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Structurally Informed Site-Directed Mutagenesis of a Stereochemically Promiscuous Aldolase To Afford Stereochemically Complementary Biocatalysts

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Abstract: 2-Keto-3-deoxygluconate aldolase from the hyperthermophile *Sulfolobus solfataricus* is a highly thermostable type I aldolase that can catalyze carbon−carbon bond formation using nonphosphorylated substrates. However, it exhibits poor diastereocontrol in many of its aldol reactions, including the reaction of its natural substrates, pyruvate and D-glyceraldehyde, which afford a 55:45 mixture of D-2-keto-3-deoxygluconate (D-KDGlu) and D-2-keto-3-deoxy-galactonate (D-KDGal). We have employed detailed X-ray crystallographic structural information of this aldolase bound to these diastereoisomeric aldol products to selectively target specific amino acids for mutation for the rapid creation of stereochemically complementary mutants that catalyze either (*Re*)- or (*Si*)-facial selective aldol reactions to afford either D-KDGlu or D-KDGal with good levels of diastereocontrol.

Introduction

Aldolases are an attractive class of enzyme for catalyzing the formation of aldol products with good levels of stereocontrol.[1] They are particularly useful as biocatalysts for the synthesis of monosaccharides[2] that have been used for the synthesis of natural products,[3] fine chemicals,[4] pharmaceutical intermediates,[5] enzyme inhibitors,[6] and complex oligosaccharides.[7] Their use for synthesis has its limitations, however, with many aldolases exhibiting narrow substrate specificity profiles for phosphorylated substrates that are difficult to prepare/handle.[8] These requirements restrict the range of aldol products that can be prepared, so there is currently much interest in using molecular biology techniques to prepare mutant aldolases with improved specificity profiles for natural/unnatural substrates.[9]

A combination of site-directed mutagenesis and/or directed evolution techniques have been employed to evolve the stereoselectivity profiles of different types of aldolase.[10] This has resulted in the creation of mutant aldolases that catalyze stereoselective aldol reactions with both enantiomers of an aldehyde substrate,[11] as well as mutants that catalyze the same aldol reaction with opposing enantioselectivities.[12,13] While these studies have proven successful in identifying mutant aldolases that selectively afford diastereoisomeric aldol products, they normally require time-consuming screening of large libraries of mutants before the desired stereoselectivity profiles were identified.

Structurally informed site-directed mutagenesis offers an alternative approach for the rapid preparation of mutants with new/improved activities, although this strategy normally requires detailed structural knowledge of an enzyme's catalytic mechanism.[14] The potential of using structural knowledge of an enzyme−substrate complex to guide mutagenesis studies has been demonstrated previously for D-2-deoxyribose-5-phosphate aldolase, which catalyzes aldol reactions of acetaldehyde with phosphorylated aldehydes.[15] A high-resolution X-ray crystal structure of its carbinolamine covalent complex with D-2-deoxyribose-5-phosphate was used as a structural template to selectively target key amino acids for site-directed mutagen-

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esis.[16] This resulted in the creation of five new mutants that displayed new/improved activities for the aldol reactions of acetaldehyde with nonphosphorylated aldehydes. Analysis of the structures of many mutant enzymes that display altered stereoselectivity profiles reveals that they are often a result of selective mutations at amino acid 'hot-spots' in their active site. Consequently, we now report how access to detailed structural information of a stereochemically promiscuous aldolase bound to diastereoisomeric aldol products has been used to guide site-directed mutagenesis studies for the rapid creation of stereochemically complementary mutants.[17]

Methods

Bacterial Strain and Plasmids. *Escherichia coli* BL21 (DE3) competent cells were obtained from Stratagene, U.K. and used in conjunction with the pET-3a expression vector as described previously.[18]

