Improving low-temperature activity of *Sulfolobus acidocaldarius* 2-keto-3-deoxygluconate aldolase

SUZANNE WOLTERINK-VAN LOO,[1] MARCO A. J. SIEMERINK,[1] GEORGIOS PERRAKIS,[1] THIJS KAPER,[2] SERVÉ W. M. KENGEN[1] and JOHN VAN DER OOST[1,3]

[1] *Laboratory of Microbiology, Wageningen University, Dreijenplein 10, 6703 HB Wageningen, The Netherlands*

[2] *Genencor International, 925 Page Mill Road, Palo Alto, CA 94304-1013, USA*

[3] *Corresponding author (john.vanderoost@wur.nl)*

Summary *Sulfolobus acidocaldarius* 2-keto-3-deoxygluconate aldolase (SacKdgA) displays optimal activity at 95 °C and is studied as a model enzyme for aldol condensation reactions. For application of SacKdgA at lower temperatures, a library of randomly generated mutants was screened for improved synthesis of 2-keto-3-deoxygluconate from pyruvate and glyceraldehyde at the suboptimal temperature of 50 °C. The single mutant SacKdgA-V193A displayed a threefold increase in activity compared with wild type SacKdgA. The increased specific activity at 40–60 °C of this mutant was observed, not only for the condensation of pyruvate with glyceraldehyde, but also for several unnatural acceptor aldehydes. The optimal temperature for activity of SacKdgA-V193A was lower than for the wild type enzyme, but enzymatic stability of the mutant was similar to that of the wild type, indicating that activity and stability were uncoupled. Valine193 has Van der Waals interactions with Lysine153, which covalently binds the substrate during catalysis. The mutation V193A introduced space close to this essential residue, and the increased activity of the mutant presumably resulted from increased flexibility of Lysine153. The increased activity of SacKdgA-V193A with unaffected stability demonstrates the potential for optimizing extremely thermostable aldolases for synthesis reactions at moderate temperatures.

Keywords: biocatalysis, directed evolution, enzyme, error-prone PCR, laboratory evolution, thermophile.

Introduction

The aldol reaction leads to the formation of a new carbon–carbon bond, generating a polyhydroxylated product with at least one new chiral center. Aldolases are enzymes that reversibly catalyze the asymmetric aldol condensation reaction and are therefore of interest for bio-organic synthesis of chiral compounds (Dean et al. 2007, Bolt et al. 2008). In contrast to traditional organic synthesis, enzymatic aldol condensation is usually stereospecific, occurs under mild reaction conditions and requires no protection and deprotection steps. Unfortunately, applications of aldolases are often hampered by high enzyme production costs, limited protein stability, and the requirement for expensive (phosphorylated) substrates (Dean et al. 2007). The 2-keto-3-deoxygluconate aldolases (KdgA) found in *Sulfolobus* species are stable enzymes with high activities on non-phosphorylated (cheap) substrates (Buchanan et al. 1999, Wolterink-van Loo et al. 2007). These class I aldolases catalyze the reversible reaction of D,L-glyceraldehyde + pyruvate D,L-2-keto-3-deoxygluconate (KDG), in which a Schiff base intermediate is formed between the substrate and the catalytic lysine residue (Lamble et al. 2005*b*). Recently, 3D structures of *S. solfataricus* and *S. acidocaldarius* KdgA have been solved with covalently bound reaction intermediates in the active site (Theodossis et al. 2004, Wolterink-van Loo et al. 2007). It has been shown that these enzymes can accept a wide variety of (aldehyde) substrates, and therefore, have great potential for biotechnological applications (Lamble et al. 2003, Lamble et al. 2005*a*, Lamble et al. 2007, Wolterink-van Loo et al. 2007).

Enzymes have been modified by natural selection to perform optimally in certain biological contexts. Using these enzymes in industrial settings (e.g., in bioreactors), often requires optimization for activity, stability, selectivity and specificity (Reetz 2001). In past decades, several techniques, including rational design and random mutagenesis, have been developed to modify enzymes for specific applications (Sen et al. 2007). Enzyme catalytic rates are determined by general protein features and can be governed by long-range interactions, which are usually difficult to address by rational protein design (Kihara 2005). Therefore, the random approach of laboratory evolution seems particularly suitable for improving enzyme catalytic rates. Generally, diversity is created by error-prone PCR, followed by an activity screen or selection to identify the best mutants. Enzyme features that can be modified by random methods include stability (Shao et al. 1998, Brouns et al. 2005), substrate specificity (Xu et al. 2003, Fishman et al. 2004, van den Heuvel et al. 2004), enantioselectivity (van Loo et al. 2004, Lipovsek et al. 2007) and low-temperature catalysis (Lebbink et al. 2000, Merz et al.

