicized) to produce the wild-type C-terminus while the sequence of the reverse primer 2 reads through to the vector sequence, adding a hexahis tag to the C-terminus. 25 Cycles of PCR amplification with *Pfu* DNA polymerase (5 U) were carried out in a 100 μL reaction containing 20 mM Tris–HCl, pH 8.75, 2 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 100 μg/mL BSA, 20 μM dNTPs, 300 ng each of 5′ and 3′ primers and 2–5 ng genomic DNA template. Each cycle consisted of 94 °C for 45 s, 55 °C for 1 min and 72 °C for 2 min, followed by a final extension at 72 °C for 7 min. The PCR product was purified using the QIAquick PCR purification kit (Qiagen) and digested with *Nco* I and *Xho* I restriction enzymes. A band of the correct size predicted for the digested KDPG aldolase gene was purified by gel electrophoresis and ligated into a T7 expression vector pET28b (Novagen) that had been digested with the same enzymes. The final DNA sequence of the *T. maritima* KDPG aldolase gene in the plasmid, pTM-eda1 (reverse primer 1) and pTM-eda2 (reverse primer 2 with His-tag), was confirmed by automated dideoxy sequencing.

Expression of active *T. maritima* KDPG aldolase

E. coli (BL-21(DE3){pTM-eda2}) was grown on 2 mL LB containing kanamycin (50 μg mL^{-1}) for 8–10 h followed by growth in a 1-L LB containing kanamycin culture in a 250 rpm shaker for 3 h at 37 °C. Protein production was induced by addition of 0.5 mM IPTG followed by incubation at 37 °C for 2 h. Cells were harvested by centrifugation, re-suspended in 20 mM KH_2PO_4, pH 6.5, and disrupted by sonication at 0 °C for 5 min. The resulting extract was filtered (0.22 μm) and applied to a nickel affinity column. The resin was washed and eluted according to the manufacturer protocol (Novagen). The final eluent was dialyzed three times against 20 mM KH_2PO_4, pH 6.5 to remove imidazole. Protein concentrations were determined either by a dye binding method using bovine serum albumin as the standard or by the method of Edelhoch.[25,26]

Enzyme kinetic parameters

KDPG aldolase activity was determined by utilizing the standard coupled assay with L-lactic dehydrogenase.[27] KH_2PO_4 (20 mM, pH 7.5, 1.0 mL), NADH (0.42 mM), KDPG (0.5–15 mM) and L-lactic dehydrogenase (EC 1.1.1.27 Type II from rabbit muscle at 40 °C, 105 U; or from *Bacillus stearothermophilus* at 70 °C, 50 U) were added to a disposable cuvette and pre-warmed to the desired temperature for 10 min. *T. maritima* KDPG aldolase (0.2 μM) was added to initiate the reaction and the disappearance of reduced cofactor was monitored for 20 min by the absorbance decrease at 340 nm. KDPG aldolase activity was determined from the initial slope of the absorbance versus time curve, and kinetic parameters were determined by fits of the Michaelis–Menten equation to velocity versus substrate concentration plot using Origin 5.0 (Microcal).

Substrate specificity

Unnatural nucleophile and electrophile assays were performed on a 500 μL scale with electrophile and nucleophile concentrations both at 50 mM. Each assay was run over a 24-h period with aliquots (50 μL) taken at intervals. Progress of the reaction was monitored by the disappearance of pyruvate. The aldolase in the aliquots was inactivated by treatment with 7% perchloric acid (20 μL), and 5 μL of each sample was then assayed

for residual pyruvate by a modification of the standard assay.[28] Buffer (KH_2PO_4, 20 mM, pH 7.5, 1.5 mL), NADH (20 μL, 15 mg mL^{-1}) and the reaction sample (5 μL) were added to a disposable cuvette. An initial UV absorbance value at 340 nm was recorded. L-lactic dehydrogenase (EC 1.1.1.27, Type II from rabbit muscle, 2.5 μL) was added to the sample. After stabilization of the absorbance, the final value was recorded. The difference in initial and final absorbance values was plotted against time to yield a curve corresponding to the loss of pyruvate. The initial slope of the curve for each unnatural nucleophile or electrophile is compared in Table 2.

Synthesis of 4-hydroxy-2-keto-4-(2′-pyridyl)butyrate (enzymatic)

Sodium pyruvate (153 mg, 1.40 mmol) and 2-pyridine carboxaldehyde (100 mg, 0.93 mmol) were dissolved in KH_2PO_4 buffer (20 mM, pH 7.5, 10 mL). *T. maritima* KDPG aldolase (466 U) was added and, following sterile filtration (22 μm), the reaction was incubated for 3 days at 37 °C and then lyophilized. The resulting solid was redissolved in hot ethanol (100 mL), the insoluble salts filtered off, and the mother liquor evaporated in vacuo. The resulting solid was used without further purification.

Synthesis of racemic 4-hydroxy-2-keto-4-(2′-pyridyl)butyrate (chemical)[29]

Oxalacetic acid (4.5 g, 35 mmol) was dissolved in deionized water and brought to pH 9.0 with NaOH (dropwise, 10 M). 2-pyridine carboxaldehyde (3.75 g, 35 mmol) was added and the pH of the resulting solution adjusted to 11.0 with NaOH (dropwise, 5 M). The pH was maintained at 11.0 with NaOH (5 M) for 2 h, and the reaction was stirred 16 h at 25 °C. The pH of the reaction was lowered to 3.5 by addition of strongly acidic (Amberlyst 131) Ion Exchange (H^+) resin. Spontaneous decarboxylation accompanied acidification. The slurry was filtered and washed with deionized water (200 mL). The pH of the eluent was adjusted to pH 7.0 and the solution was lyophilized. The residual solid was redissolved in hot ethanol (250 mL), filtered, and evaporated in vacuo. The resulting solid was used without further purification.

Synthesis of (2,2-dithioethyl-4-(2′-pyridyl)-4-butyro-γ-lactone)

Crude 4-hydroxy-2-keto-4-(2′-pyridyl)butyrate was dissolved in ethane thiol (6 mL) and concentrated HCl (2 mL) and stirred for 18 h at 22 °C. Excess ethane thiol was removed by distillation. The resulting solid was extracted with methylene chloride (3×10 mL), the combined organic extracts were washed with water (2×20 mL) and saturated bicarbonate (2×20 mL), dried ($MgSO_4$) and evaporated. Silica gel chromatography (petroleum ether/ethyl acetate, 3:1) yielded the desired dithioacetal. ^1H NMR ($CDCl_3$) δ 8.59 (d, 1H, $J=4.94$ Hz), 7.76 (dt, 1H, $J=1.92$, 7.80 Hz), 7.50 (d, 1H, $J=8.0$ Hz), 7.21 (dd, 1H, $J=4.12$, 6.99 Hz), 5.64 (dd, 1H, $J=6.32$, 9.31 Hz), 2.94–2.56 (m, 6H), 1.21 (q, 6H, $J=7.691$ Hz); ^{13}C NMR ($CDCl_3$) δ 168.290, 155.940, 148.840, 137.461, 123.468, 120.592, 78.059, 58.268, 46.031, 24.799, 24.321, 13.913, 13.750.

Chiral GC

Chiral GC was performed on a Hewlett Packard 5890A gas chromatograph using a Chrompack Chirasil-L-Val column (25 m×0.25 mm ID) at a temperature of 160 °C and pressure of 15 psi.

Acknowledgements

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