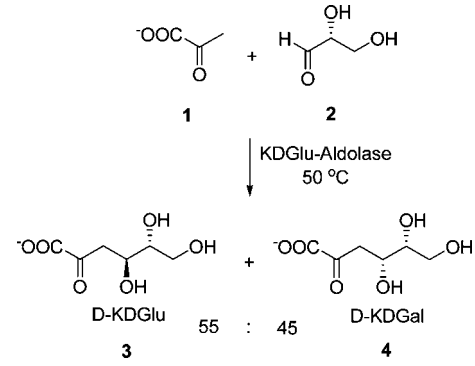
Site-Directed Mutagenesis and Saturation Mutagenesis at Thr-157. Mutations were introduced using a QuikChangeII PCR kit obtained from Stratagene according to the manufacturer's protocol. Primers were synthesized by MWG Biotech AG, Germany. Plasmids from isolated colonies were purified using the Wizard Plus SV Minipreps DNA Purification System from Promega, U.K. and sequenced by GeneService, U.K. to confirm that the correct mutation(s) had been introduced into each gene.

Expression and Purification of Recombinant KDG Aldolase and Variants. Plasmids carrying mutant genes were transformed into and expressed in *E. coli* BL21 (DE3) using our previously described procedures.[18] A total of 50 mL of cell culture was centrifuged at 12 500*g* and the resultant cellular mass resuspended to a concentration of ~0.2 mg/mL in Tris-HCl, pH 8.5, followed by 5 × 30 s bursts of sonication at room temperature. Cell debris was then removed by centrifugation and the resultant extract semipurified by heat treatment at 70 °C for 30 min, with any precipitated proteins being removed by further centrifugation. For determination of kinetic data, the semipurified enzymes underwent two further purification steps: anion exchange chromatography on a HiTrap Q HP column from GE Healthcare (20 mM Tris-HCl, pH 8.5, with a 0−2 M NaCl elution gradient), followed by gel filtration on Superdex 200 10/300GL from GE Healthcare (20 mM Tris-HCl, pH 8.5, 200 mM NaCl). Protein concentrations were determined by the standard Bradford assay.[19]

Determination of Diastereoselectivity of Aldolase Catalyzed Reactions. Each recombinant mutant aldolase (10 μg) was added to a solution of 600 mM pyruvate **1** and 100 mM D-glyceraldehyde **1** in water (17 mL) and the reaction mixture incubated at 70 °C overnight. An aliquot of each biotransformation was then subjected to HPLC analysis using an Agilent 1200 machine fitted with a Bio-Rad Aminex HPX-87H column (300 mm ×7.8 mm) (0.6 mL/min, 8 mM H_2SO_4, 60 °C) with peak detection using a refractive index detector.[18]

Preparative Reactions. Preparative reactions were carried out using our previously described procedure:[18] 10 mg of purified aldolase (D-KDGlu mutant-2 or D-KDGal mutant-1) were used to catalyze the biotransformation of 1.0 g of pyruvate **1** and 149 mg of D-glyceraldehyde **2** in 1 M formic acid to afford 0.75−1.0 g of crude product. A portion of each crude product (100 mg) was purified by semipreparative HPLC (Bio-Rad Aminex HPX-87H) to remove excess pyruvate resulting in 25−30 mg of D-KDGlu **3** or D-KDGal **4**, respectively, in >90% diastereoisomeric ratio (dr).[18]

Scheme 1. KDGlu-Aldolase Catalyzed Nonstereoselective Aldol Reaction between Pyruvate **1** and D-Glyceraldehyde **2**



Enzyme Assay. Kinetic parameters of key mutants (D-KDGlu mutant-2 and D-KDGal mutant-1) for pyruvate **1** and D-glyceraldehyde **2** were determined using a thiobarbituric acid assay as described previously.[20]