2000, Sriprapundh et al. 2003, Machielsen et al. 2008). Many laboratory studies of aldolase evolution have been directed toward broadening substrate specificity improving enantio-selectivity (Bolt et al. 2008) or increasing thermostability (Hao and Berry 2004).

In this study, random mutations were introduced into the thermostable KDG aldolase of *Sulfolobus acidocaldarius* using error-prone PCR (ep-PCR). A library of 1500 variants was screened for both enhanced condensation activity with pyruvate and D,L-glyceraldehyde at 50 °C and retention of kinetic stability. A single mutant, SacKdgA-V193A, showed enhanced activity and was analyzed in detail for its biochemical properties and stability, demonstrating the feasibility of optimizing extreme thermostable aldolases for application in cascade synthetic reactions with mesophilic enzymes. To our knowledge, it is the first report of increased low-temperature activity of (hyperthermophilic) aldolases through laboratory evolution.

Materials and methods

Chemicals, strains, plasmids, and media

All chemicals were of analytical grade and were purchased from Sigma-Aldrich (Zwijndrecht, Netherlands). A previously constructed vector pWUR193 (Wolterink-van Loo et al. 2007), a pET24d derivative containing the SacKdgA gene (Saci_0225), was used to produce SacKdgA. *Escherichia coli* XL1-Blue cells were used for cloning and *E. coli* BL21(DE3) cells were used for expression. The growth medium consisted of LB supplemented with 50 µg ml^{-1} kanamycin, unless stated otherwise. Cells were grown at 37 °C and liquid cultures were incubated in Erlenmeyer flasks in a rotary shaker at 100 rpm.

Construction of SacKdgA random library

Random mutations were introduced by PCR using two DNA polymerases in parallel to generate a library with balanced mutation ratio. *Taq* polymerase, which preferentially mutates As and Ts, and Mutazyme DNA polymerase, which mutates more Gs and Cs, were used. Error-prone PCR with *Taq* DNA polymerase was performed with skewed nucleotide concentrations, with the following primers: 5′-CTTTAAGAAGGAGA-TATACCATG-3′, and 5′-GGCCGCAACCTTGTCGAC-TTA-3′ and the pWUR193 plasmid as template DNA. These primers flank the *kdgA* gene and include the start and stop codons. As such only the *kdgA* coding sequence was targeted in the ep-PCR procedure. The 50-µl reaction mixtures contained 10 ng template DNA, 200 ng forward primer, 200 ng reverse primer, 0.2 mM dATP and dGTP, 1 mM dCTP and dTTP, 0.5 mM MnCl$_2$ and 5U REDTaq DNA polymerase (Sigma) in REDTaq reaction buffer. Additionally, the GeneMorph Random mutagenesis kit (Stratagene) was used for the ep-PCR with the Mutazyme DNA polymerase using the same primers and template DNA according to the manufacturer's instructions, aiming for a mutation rate of 3–7 mutations per kb.

The PCR products obtained were purified, double digested with *NcoI* and *SalI* and ligated into equally digested pET24d

with T4 DNA ligase. The different ligation mixtures were transformed to *E. coli* XL-1 blue cells, plated and grown overnight. We used this method of generating a library, because transformation to XL-1 blue cells appeared more efficient than to BL21(DE3) cells. Finally, two *kdgA* gene libraries with random mutations were obtained. One library consisted of 1084 clones originating from the *Taq* DNA polymerase PCR reaction. The other library consisted of 430 clones from the Mutazyme DNA polymerase PCR reaction. For each library, plasmid DNA was purified from pooled cell suspensions. Random sequencing of the constructs revealed a mean mutation rate of one base change per gene for both libraries.

