Mutagenesis of the phosphate-binding pocket of KDPG aldolase enhances selectivity for hydrophobic substrates

MANOJ CHERIYAN,¹ ERIC J. TOONE,² AND CAROL A. FIERKE¹

¹Department of Chemistry, University of Michigan, Ann Arbor, Michigan 48109, USA

²Department of Chemistry, Duke University, Durham, North Carolina 27708, USA

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Abstract

Narrow substrate specificities often limit the use of enzymes in biocatalysis. To further the development of *Escherichia coli* 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase as a biocatalyst, the molecular determinants of substrate specificity were probed by mutagenesis. Our data demonstrate that S184 is located in the substrate-binding pocket and interacts with the phosphate moiety of KDPG, providing biochemical support for the binding model proposed on the basis of crystallographic data. An analysis of the substrate selectivity of the mutant enzymes indicates that alterations to the phosphate-binding site of KDPG aldolase changes the substrate selectivity. We report mutations that enhance catalysis of aldol cleavage of substrates lacking a phosphate moiety and demonstrate that electrophile reactivity correlates with the hydrophobicity of the substituted side chain. These mutations improve the selectivity for unnatural substrates as compared to KDPG by up to 2000-fold. Furthermore, the S184L KDPG aldolase mutant improves the catalytic efficiency for the synthesis of a precursor for nikkomycin by 40-fold, making it a useful biocatalyst for the preparation of fine chemicals.

Keywords: nikkomycin; aldolase; KDPG; substrate specificity; rational redesign; active site; directed evolution; protein engineering; biocatalysis

The development of catalysts that effect synthetic transformations with predictable behavior is among the most challenging task facing synthetic chemistry. Biocatalysis—the use of enzymes to carry out synthetic transformation—is a field dating to the late nineteenth century. Enzymes are especially attractive catalysts for organic synthesis, given their ability to catalyze reactions with remarkable efficiency, substrate specificity, and stereoselectivity. The broader use of enzymes in biocatalysis is limited by the defined substrate specificities displayed by enzymes derived from natural sources; the *de novo* design of protein catalysts with predictable, defined substrate specificities is a vigorous area of research. Despite over 30 years of

effort in the field, an ability to rationally redesign enzyme active sites remains only rudimentary, a reflection of the complex structural basis of enzyme activity.

The aldolases are a large group of enzymes that catalyze reversible stereoselective aldol addition and have demonstrated utility in organic synthesis (Fessner and Walter 1997; Samland and Sprenger 2006). 2-Keto-3-deoxy-6-phosphogluconate (KDPG) aldolase is an especially attractive enzyme for development as a biocatalyst, showing good thermal stability (Griffiths et al. 2002), broad pH optimum, high tolerance of organic cosolvent (Shelton et al. 1996), high stereoselectivity, moderately broad substrate specificity, and ready availability. Together, these properties make KDPG aldolase a prime candidate for use as a catalyst in a number of synthetic applications (Henderson et al. 1997; Cotterill et al. 1998).

In vivo, KDPG aldolase functions as part of the Entner–Doudoroff glycolytic pathway, catalyzing the reversible retro-aldol cleavage of KDPG to pyruvate and

Reprint requests to: Carol A. Fierke, Department of Chemistry, University of Michigan, Box 1055, Ann Arbor, MI 48109, USA; e-mail: fierke@umich.edu; fax: (734) 647-4865.

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Figure 1. Reaction catalyzed by KDPG aldolase.