Results and Discussion

2-Keto-3-deoxygluconate aldolase from the hyperthermophile *Sulfolobus solfataricus* is a type I aldolase with broad specificity for the aldol reaction of pyruvate with nonphosphorylated aldehydes.[21] It is extremely thermostable, can be efficiently expressed in *E. coli*, but exhibits poor diastereocontrol in many of its aldol reactions.[21] Indeed, this aldolase is a rare example of an enzyme that exhibits no stereocontrol when catalyzing the aldol reaction of its natural substrates,[22] with pyruvate **1** and D-glyceraldehyde **2** affording a 55:45 mixture of D-2-keto-3-deoxygluconate (D-KDGlu) **3** and D-2-keto-3-deoxy-galactonate (D-KDGal) **4** (Scheme 1).[23] X-ray crystal structures of imine covalent complexes of this aldolase with pyruvate **1**, D-KDGlu **2**, and D-KDGal **3** have been determined previously, and the resultant structural information used to understand its catalytic mechanism.[24] Access to this structural information enabled us to devise a substrate engineering approach to *indirectly* cure its stereochemical promiscuity for D-glyceraldehyde **2**, with the wild-type aldolase being shown to catalyze the aldol reaction of pyruvate **1** and structurally rigid D-

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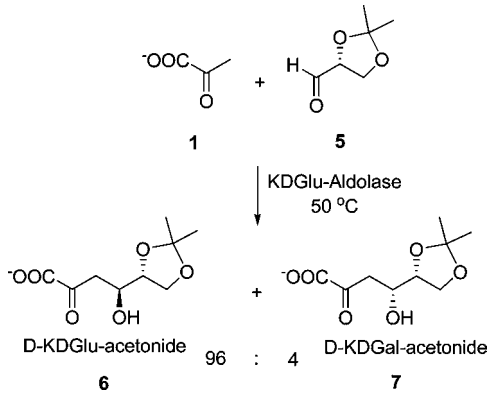
Scheme 2. KDGlu-Aldolase Catalyzed Stereoselective Aldol Reaction between Pyruvate **1** and D-Glyceraldehyde Acetonide **5**



glyceraldehyde acetonide **5** to give D-KDGlu-5,6-acetonide **6** and D-KDGal-5,6-acetonide **7** in a dr of 96:4 (Scheme 2).[25] We reasoned that this structural information might also be used to mutate key active site residues involved in the selective binding of D-KDGlu **3** or D-KDGal **4**, with the aim of creating stereocomplementary mutants that could catalyze opposing (*Re*)- and (*Si*)-enantiofacial aldol reactions with high levels of diastereocontrol.

X-ray crystallographic analysis of the active site of this Type I aldolase bound to pyruvate **1**, D-KDGlu **2** and D-KDGal **3** at 4 °C, reveals that its catalytic mechanism is typical of the *N*-acetylneuraminic acid lyase superfamily.[26] Briefly, the ω-amino functionality of its Lys-155 residue forms a Schiff base with the α-keto residue of pyruvate, which tautomerizes to afford an enamine intermediate that can attack either the (*Re*)- or (*Si*)-face of the carbonyl of D-glyceraldehyde. This aldol reaction is mediated by the phenolic substituent of a highly conserved Tyr-130 residue, which plays a key catalytic role in shuttling protons between reactive enzyme bound intermediates (Figure 1).

Analysis of the X-ray crystal structures of the aldolase bound to D-KDGlu **3** (Figure 2) and D-KDGal **4** (Figure 3) reveals a series of amino acid residues in the active site that confer sufficient functionality/redundancy to bind both diastereoisomers.[24] The carboxylates of bound D-KDGlu **3** and D-KDGal **4** exhibit common interactions with the amidic backbone residues of Thr-43/Thr-44, the hydroxyl residue of Thr-44, and the phenolic residue of Tyr-130. Their respective (*R*)-C4-OH and (*S*)-C4-OH residues form common hydrogen bonding interactions with the phenolic and hydroxylic residues of Tyr-130 and Thr-157. However, their respective C5-OH residues are stabilized by significantly different interactions. The C5-OH residue of bound D-KDGlu **3** makes bridging water mediated hydrogen bonding interactions with the amidic residues of Gly-179, Ala-198, and the side chains of Thr-44, Ser-241,