Screening of random SacKdgA variants

For construction of the expression library, BL21(DE3) expression cells were transformed with the plasmid library, plated on Qtray LB-agar plates (25 cm × 25 cm) and incubated overnight. Single colonies were picked with a colony picker (QPix, Genetix) and used to inoculate 96-wells plates (Costar 3370; Corning) with 200 µl of medium per well supplemented with 0.1 mM IPTG. In each plate, two wells were manually inoculated with the negative control (pET24d in BL21(DE3)) and positive control (pWUR193 in BL21(DE3)). After 16 h of incubation at 37 °C, with shaking, the OD$_{600}$ (Spectramax Plus384; Sopachem b.v.) was recorded. A replica plate was made by duplication of the induced plates in fresh 96-well plates containing 200 µl of medium with 10% glycerol per well, incubation overnight (37 °C, and shaking) and subsequent storage at −80 °C for future use. The induced plates were centrifuged to pellet the cells in a Sigma 4-15C centrifuge for 30 min at 1000 *g*. The pellets were stored at –20 °C until further use. Cells were lysed by addition of 100 µl 25% B-PER (Pierce) in 50 mM sodium phosphate buffer (pH 6.0) to the pellets and additional shaking at 700 rpm for 15 min at 37 °C.

Aldolase reactions were performed in 2-ml-deep well plates (Greiner) containing, per well, 250 µl of 50 mM sodium phosphate buffer, pH 6.0, 10 mM pyruvate, 20 mM D,L-glyceraldehyde, and 40 µl of lyzed cells. The plates were incubated for 60 min at 50 °C and then placed in a freezer (–20 °C) to stop the reaction. Reactions were diluted three times by addition of 500 µl of water. The remaining amount of pyruvate was determined by the L-lactate dehydrogenase (LDH) / NADH assay. Briefly, 190 µl of LDH assay buffer (containing 250 mM Tris-HCl buffer, pH 7.5, 0.2 mM NADH, 5 U ml^{-1}, LDH (in excess)) was transferred to each well of a 96-well plate. The OD$_{340}$ was measured, and then 10 µl of diluted sample of the aldolase reaction was added and incubated at 37 °C for 15 minutes. An end measurement of the OD$_{340}$ was made to determine the difference in NADH absorption and hence the difference in pyruvate concentration of each sample.

DNA sequencing

Plasmids were isolated using the Qiagen plasmid isolation kit according to the manufacturer's protocol. Inserts of the isolated plasmids were sequenced by single primer extension reaction with the T7 primer set (Forward: 5′-AATACGAC-

TCACTATAG-3′, reverse: 5′-GCTAGTTATTGCTCAG-CGG-3′).

Production and purification of SacKdgA variants

SacKdgA and variants were produced to obtain a heat stable cell-free extract (HSCFE) and purified enzyme as described previously (Wolterink-van Loo et al. 2007). Fractions containing aldolase were pooled. Enzyme purity was checked by SDS-PAGE, staining the proteins with Coomassie brilliant blue. Protein concentrations were determined by the BioRad Bradford method.

Aldol condensation activity measurements

We assayed KDG aldolase activity by detection of the condensation product, 2-keto-3-deoxygluconate, with the thiobarbituric acid (TBA) assay as described previously (Wolterink-van Loo et al. 2007) and performing the standard assay at pH 6.0 and 50 °C, with 50 mM pyruvate and 20 mM D,L-glyceraldehyde. The temperature curve was made by using 10-min incubations for each temperature analyzed.

Aldolase activities with different aldehyde acceptors were measured by assaying the remaining pyruvate in an LDH assay in triplicate. Reactions of 150 μl volume containing 50 mM sodium phosphate buffer, pH 6.0, 50 mM pyruvate and 100 mM aldehyde and 0.5–1.5 μg enzyme were incubated at 50 °C for 10–60 min. The reactions were stopped by rapidly cooling the reaction mixtures on ice. From 985 μl of assay buffer (containing 100 mM Tris-HCl, pH 7.5, 0.16 mM NADH and ~3 units LDH) the starting absorbance at 340 nm was read. After addition of 15 μl of sample and incubation for 5 minutes the absorbance was remeasured. The absorbance difference in NADH (molecular extinction coefficient 6.22 mM^{-1} cm^{-1} (Horecker and Kornberg 1948)) was taken to calculate the amount of unconsumed pyruvate.

Thermostability of the enzymes at 90 °C was determined as follows. The KDG aldolase was diluted to 40 μg ml^{-1} in 50 mM sodium phosphate, pH 6.0, and 175-μl aliquots and were placed in sealed HPLC-vials. Vials were immersed in a 90 °C water bath and sampled at different times and immediately cooled on ice. Residual activity of each sample was measured by the TBA assay.