D-glyceraldehyde-3-phosphate (GAP) (Fig. 1; Peekhaus and Conway 1998). The reverse reaction, the stereo-specific formation of a carbon–carbon bond between pyruvate and a range of electrophilic aldehydes, is of significant interest for the synthesis of fine chemicals. However, as with most enzymatic catalysts, substrate selectivity ultimately limits the broad use of KDPG aldolase as a synthetic reagent. Recently, both rational design and directed evolution have been used to alter the substrate specificity of various aldolases with improvements in k_{cat}/K_M of up to 25-fold (DeSantis et al. 2003; Wada et al. 2003; Franke et al. 2004; Hsu et al. 2005; Ran and Frost 2007); similar efforts to alter the substrate specificity of KDPG aldolase have also been reported (Fong et al. 2000; Wymer et al. 2001). Further rational redesign of KDPG aldolase is limited by the lack of a detailed model of the substrate-binding site. We have reported crystallographic studies of various bound forms of KDPG aldolase from *Thermatoga maritima* (Fullerton et al. 2006) and *Escherichia coli* (Wymer et al. 2001), both of which crystallized bound to a dibasic inorganic anion. With the assumption that this ion reveals the phosphate-binding site, we proposed a binding model for full length substrate (Fig. 2). Here, we provide biochemical evidence for the validity of this model and consider the identity of the binding site for the electrophilic substrate through the preparation of a series of mutant proteins. We demonstrate that alteration of the phosphate-binding pocket significantly changes the substrate selectivity of this enzyme, providing biocatalysts with increased activity toward nonphosphorylated electrophiles. Furthermore, the mutant enzymes described here are useful for the synthesis of nikkomycins (Henderson et al. 1997), a group of potent inhibitors of chitin synthase (Schlüter 1982). These studies provide an essential background for structure-based remodeling of the KDPG aldolase active site and for the rational development of novel biocatalysts with predetermined substrate specificities.

Results

The first step in both aldol addition and retro-aldol cleavage by KDPG aldolase is the formation of a Schiff-base between an active site lysine and the C2 carbonyl of the nucleophilic substrate (Fig. 3). In *E. coli* KDPG aldolase this residue is K133, an assertion verified both by crystallographic analysis of pyruvate-bound protein (both Schiff base and carbinolamine intermedi-

ates) (Allard et al. 2001; Fullerton et al. 2006) and by mutagenesis (Wymer et al. 2001). A general base catalyzes the formation of the nucleophilic enamine, which, in turn, reacts with the aldehydic carbon of the electrophilic substrate, glyceraldehyde-3-phosphate, to form KDPG. Glutamate 45, located proximal to Lys133 in space, was proposed as the catalytic general acid/general base, functioning through the intermediacy of bound water molecules (Fullerton et al. 2006).

The structure of KDPG aldolase, both apoenzyme and various bound forms, has been studied extensively (Mavridis et al. 1982; Allard et al. 2001; Wymer et al. 2001; Fullerton et al. 2006). Despite a wealth of crystallographic data regarding the location of the pyruvate-binding site, little structural or biochemical information exists that informs the location or nature of the glyceraldehyde-3-phosphate-binding site. A plausible location of the electrophilic binding site was proposed based on crystal structures of the *E. coli* and *T. maritima* KDPG aldolases, both of which crystallize with sulfate ions bound to a solvent-exposed cleft roughly 10 Å from the catalytic residues (Fig. 2; Allard et al. 2001; Fullerton et al. 2006). The site is a highly conserved region of KDPG aldolase and is formed by G162, G163, I164, and S184. These residues are found in the loop regions between β -sheet 7/helix 7 and β -sheet 8/helix 8, a location where phosphate binding motifs are observed in other TIM barrel proteins (Wilmanns et al. 1991; Nagano et al. 2002). Based on these data, we proposed a model of full-length substrate binding that locates the KDPG phosphate moiety in the place of the sulfate ions observed in pyruvate-bound aldolase. To test the validity of this model we examined

Figure 2. Model of KDPG bound to KDPG aldolase. Structural model of KDPG bound to KDPG aldolase based on the crystal structure of *T. maritima* aldolase (IWA3; blue) and the *E. coli* aldolase (1EUA; red). KDPG bound as a Schiff base was modeled as described by Fullerton et al. (2006). Residues that line the phosphate-binding site G162, G163, I164, and S184 are shown with possible hydrogen bond contacts to the substrate drawn with yellow lines. The catalytic residues E45 and K133 are also shown.