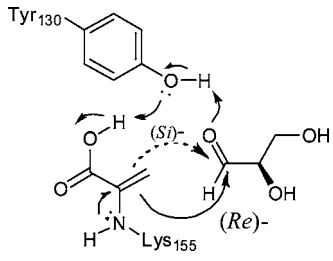


Figure 1. A Lys-155 derived enamine derivative of pyruvate **1** attacks either the *Re*- or *Si*-face of D-glyceraldehyde **2** to afford a 55:45 mixture of D-KDGlu **3** and D-KDGal **4**.

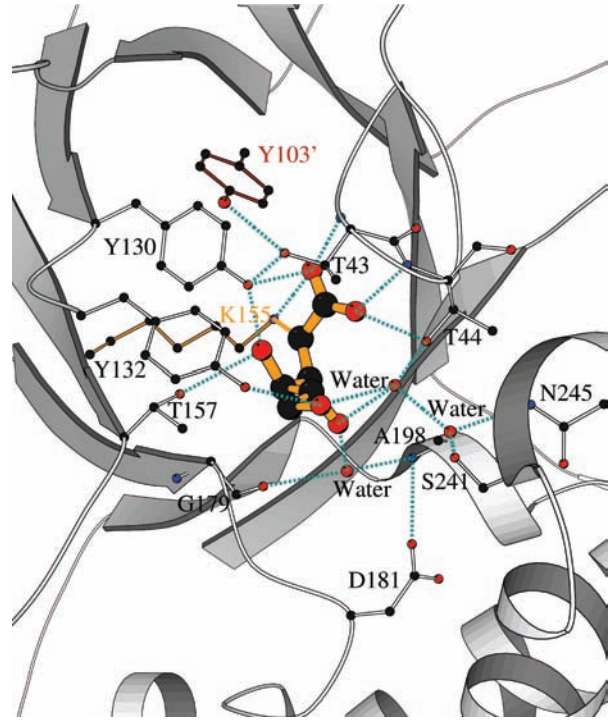


Figure 2. X-ray crystal structure of aldolase bound to D-KDGlu **3**, showing primary hydrogen bonding interactions with active site residues and conserved water molecules.

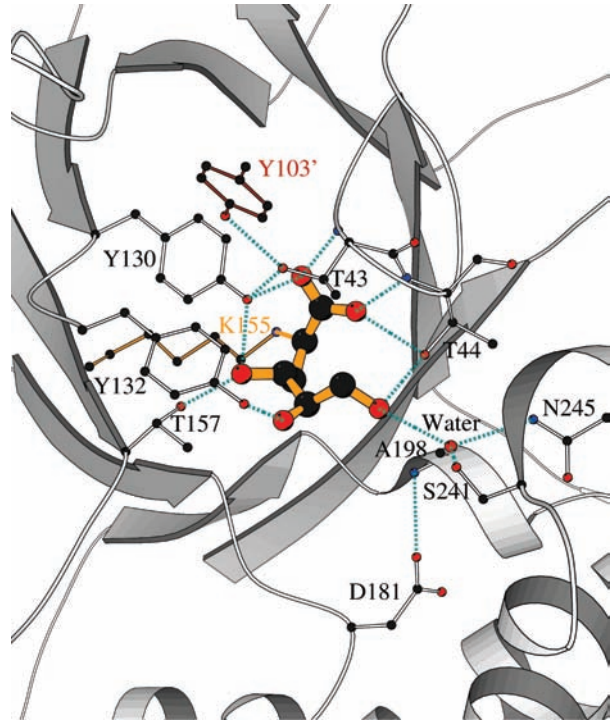
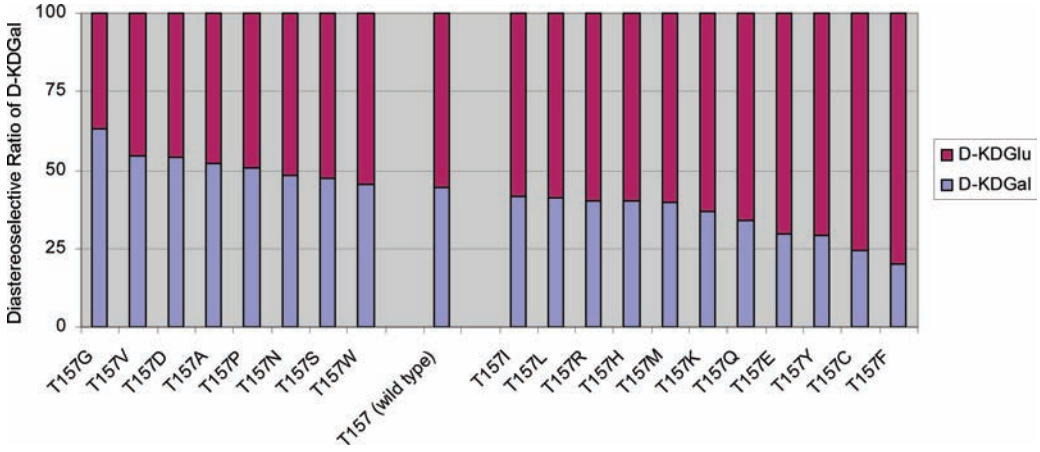