Results and discussion

Construction of a random mutant SacKdgA library

Synthetic cascade reactions in which aldolases are involved are performed either separately or as one-pot reactions (Sugiyama et al. 2007, Yu et al. 2006). Combinations of different enzymes often require establishing a compromise of the chemical and physical reaction conditions (Jennewein et al. 2006, Littlechild et al. 2007, Pollard and Woodley 2007, Straathof et al. 2002). When combining enzymes from thermophilic and mesophilic organisms, optimization of conditions for one type of enzyme or the other is required.

The KDG aldolase from *S. acidocaldarius* (SacKdgA) is an extremely stable pyruvate-dependent aldolase, with a broad substrate specificity and high affinity for non-phosphorylated substrates (Wolterink-van Loo et al. 2007). A potential application of this enzyme would be in a cascade with an available mesophilic alditol oxidase (Heuts et al. 2007), in which the latter enzyme provides the aldehyde substrate for the aldolase. The SacKdgA has maximal activity at 95 °C and at 37 °C its activity is reduced to approximately 5% of its rate at 95 °C. We therefore attempted to improve SacKdgA activity at suboptimal temperatures, while seeking to maintain its stability. As little is known about the molecular basis of catalytic temperature dependence, a random approach was used to optimize low-temperature activity of SacKdgA. Diversity was generated in the SacKdgA gene by ep-PCR, resulting in a library with a mean of one mutation per gene. A plasmid library of 1500 clones was isolated from *E. coli* XL-1 Blue cells, which was used in the transformation of *E. coli* BL21(DE3) for expression. A total of 5000 transformants were screened to ensure sufficient coverage of the genetic diversity in the *kdgA* mutant library.

Screening for increased condensation activity at 50 °C of SacKdgA

The desired operating temperature for bio-cascade reactions in combination with mesophilic enzymes is 37 °C (or lower). The incubation time necessary to detect any wild-type SacKdgA activity at this temperature was in the order of hours, which was deemed incompatible with a high-throughput screening strategy. Therefore, the mutant KdgA library was screened for aldol condensation activity at 50 °C, because at this temperature a small but significant amount of activity of the wild type could be detected in a relatively short assay time (60 min). The mutants were screened for their ability to convert D,L-glyceraldehyde and pyruvate to D,L-2-keto-3-deoxygluconate. Because protein expression levels were not taken into account, winners in the screening procedure would be more active than the wild type due to either increased activity or increased enzyme content, or both.

Determination of pyruvate consumption was chosen as a primary screen for KdgA activity for ease of execution and because it provided the opportunity to screen for activity with other aldehydes in the same assay. The activities of the KdgA variants in the libraries were corrected for effects of other pyruvate consuming or producing activities in the *E. coli* extract by subtracting the activity of BL21(DE3) harboring a pET24d vector without insert. This way, 162 mutants with up to 4-fold increased activities were identified. However, consecutive screening in triplicate (using the pyruvate depletion assay) proved most candidates to be false positives. This was attributed to the indirect screening method of measuring residual pyruvate, which resulted in more frequent errors compared with screening for condensation product. Finally 12 clones were selected for further testing and were recultivated, their plasmid DNA was isolated for sequencing, and used to re-transform BL21(DE3) to obtain the proteins for further analyses. Wild-type SacKdgA and the 12 selected clones were produced on a one-liter scale, heat-stable cell-free extracts

(HSCFE) were prepared from the cultures, and the variants were compared with the activity of the wild type using the TBA assay (measuring the amount of condensation product formed).

Most clones had equal or less activity than wild-type KdgA and sequencing revealed that these clones had either no amino acid change or only a single mutation of a solvent exposed residue (data not shown). Variants with lower activity had less protein in the HSCFE (data not shown). Variant t34D12 appeared to be on average 1.5 times more active than wild-type KdgA, included no mutations. Variant t34B9 was 1.5 times more active and contained mutation N168D, located at the protein surface. The most active variant, t37F3 was 3 times more active than the wild-type SacKdgA, with a specific activity of 28 U mg^{-1} versus 9 U mg^{-1}. This variant contained a single mutation of the buried Val193 to alanine.