Figure 3. Abbreviated mechanism for KDPG aldolase.

the effect of perturbing one of the residues in this cleft, serine 184, on the reactivity of this enzyme.

The sulfate ion observed in the crystal structure forms hydrogen bonds with the backbone amides of G162, G163, I164, and the side chain hydroxyl of S184. The two glycine residues create a tight turn between β -sheet 7 and helix 7; mutation of these residues would likely cause significant structural reorganization, precluding these sites as targets for mutagenesis. We thus considered the functional effect of altering S184, changing the residue to alanine, leucine, and aspartate. Alanine mutation probes the role of hydrogen bonding, while the leucine substitution explores the effect of partially occluding the binding site with a hydrophobic residue. Finally, mutation of position 184 to aspartate investigates the effect of inserting negative charge into the putative phosphate-binding pocket. The functional consequences of these mutations are quantified by evaluating the effect of the mutation on catalysis of aldol cleavage of several substrates.

The steady-state kinetic parameters for KDPG cleavage catalyzed by each mutant enzyme were determined to analyze the importance of the interaction of the phosphate moiety with the phosphate-binding pocket. Kinetic parameters were also determined for the cleavage of 2-keto-3-deoxy-gluconate (KDG), an analog of KDPG that lacks the C6 phosphate, to isolate effects that are specific to an interaction with the phosphate moiety. And for 2-keto-4-hydroxyoctonate (KHO), a substrate analog in which the C5 alcohol and C6 phosphate are replaced with a hydrogen and an ethyl group, respectively, was studied to probe the potential for new hydrophobic interactions between substrates and the side chain at position 184 that could enhance catalytic activity toward unnatural substrates (Fig. 4; Table 1).

Wild-type KDPG aldolase efficiently catalyzes retro-aldol cleavage of KDPG with values of $k_{cat}/K_M^{\text{KDPG}}$ and k_{cat} of $8.3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and 83 s^{-1} , respectively. Removal of the C6 phosphate in the substrate (KDG) decreases the catalytic efficiency of KDPG aldolase enormously, by 8.6×10^4 -fold, a loss due to both a 520-fold decrease in the value of k_{cat} and a 160-fold increase in the

value of K_M . The efficiency of retro-aldol cleavage of the hydrophobic substrate analog KHO is comparable to that of KDG, with a decrease in k_{cat}/K_M of 6×10^4 relative to KDPG. In this instance, however, the loss in activity is primarily the result of a 1400-fold increase in K_M , presumably reflecting mainly weaker substrate binding.

Mutation of S184 in KDPG aldolase to alanine decreases the efficiency of KDPG retro-aldol cleavage only modestly, reducing $k_{cat}/K_M^{\text{KDPG}}$ by 27% to $6.1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. Deletion of this single interaction with the phosphate group clearly does not recapitulate the low catalytic activity of the KDG substrate, presumably reflecting the ability of the remaining residues in the putative phosphate-binding site to effectively bind and orient the substrate for catalysis. The S184A mutation has only a modest effect on the catalytic efficiency for cleavage of KDG, and increases the value of k_{cat}/K_M^{KHO} for cleavage of KHO by threefold.

Figure 4. Substrates for KDPG aldolase. (A) KDPG is the natural substrate. (B) 2-keto-3-deoxygluconate (KDG) lacks the C6 phosphate. (C) 2-keto-4-hydroxy-octonate (KHO) is a hydrophobic analog of KDPG. (D) 2-keto-4-hydroxy-4-(2'-pyridyl)butyrate (KHPB) is a reactive analog. (E) 2-keto-3-deoxy-6-phosphogalactonate (KDPGal) differs from KDPG by only the stereochemistry at C4.