Figure 3. X-ray crystal structure of aldolase bound to D-KDGal **4**, showing primary hydrogen bonding interactions with active site residues and conserved water molecules.

and Asn-245. Its terminal C6-OH residue is stabilized by a direct hydrogen bonding interaction with the phenolic residue of Tyr-132 and bridging water interactions with the side chains of Thr-44, Ser-241, and Asn-245 (Figure 2). Alternatively, the C5-OH residue of bound D-KDGal **4** hydrogen bonds directly with the phenolic residue of Tyr-132, while its terminal C6-OH

Table 1. Diastereoselectivities Obtained for Aldol Reaction of Pyruvate **1** and D-Glyceraldehyde **2** Catalyzed by 20 Mutant KDG-Aldolases Obtained from Saturation Mutagenesis at Thr-157



residue interacts directly with the hydroxyl residue of Thr-44, as well as making water-mediated bridging interactions with the side chains of Ser-241 and Asn-245 (Figure 3). Overall, this means that stabilization of the C5-OH and C6-OH residues of bound D-KDGlu **3** is highly dependent upon hydrogen bonding to a conserved network of active site water molecules,[27] which results in its C4−C6 backbone presenting a relatively hydrophobic region toward Thr-157. In contrast, the vicinal C5-OH and C6-OH residues of bound D-KDGal **4** are oriented in opposite directions, with its C5-OH residue creating a relatively hydrophilic region within the vicinity of Thr-157.

The alternative hydrogen bonding opportunities and water bridging interactions observed when this aldolase binds D-KDGlu **3** and D-KDGal **4** may also be used to explain why it catalyzes the aldol reaction of its natural substrates with essentially no stereoselectivity.[24] It follows, from the principle of microscopic reversibility, that D-glyceraldehyde **2** can bind in the two different conformational positions available to the C_4-C_6 fragments of bound D-KDGlu **3** and D-KDGal **4**, which enables attack of the Lys-155 bound enamine equivalent of pyruvate at either the *Re*- or *Si*-face of its carbonyl residue. Since the resulting D-KDGlu **3** and D-KDGal **4** products are formed via competing diastereoisomeric transition states, different combinations of substrate−protein interactions are employed to facilitate their formation. Therefore, it was reasoned that disruption of stabilizing active site interactions that are responsible for selectively binding D-KDGlu **3** might result in a mutant aldolase that catalyzes the preferential formation of D-KDGal **4**, and vice versa.