Structural analysis of SacKdgA V193A

Variant t37F3, containing mutation V193A, was 3 times more active than the wild-type SacKdgA. Valine 193 is well conserved in KdgA aldolases from hyperthermophilic crenarchaea (Figure 1). A valine at position 193 in SacKdgA appears to be specific for KdgA-type aldolases from hyperthermophilic crenarchaea and euryarchaea. Homologous sequences of other (putative) pyruvate aldolases have an isoleucine at this position and have been characterized as, or predicted to be, dihydrodipicolinate synthases (DHDPS) and neuraminate lyases (NAL) (Figure 1). Other archaeal (both crenarchaeal and euryarchaeal) hypothetical KdgAs or hypothetical DHDPS have a very different genetic environment (Ahmed et al. 2005) and are unlikely to be true KdgAs. In wild-type SacKdgA, Val193 has Van der Waals interactions with the catalytic lysine (Lys153) that forms a Schiff base intermediate with the substrate (Figure 2). Mutation V193A created space in close proximity to Lys153 and possibly allowed a higher degree of mobility of the active site compared with wild-type SacKdgA. Similar mutations, creating space in close proximity to the active site, have resulted in increased activity at suboptimal temperatures of various proteins such as *Pyrococcus furiosus* β-glucosidase (Lebbink et al. 2000), *Thermotoga neapolitana* xylose isomerase (Sriprapundh et al. 2003), and *Sulfolobus solfataricus* indoleglycerol phosphate synthase (Merz et al. 2000). In addition, a natural cold-adapted elastase (Papaleo et al. 2006) has increased local flexibility (in loop regions) compared with its mesophilic counterparts.

Optimal temperature for activity and kinetic stability

Wild-type SacKdgA and variant V193A were purified to homogeneity for characterization (data not shown). To study the effect of temperature on the activity of SacKdgA-V193A and to determine whether its activity was also increased at moderate temperatures, SacKdgA-V193A and wild-type SacKdgA were assayed in the temperature range of 30 °C to 90 °C (Figure 3). SacKdgA-V193A was more active than wild-type

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                        ↓
SacKdgA    :  LSLDGVVASFTN 199
SsoKdgA    :  TGLDGNVAAGSN 202
StoKdgA    :  TSLDGSVTAASN 201
TteKdgA    :  VRLDGVVASSAN 200
Paedhdps*:   VGAHGGILAGPN 209
PtoKdgA*   :  SGVDGTVSAAGN 197
TacKdgA*   :  NGLDGAVAAAGN 195
Tvodhdps*:   SGVDGSVAAAGN 197
Pfudhdps*:   LGGDGGIMACAN 209
Phodhdps*:   LGGDGGIMACAN 208
Tmadhdps   :  AGGDGVISVVSN 212
Ecodhdps   :  LGGHGVISVTAN 209
EcoNAL     :  AGADGGIGSTYN 212
HinNAL     :  LGVDGAIGSTFN 211
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Figure 1. Partial alignment of different (hypothetical) pyruvate-dependent aldolases (KdgAs, dihydrodipicolinate synthases (DHDPS) and neuraminate lyases (NAL)) from *Sulfolobus acidocaldarius* (Sac), *S. solfataricus* (Sso), *S. tokodaii* (Sto), *Thermoproteus tenax* (Tte), *Pyrobaculum aerophilum* (Pae), *Picrophilus torridus* (Pto), *Thermoplasma acidophilum* (Tac), *T. volcanium* (Tvo), *Pyrococcus furiosus* (Pfu), *P. horikoshii* (Pho), *Thermotoga maritima* (Tma), *E. coli* (Eco) *and Heamophilus influenzae* (Hin). The valine residue (at the arrow) is conserved in crenarchaeal KdgAs and some euryarchaea (*Thermoplasma*) and otherwise conservatively replaced by an isoleucine in (hypothetical, indicated by an asterisk) dihydrodipicolinate synthases (DHDPS) and neuraminate lyases (NAL).