Table 1. Kinetic parameters catalyzed by S184 mutants of *E. coli* KDPG aldolase

	KDPG			KDG			KHO ^a		
	k_{cat} s ⁻¹	K_M mM	k_{cat}/K_M M ⁻¹ s ⁻¹	k_{cat} s ⁻¹	K_M mM	k_{cat}/K_M M ⁻¹ s ⁻¹	k_{cat} s ⁻¹	K_M mM	k_{cat}/K_M M ⁻¹ s ⁻¹
Wild type	83 ± 2	0.10 ± 0.01	830,000 ± 2000	0.019 ± 0.001	1.9 ± 0.1	9.7 ± 0.4	2 ± 0.6	150 ± 50	14 ± 1
S184A	97 ± 2	0.16 ± 0.01	610,000 ± 5000	0.040 ± 0.001	2.3 ± 0.1	17 ± 1	2.6 ± 0.2	56 ± 7	47 ± 2
S184L	74 ± 7	0.35 ± 0.10	210,000 ± 10,000	0.080 ± 0.002	1.5 ± 0.1	53 ± 3	2.9 ± 0.2	26 ± 4	110 ± 10
S184D	3.2 ± 0.1	1.2 ± 0.1	2,700 ± 100	0.110 ± 0.003	1.8 ± 0.1	62 ± 4	1.8 ± 0.1	51 ± 5	35 ± 2

Initial velocity for pyruvate formation was measured using a lactate dehydrogenase coupled assay. Steady-state kinetic parameters were determined by fit of the Michaelis–Menten equation to the initial velocity as a function of the substrate concentration (Michaelis and Menten 1913).

^aKHO is synthesized in racemic form and the indicated concentration includes both C4 stereoisomers; however, KDPG aldolase preferentially catalyzes the cleavage of (S)-KHO. Therefore, the observed K_M is likely to be higher than the actually value.

Similar trends in reactivity are observed for the S184L mutation, although the magnitude of the effect is larger; the catalytic efficiency of KDPG cleavage decreases nearly fourfold as compared to the wild-type enzyme. This effect is due mainly to a threefold increase in K_M , suggesting that the leucine substitution further disrupts interactions with the phosphate-binding pocket. In contrast to the S184A mutant, S184L KDPG aldolase *increases* the efficiency for retro-aldol cleavage of both KDG and KHO, with improvements in k_{cat}/K_M , of 5.5- and 8-fold, respectively. The increase in efficiency for KDG cleavage occurs mainly through an increase in k_{cat} while for KHO cleavage the K_M is reduced. Therefore, the S184L mutant has an enhanced selectivity for KHO over KDPG of 32-fold relative to wild-type KDPG aldolase¹ (Table 2).

In contrast to the modest effects of the Ala and Leu substitutions, mutation of S184 to aspartate significantly abrogates KDPG cleavage activity, diminishing k_{cat}/K_M^{KDPG} by over 300-fold; both K_M and k_{cat} are affected, by 12- and 26-fold, respectively. Surprisingly, this mutation increases the catalytic efficiency of aldol cleavage of substrates lacking a negatively charged phosphate group; the values of k_{cat}/K_M for catalysis of KDG and KHO cleavage increase by 6.5- and 2.5-fold, respectively, as compared to the wild-type enzyme. The S184D mutant also enhances the selectivity for reaction with KHO compared to KDPG by 780-fold relative to the wild-type KDPG aldolase (Table 2).

To further consider the effect of mutations to the phosphate-binding pocket on the reactivity and selectivity of KDPG aldolase, we measured steady-state kinetic parameters for retro-aldol cleavage of 2-keto-4-hydroxy-4-(2'-pyridyl)butyrate (KHPB). KDPG aldolase efficiently cleaves this substrate (Henderson et al. 1997) despite the structural dissimilarity to KDPG (Fig. 4). Kinetic analysis

of cleavage of KHPB catalyzed by wild-type KDPG aldolase (Table 3) indicates that k_{cat}/K_M^{KHPB} is reduced by 2500-fold compared to that of KDPG; however, the catalytic efficiency for this substrate is still more than one order of magnitude greater than either KDG or KHO. Aldol addition proceeds with *si*-facial stereochemical specificity, identical to natural substrates (Henderson et al. 1998), suggesting that KHPB and KDPG may share a common binding orientation. The rapid turnover of this substrate presumably reflects, at least in part, the intrinsic reactivity of the electrophilic pyridyl-aldehyde.