Analysis of the crystal structures of KDG-aldolase bound to D-KGlu **3** and D-KDGal **4** revealed that Thr-157 plays a critical role in hydrogen bonding to the epimeric C4-OH residues of both diastereoisomers, and as a consequence, this residue was chosen as a logical starting point for mutation.[28] Tyr-130 also binds to the C4-OH residues of D-KDGlu/D-KDGal; however, this residue plays a critical catalytic role in facilitating the aldol reaction (see Figure 1), so it was reasoned that any changes at this site would be likely to result in poorly active mutants. Therefore, a series of 20 aldolase mutants were prepared by saturation mutagenesis of Thr-157, that were then screened as new biocatalysts for the aldol condensation reaction of pyruvate **1** with D-glyceraldehyde **2**.[29] This revealed a number of mutants with improved selectivity for formation of either D-KDGlu **3** or D-KDGal **4**, with five single mutants identified with an improved diastereoisomeric ratio (≥10%) for D-KDGlu **3** (>65% dr), and two single mutants with improved diastereoselectivity for D-KDGal **4** (>55% dr) (Table 1).

The two best single mutants that gave improved stereocontrol for D-KDGlu **3** were Thr-157-Cys with a 75% dr, and Thr-157-Phe with a 79% dr. Analysis of crystal structures of the wild-type aldolase reveals that Thr-157 makes an optimal hydrogen bond with the C4-OH residue of D-KDGlu **3** of 3.07 Å, while it makes a much shorter hydrogen bond with D-KDGal **4** of only 2.47 Å. Therefore, it was rationalized that the improved selectivity of the Thr-157-Cys mutant for D-KDGlu **3** might be due to the increased steric demand of its thiol residue, that would result in greater destabilizing interactions with the carbonyl residue of the D-glyceraldehyde conformer leading to D-KDGal **4**. Alternatively, it was reasoned that the aryl ring of the Thr-157-Phe mutant would make favorable interactions with the hydrophobic carbon backbone of the D-glyceraldehyde conformer leading to D-KDGlu **3**, while making destabilizing interactions with the hydrophilic C2-OH residue of the D-glyceraldehyde conformer leading to D-KDGal **4**.

The phenolic residue of Tyr-132 makes a single hydrogen bond with the C5-OH residue of D-KDGal **4**, while it makes a hydrogen bond interaction with C6-OH residue of D-KDGlu **3**,

(25) This aldolase also catalyzes the highly stereoselective aldol reaction of pyruvate with L-glyceraldehyde acetonide to afford L-KDGal-5,6-acetonide in >95% de, see Lamble, H. J.; Danson, M. J.; Hough, D. W.; Bull, S. D. *Chem. Commun.* **2005**, 124–126.

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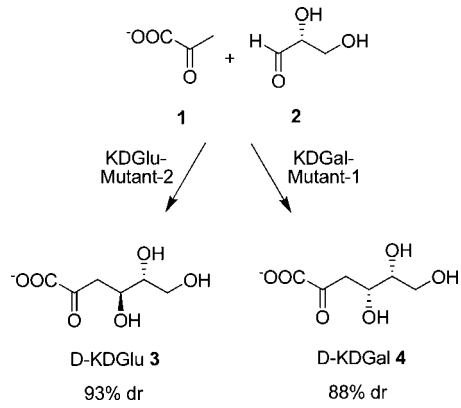
(27) X-ray crystallographic analysis of D-2-deoxyribose-5-phosphate aldolase bound to D-2-deoxyribose-5-phosphate reveals a similar hydrogen bonding network between the hydroxyl residues of its bound sugar and conserved active site water molecules that play a critical role in catalysis and stereocontrol, see ref 15.

(28) Berry and Nelson used our X-ray crystal structures of KDG-aldolase bound to D-KDG and D-KDGal to target the structurally analogous Thr-167 residue of *N*-acetylneuraminic acid lyase for mutation, which was shown to play a key role in determining the stereoselectivity of its aldol reactions for the preparation of diastereoisomeric sialic acid analogues, see refs 12 and 24.