SacKdgA between 30 °C and 60 °C. The optimum temperature for activity shifted from 90 °C in wild-type SacKdgA to 70 °C for the mutant, similar to results obtained for a *P. furiosus* β-glucosidase CelB variant reported by Lebbink et al. (2000). Although at 30 °C the activities of both enzymes were just detectable above background level, it was found that mutant V193A was significantly more active between 40 and 60 °C. The highest relative increase in activity was found at 40 °C, close to the desired "mesophilic" 37 °C. In addition, it was concluded that V193 contributes significantly to the activity of SacKdgA at the optimal growth temperature of *S. acidocaldarius* (80 °C), which agrees well with the fact that it is well conserved in homologous enzymes from hyperthermophiles; however, in some orthologs valine is replaced by isoleucine, which probably gives comparable restricted flexibility of the active site lysine residue. An attractive feature of SacKdgA in biotechnological applications is its extreme thermostability. Improving the enzyme's low-temperature activity should not interfere with protein stability. Variant SacKdgA-V193A remained soluble during purification, which included a heat incubation step of 30 min at 75 °C, indicating that the variant had retained significant residual thermostability. Furthermore, inactivation studies at 90 °C of both wild-type and variant aldolase showed that they had similar activity half-lives of 100 and 120 minutes, respectively. Therefore, mutation V193A affected only the activity of SacKdgA,
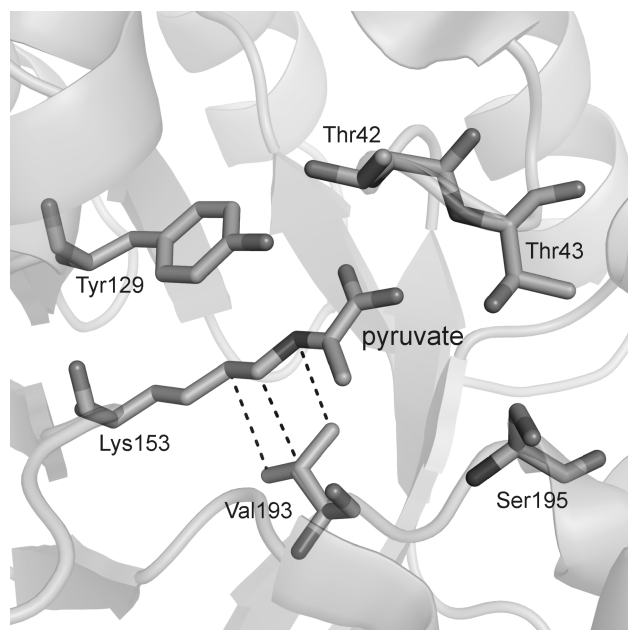
had already decreased at temperatures above 70 °C.

Kinetic characterization of mutant and wild-type SacKdgA at 50 °C

To study the effect of mutation V193A on catalysis, the kinetic reaction constants of SacKdgA-V193A and wild-type aldolase for condensation of pyruvate and D,L-glyceraldehyde were determined at 50 °C (Table 1). The turnover, k_{cat}, of the mutant enzyme, had increased three times. The K_M had increased as well, three times for glyceraldehyde and five times for pyruvate. The kinetic constants confirm the validity of the high-throughput screen, which was performed under saturating substrate conditions. Despite the increase in K_M, the catalytic rate of the mutant enzyme, as a function of the substrate concentration, was always higher than the catalytic rate of the wild-type enzyme (not shown); only at low pyruvate concentrations (< 2 mM) did the wildtype enzyme shows a higher activity than the mutant enzyme. This mutant V193A would provide a benefit over wild-type SacKdgA in biotechnological applications, in which saturating substrate conditions are typically used.

Substrate specificity of mutant and wild type SacKdgA

Sulfolobus acidocaldarius KdgA is part of the Entner-Doudoroff pathway for degradation of glucose (Selig et al. 1997) and probably galactose (Lamble et al. 2003) to pyruvate and glyceraldehyde. The enzyme displays broad substrate specificity and can accept various non-natural acceptors in the condensation reaction with pyruvate (Wolterink-van Loo et al. 2007). Therefore, we investigated the effect of mutation V193A on the condensation activity at 50 °C using pyruvate and alternative aldehyde acceptors (Figure 4). Under substrate conditions far above the K_M (at least 9 times), mutant V193A was more active than the wild-type enzyme on all tested acceptor substrates. The highest relative increases in activity were measured with glyceraldehyde, glycolaldehyde and 3-azido-2-hydroxypropanal as acceptors. Glyceraldehyde was the acceptor used in the screening, and glycolaldehyde and 3-azido-2-hydroxypropanal are the tested acceptors that are most similar to glyceraldehyde. As such, this study confirms the first law of directed evolution "You get what you screen for" (Schmidt-Dannert and Arnold 1999).