Mutation of S184 to A, L, or D enhances the catalytic efficiency for retro-aldol cleavage of KHPB by 6- to 40-fold and with the same order of reactivity observed for catalysis of KHO cleavage, i.e., S184L > S184A > S184D > wild type. All of the mutations improve K_M by 10-fold relative to wild-type enzyme. Remarkably, the S184L mutation also enhances turnover fourfold, which, coupled with the tighter apparent binding affinity, leads to a 40-fold enhancement in catalytic activity against this substrate. The S184D mutation also significantly alters the substrate selectivity such that the values of k_{cat}/K_M are comparable for KHPB and KDPG (Tables 1 and 3).

The turnover number of the S184L enzyme for cleavage of KHPB is sufficiently rapid to make this enzyme a viable candidate for practical biocatalyst development. To further examine the suitability of S184L KDPG aldolase as a biocatalyst, we evaluated the stereoselectivity of

Table 2. Improvements in selectivity for unnatural substrates as compared to KDPG

Mutants	Substrate selectivity compared to KDPG cleavage ^a		
	KDG	KHO	KHPB
S184A	2.5	4.6	37
S184L	22	32	160
S184D	2000	780	1800

¹Selectivity ratio = $[(k_{cat}/K_M^{KDPG})/(k_{cat}/K_M^{KHO})]^{WT}/[(k_{cat}/K_M^{KDPG})/(k_{cat}/K_M^{KHO})]^{Mutant}$.

^aThe equation for selectivity ratio = $[(k_{cat}/K_M^{KDPG})/(k_{cat}/K_M^S)]^{WT}/[(k_{cat}/K_M^{KDPG})/(k_{cat}/K_M^S)]^{Mutant}$.

Table 3. Kinetic parameters for cleavage of KHPB catalyzed by *E. coli* KDPG aldolase mutants

	KHPB		
	k_{cat} s ⁻¹	K_M mM	k_{cat}/K_M M ⁻¹ s ⁻¹
Wild type	11 ± 1	32 ± 2	330 ± 9
S184A	27 ± 3	3 ± 1	9000 ± 3000
S184L	40 ± 1	3 ± 0.2	13,300 ± 600
S184D	6.0 ± 0.6	3 ± 1	2000 ± 600

The kinetics were measured as described in Table 1.

enzyme-catalyzed aldol addition of pyruvate to 2-pyridine carboxaldehyde. The KHPB product was converted to the corresponding dithiolactone and analyzed by chiral GC, which showed a single peak. These data demonstrate that the S184L mutant behaves like wild type (Henderson et al. 1998) with regard to enantioselectivity, achieving greater than 99.7% *si*-facial addition product. The ability of this mutant to more efficiently catalyze KHPB cleavage and yet still retain precise stereochemical control makes it an excellent candidate as a practical biocatalyst for the synthesis of nikkomycins.

To further analyze the effect of mutations at S184 on the stereoselectivity of KDPG aldolase, we measured the steady-state kinetic parameters for retro-aldol cleavage of 2-keto-3-deoxy-6-phosphogalactonate (KDPGal; Table 4). Since KDPGal and KDPG differ only by the identity of the chiral center at C4 (Fig. 4), the enantioselectivity of each enzyme can be measured by the ratio of (k_{cat}/K_M^{KDPG})/(k_{cat}/K_M^{KDPGal}). Wild-type KDPG aldolase has an enantiomeric selectivity for cleavage of KDPG compared to KDPGal of 1.4×10^4 -fold. The S184L mutation in KDPG aldolase causes a larger decrease in the value of k_{cat}/K_M^{KDPGal} than the value of k_{cat}/K_M^{KDPG} , thereby increasing the enantiomeric selectivity ratio to 7.8×10^4 (Table 4). The S184D mutant has an enantiomeric selectivity ratio of 1.1×10^4 , indicating that the stereoselectivity of this mutant is the same as wild type within error. These data confirm that mutation at S184 does not directly alter the stereoselectivity of KDPG aldolase.