(29) All aldolase catalyzed reactions were carried out using a large excess of pyruvate **1** that resulted in complete consumption of D-glyceraldehyde **2**, thus, ensuring that any mixtures of D-KDGlu **3** and D-KDGal **4** were formed under kinetic control.

Scheme 3. Mutant Aldolases Catalyze Stereoselective Aldol Reaction of Pyruvate **1** and D-Glyceraldehyde **2** To Afford D-KDGlu **3** and D-KDGal **4**, Respectively



which is also stabilized by hydrogen bonding to the network of active site water molecules. Therefore, it was reasoned that replacement of this phenolic residue with a hydrophobic amino acid might selectively disfavor binding of D-glyceraldehyde **2** in the conformation leading to D-KDGal **4** because its C2-OH residue would no longer be stabilized by hydrogen bonding to the phenolic residue of Tyr-132. In contrast, binding of D-glyceraldehyde **2** in the conformation leading to D-KDGlu **3** would still be relatively stabilized by hydrogen bonding of its C2-OH/C3-OH residues to the network of active site water molecules. To test this hypothesis, the single mutant Tyr-132-Val was created and shown to catalyze the selective formation of D-KDGlu **3** in 79% dr.

It was then decided to combine these 'hot-spots' into double mutants to determine whether further improvements in diastereoselectivity for D-KDGlu **3** would be observed. Therefore, the double mutants Thr-157-Cys|Tyr-132-Val (KDGlu Mutant-1) and Thr-157-Phe|Tyr-132-Val (KDGlu Mutant-2) were prepared and shown to catalyze the stereoselective formation of D-KDGlu **3** in much improved 91% and 93% drs, respectively (Scheme 3).

Having used structural information to rapidly create two double mutants with selectivity for D-KDGlu **3**, our attention then turned toward creating a mutant aldolase with good D-KDGal **4** selectivity. Saturation studies at Thr-157 had revealed that two mutant aldolases, Thr-157-Val and Thr-157-Gly, catalyzed formation of D-KDGal **4** in 55% dr and 62% dr, respectively. To rationalize these improvements, it was proposed that the energy loss arising from removal of an optimal hydrogen bond to the carbonyl residue of the D-glyceraldehyde conformer leading to D-KDGlu **3** for these mutants must be greater than the corresponding energy loss from removal of the nonoptimal hydrogen bond to the carbonyl of the D-glyceraldehyde conformer leading to D-KDGal **4**.

It was then decided to attempt to improve KDGal selectivity further by disrupting the network of active site water molecules that are known to contribute more hydrogen bond interactions to D-KDGlu **3** than D-KDGal **4**. Therefore, it was decided to create a double mutant in which Ala-198 was transformed into an amino acid containing a hydrophobic side chain such as leucine, since this replacement would serve to disrupt the network of active site water molecules in its vicinity. Exclusion of one or more of these water molecules should therefore serve to preferentially disfavor the transition state leading to D-KDGlu **3**. Disappointingly, creation of the double mutant Thr-157-Gly|Ala-198-Leu only resulted in a small improvement in diastereoselectivity, affording D-KDGal **4** with a 66% dr. However, the double mutant Thr-157-Val|Ala-198-Leu did result in a significant improvement in diastereocontrol, affording D-KDGal **4** in a much improved 72% dr.