Figure 2. Diagram of the active site of SacKdgA with pyruvate covalently bound to Lys153 (pdb: 2nuy). Van der Waals interactions between the Lys153 side chain and Val193 are indicated with dotted lines. The figure was generated with PyMol.

demonstrating that activity and thermostability can be uncoupled for thermostable Class I aldolases. Previously, similar observations have been made for mesophilic class II fructose-bisphosphate aldolase; aldolase thermostability was improved by directed evolution without reduction in catalysis at mesophilic temperatures (Hao and Berry 2004). Moreover, a model has been proposed that describes the apparent uncoupling of stability and activity (Daniel et al. 2008) in enzymes, and in mutant V193A stability is unaffected, although activity
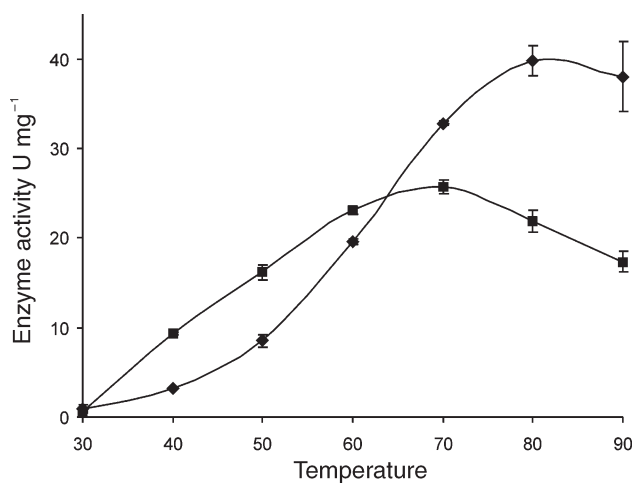

Figure 3. Temperature dependent activity of purified SacKdgA wild type (◆) and SacKdgA-V193A (■). Activities were determined in 50 mM sodium phosphate buffer (pH 6.0), 10 mM pyruvate and 20 mM glyceraldehyde.

Table 1. Biochemical data for SacKdgA and mutant V193A, determined at 50 °C. Values between brackets are standard deviations.

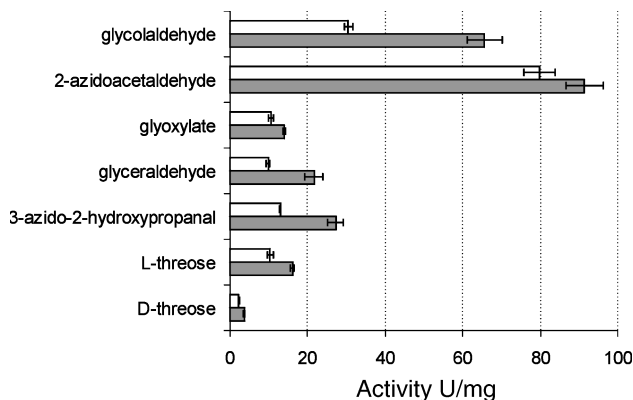|	SacKdgA	SacKdgA-V193A
$V_{max\,(GA)}$ (U/mg)	8.1 (0.4)	29.1 (0.9)
$k_{cat\,(GA)}$ (s^{-1})	4.4 (0.2)	15.7 (0.5)
$K_{M(GA)}$ mM	2.1 (0.3)	5.4 (0.5)
$V_{max\,(pyr)}$ (U/mg)	10.1 (0.1)	27.8 (1.0)
$k_{cat\,(pyr)}$ (s^{-1})	5.5 (0.1)	15.0 (0.5)
$K_{M(pyr)}$ mM	0.8 (0)	5.4 (0.6)

Figure 4. Activity of purified SacKdgA (white bars) and SacKdgA-V193A (gray bars) on different (unnatural) aldehyde substrates at 50 °C. Activities were determined in 50 mM sodium phosphate buffer (pH 6.0), 50 mM pyruvate and 100 mM aldehyde.

Conclusions

In this study, the extremely thermostable Class I KDG aldolase of *S. acidocaldarius* with maximal activity at 95 °C was successfully improved for catalysis at the suboptimal temperature of 50 °C to enhance its potential for use in one-pot synthesis reactions together with mesophilic enzymes. Screening of 1500 variants resulted in isolation of a single variant Sac-KDG-V193A that was three times more active than wild-type SacKdgA for condensation of pyruvate and glyceraldehyde at 40 °C. The mutation increased the activity of the enzyme but did not affect its extreme thermostability. Despite the relatively small size of the generated library, the chosen laboratory evolution approach has proved to be an efficient strategy for improving the desired property of SacKdgA. To our knowledge this is the first report on improving aldolase activity at suboptimal temperatures.

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