Discussion

Phosphate-binding site

Phosphate-binding motifs in many enzymes contain one or more positively charged residues that directly hydrogen bond to the substrate (Copley and Barton 1994). However, the proposed binding site in KDPG aldolase lacks a nearby positively charged residue, relying mainly on backbone amide interactions to bind the phosphate. Copley and Barton (1994) found that a phosphate anion bound close (<5 Å) to the N terminus of an α -helix often

interacts with the protein exclusively by backbone contacts, highlighting the stabilizing effect of the macrodipole of α -helices. Recently Kinoshita and coworkers (1999) identified an important motif for binding phosphate-containing substrates, the “structural P-loop” (GXXX), where all interactions are made with backbone amides. The phosphate-binding site in KDPG aldolase shares similarities with the structural P-loop, including the location of a glycine residue and four hydrogen bond contacts with backbone amides.

Functional importance of the KDPG phosphate moiety

A structural model of KDPG bound in open chain form to KDPG aldolase was developed based on crystallographic data of the pyruvate-bound enzyme that shows a phosphate and/or sulfate ion bound to both the *T. maritima* and *E. coli* enzymes (Fig. 2). This model required only minimal adjustments to the experimentally determined positions of the covalent pyruvyl intermediate and bound anion to accommodate the full-length substrate. The low catalytic activity (k_{cat}) of substrates lacking a phosphate group (KDG and KHO) suggests that productive interactions between the phosphate moiety and the enzyme are crucial for efficient turnover of the bound substrate ($\Delta\Delta G^\ddagger = 3.9$ kcal/mol, estimated from the values of k_{cat}),² and that the phosphate moiety is apparently critical for productive orientation of the bound substrate. The model of bound KDPG suggests that a minimum of three interactions enforce substrate orientation: a covalent imine bond between Lys33 and C2 of KDPG, hydrogen-bond interactions between the side chain of Arg47 and the carboxylate oxygens of KDPG, and hydrogen bonding between the phosphate moiety and the anion-binding site of the enzyme. In the absence of the phosphate anchor, the various substrate-bound intermediates (carbinol-amine, imine, enamine) are mobile in space and/or not properly positioned for efficient protonation/deprotonation by the general base, E45, through the intermediacy of a water molecule (Fullerton et al. 2006).

A complicating facet for interpreting the KDG turnover data involves the solution form of the substrate. KDPG aldolase is specific for the open-chain form of KDPG, and this open-chain form is roughly 9% of the material in solution (Midelfort et al. 1977). However, KDG is almost entirely in the pyranose and furanose cyclic forms (Fong et al. 2000), with no open-chain ketose form visible by either ¹³C or ¹H NMR. The increase in K_M^{KDG} compared to K_M^{KDPG} arises at least in part, and perhaps entirely, from the lowered concentration of the open-chain substrate. Because binding is a coupled equilibrium, preferential

² $\Delta\Delta G^\ddagger = -2.303RT \log(k_{cat}^{KDPG}/k_{cat}^{KDG})$.

Table 4. Kinetic parameters for cleavage of KDPGal catalyzed by *E. coli* KDPG aldolase mutants and the enantiomeric selectivity ratio

Enzyme	KDPPGal			$\frac{k_{cat}/K_M^{KDPPGal}}{k_{cat}/K_M^{KDPPGal}}$
	$k_{cat} \text{ s}^{-1}$	$K_M \text{ mM}$	$k_{cat}/K_M \text{ M}^{-1} \text{ s}^{-1}$	
Wild type	0.0063 $\pm$ 0.0001	0.10 $\pm$ 0.01	61 $\pm$ 3	14,000
S184L	0.0026 $\pm$ 0.0001	0.95 $\pm$ 0.17	2.7 $\pm$ 0.4	78,000
S184D	0.00029 $\pm$ 0.00001	1.2 $\pm$ 0.1	0.24 $\pm$ 0.01	11,000