Table 2. Kinetic Data for Wild-Type and Mutant Aldolases for Pyruvate **1** and D-Glyceraldehyde **2**

	K_M(mM)[a]	V_{max}[a](μmol/(min/mg))	k_{cat} (min^{-1})[a]
Wild-Type			
Pyruvate	1.1	8.3	274
D-glyceraldehyde	5.3	8.9	328
KDGlu Mutant-2			
Pyruvate	4.1	0.13	4.5
D-glyceraldehyde	8.0	0.11	3.4
KDGal Mutant-1			
Pyruvate	39.6	0.12	4.0
D-glyceraldehyde	9.1	0.13	4.3

[a] Kinetic parameters determined at 70 °C using a thiobarbituric acid assay.[20]

Further examination of the aldolase crystal structures revealed that the carboxylate of Asp-181 makes a hydrogen bonding interaction with the amide carbonyl of Ala-198. It was reasoned that creating a triple mutant in which this Asp-181 residue was replaced with an amino acid that contained a long polar side chain might serve to restrict the conformational mobility of the alkyl side chain of Leu-198. This would result in its hydrophobic side chain being further directed toward the D-glyceraldehyde binding site, thus, maximizing the chances that one or more of the conserved water molecules would be displaced from the active site. This would maximize the chances of stabilizing hydrophobic interactions occurring between the Leu-198 side chain and the C1−C3 hydrophobic face of the D-glyceraldehyde conformer leading to D-KDGal **4**. Conversely, the conformer of D-glyceraldehyde leading to D-KDGlu **3** should be destabilized due to its hydrophilic C2-OH residue being repelled by the hydrophobic side chain of Leu-198. Consequently, a triple mutant Thr-157-Val|Ala-198-Leu|Asp-181-Gln (KDGal Mutant-1) was prepared and shown to catalyze the diastereoselective formation of D-KDGal **4** with a much improved 88% dr (Scheme 3).

The kinetic parameters of the best mutants for the diastereoselective formation of D-KDGlu **3** and D-KDGal **4** were then determined using our previously described thiobarbituric assay (Table 2),[20] which revealed that their activity was much decreased when compared to the wild-type aldolase. For KDGlu mutant-2, its K_M values for pyruvate **1** and D-glyceraldehyde **2** were increased by 4 and 1.5 times, respectively, with a 60- to 100-fold drop in k_{cat} for each substrate. Similarly, D-KDGal mutant-1 had K_M values for pyruvate **1** and D-glyceraldehyde **2** that were 8−9 times greater than those for the wild-type aldolase, with a similar 60- to 70-fold reduction in k_{cat} for each substrate. Although these kinetic data clearly reveal that the improved stereoselectivity of both these mutants has occurred at a 'kinetic price', both mutants retained sufficient activity to enable their use in preparative biotransformations to afford either D-KDGlu **3** (93% dr) or D-KDGal **4** (88% dr), respectively, on a millimole scale.[30] Furthermore, both these mutant aldolases exhibited similar thermal stability profiles to that of the wild-

(30) Spectroscopic data for D-KDGlu **3** and D-KDGal **4** were identical to those reported previously, see refs 18 and 25, and references contained therein.

type aldolase, demonstrating essentially unchanged activity at 70 °C over a period of 1 h.

Conclusion

We have shown how structural information obtained from a 'stereochemically promiscuous' aldolase bound to diastereoisomeric aldol products can be used to rapidly create stereocomplementary mutants. This structurally informed site-directed mutagenesis approach required screening of less than 30 mutants to identify a pair of mutant aldolases capable of catalyzing enantiofacial aldol reactions to give diastereoisomeric aldol products. This approach compares favorably with many other directed evolution studies in this area that often require screening of thousands of aldolase mutants to achieve the same aim.[10−12,17] Our results further illustrate how relatively few mutations at 'hot-spot' amino acids within an enzyme's active site can result in large changes in the stereoselectivity of the reactions that it catalyzes.[14] In this respect, this study may also provide an important insight into the ease with which an ancestral protein with nonstereoselective catalytic activity might have evolved into an enzyme that catalyzes the same reaction with higher levels of stereocontrol.

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Supporting Information Available: HPLC traces used to calculate diastereoselectivities of aldolase catalyzed reactions of pyruvate and D-glyceraldehyde. This material is available free of charge via the Internet at http://pubs.acs.org.

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