The kinetics were measured as described in Table 1.

population of the cyclic forms decreases the apparent substrate binding affinity. In addition to a thermodynamic preference for cyclic forms of the substrate, kinetic inter-conversion of the variously populated cyclic forms and the open-chain productive substrate may become partially rate limiting. However, these factors should not contribute to variations in catalytic efficiency between the various mutant enzymes, since interconversion of the substrate occurs independent of the enzyme catalyzed retro-aldol reaction (Midelfort et al. 1977). Furthermore, these complications are not relevant for KHO and KHPB, which populate only open-chain forms.

On the other hand, the decrease in k_{cat} reflects a diminished efficiency for turnover of the bound, open-chain substrate. Because the phosphorylation site is far from the active site, it is unlikely that the phosphate moiety participates directly in catalysis. Instead, the phosphate group presumably interacts with the protein to position the bound substrate for optimal catalysis.

Importance of the S184 side chain

In our model of KDPG-bound aldolase (Fullerton et al. 2006), the hydroxyl group of S184 lies within hydrogen-bonding distance of one of the phosphoryl oxygens of KDPG. However, the modest changes in both catalytic efficiency and apparent substrate-binding affinity ($\Delta\Delta G_{\text{Bind}} = 0.3 \text{ kcal/mol}$) observed on deletion of the S184 hydroxyl group suggests that this hydrogen bond plays only a minor role in the overall catalytic efficiency of the enzyme. This observation is likely due to the redundant nature of the hydrogen-bond network in the phosphate-binding site, made up of the backbone amide groups of G162, G163, I164, and the residue at position 184. Presumably, the S184 hydroxyl moiety is replaced by a water molecule in the mutant enzymes, providing an intact phosphate-binding pocket. Leucine replacement at position 184 potentially offers significant steric encumbrance to the anion-binding site. The high catalytic activity of S184L mutant seemingly discounts this concern and suggests that the leucine side chain swings away from the hydrophilic-binding pocket.

In contrast, conversion of S184 to aspartate decreases the catalytic efficiency for cleavage of KDPG by 300-fold,

due to both lower apparent substrate affinity ($\Delta\Delta G_{\text{Bind}} = 1.5 \text{ kcal mol}^{-1}$) and increased activation energy to form the transition state from the E•S complex ($\Delta\Delta G^\ddagger = 2 \text{ kcal mol}^{-1}$). A decreased binding affinity is consistent with electrostatic repulsion between the like negative charges on the KDPG phosphate moiety and the aspartyl side chain. Again, productive binding by the phosphate-binding site apparently orients the bound substrate for efficient turnover; the introduction of a negatively charged side chain disrupts this orientation.

Mutations at position 184 enhance the reactivity of KDPG aldolase for cleavage of substrates lacking a phosphate group, including KDG, KHO, and KHPB, and decrease the reactivity against KDPG. Excluding S184D, the reactivity of the mutant enzymes correlates with the hydrophobicity of the substituent at position 184. Values of $\Delta\Delta G^\ddagger$ (k_{cat}/K_M) for reaction of aldolases with A, S, and L at position 184 show a linear correlation with $\Delta G_{\text{transfer}}^0$,³ with reactivity increasing with increasing hydrophobicity for KDG (slope = -0.40 ± 0.06 , $R = 0.98$) and KHO (slope = -0.45 ± 0.20 , $R = 0.82$) (Fig. 5). The opposite correlation is observed for catalysis of KDPG cleavage (slope = 0.34 ± 0.01 , $R = 0.99$), with increased hydrophobicity at position 184 correlating with decreased reactivity. These data suggest that further improvements in both reactivity and selectivity for KDG and KHO could be achieved by incorporation of other hydrophobic residues in the phosphate-binding region of KDPG aldolase.

However, not all of the observed changes in reactivity (Tables 1 and 3) can be explained by hydrophobicity of the S184 side chain. For all substrates, aspartate substitution has an effect on reactivity greater than that predicted by side chain hydrophobicity; the S184D mutant reacts more rapidly with KDG and KHO and less rapidly with KDPG than predicted by the hydrophobicity correlation. In the case of KDPG, this observation undoubtedly reflects electrostatic repulsion with the negatively charged phosphate. The origin of the enhanced reactivity

³Hydrophobicity of the amino acid is indicated by the $\Delta G_{\text{transfer}}^0$ between octanol and water (Karplus 1997).

Figure 5. Correlation between activity and hydrophobicity. The $\Delta\Delta G^\ddagger$ (k_{cat}/K_M) values for catalysis of retro-aldol cleavage of KDPG (Δ), KDG ($\circ$), and KHO ($\square$) by wild-type and mutant KDPG aldolases are plotted against the $\Delta G^\circ_{\text{Transfer}}$ for the side chain (S, A, or L) at position 184 (Karplus 1997).

of S184D with KDG and KHO is less clear. It is possible that the aspartate side chain forms hydrogen bonds with the amide hydrogen-bond donors in the anion-binding site, increasing the overall hydrophobicity of the region.

The reactivity of aldolase mutants for catalyzing cleavage of KHPB also increases with increasing hydrophobicity of the residue at position 184; values of $\Delta\Delta G^\ddagger$ (k_{cat}) for enzymatic cleavage of KHPB with A, S, L, and D at position 184 show a linear correlation with $\Delta G^\circ_{\text{transfer}}$ (slope = -0.33 ± 0.09 , $R = 0.85$; plot not shown). In this analysis, $k_{cat}/K_M^{\text{KHPB}}$ for the wild-type enzyme is significantly lower than would be predicted based on the side chain hydrophobicity. These data suggest that the serine hydroxyl group has a specific unfavorable interaction with KHPB that increases K_M to 32 mM, compared to the 3 mM value measured for the three mutant enzymes. In total, the behavior of these mutants suggests that KHPB interacts with the anion-binding site similar to our model of bound KDPG. Additionally, these data demonstrate that substitution in the anion-binding pocket generally enhances the reactivity of KDPG aldolase toward nonphosphorylated substrates.

Development of KDPG aldolases with altered substrate selectivity

Directed evolution of KDPG aldolase to identify mutants with enhanced substrate selectivity for hydrophobic substrates, such as KHO, has generally identified mutations distal to the active site (M. Cheriyian, M.J. Walters, E.J. Toone, and C.A. Fierke, unpubl. data). Similar observations have been reported during the directed evolution of other enzymes (Franke et al. 2004; Hsu et al. 2005; Morley and Kazlauskas 2005). In contrast, a previous selection to

enhance the reactivity of KDPG aldolase using benzaldehyde as the electrophilic substrate identified a mutation that altered the position of the active site lysine, demonstrating that changes directly in the active site can lead to beneficial alterations in substrate selectivity (Wymer et al. 2001). Morley and Kazlauskas (2005) have suggested that the preponderance of non-active-site mutations recovered in directed evolution experiments is a simple probability effect, since the number of amino acids located near the active site is smaller than the number of distal residues. An alternative explanation is that mutations near the active site cause perturbations to the binding pocket and the positions of catalytic residues too large to produce productive alterations in activity, while mutations far from the active site produce the subtle effects on active-site structure, dynamics, and electrostatics required for enhanced activity. Here we have used a structure-based approach to remodel the active site of KDPG aldolase. The phosphate-binding pocket was identified crystallographically (Fullerton et al. 2006), and we postulated that mutations in this region of the protein should diminish recognition of phosphorylated substrates and enhance the reactivity of more hydrophobic substrates. This prediction is borne out, as mutations at S184 alter the substrate selectivity in the predicted fashion. Similarly, recent directed evolution experiments focused on active site residues in other enzymes also indicate that mutations near the active site can rapidly alter substrate specificity (Geddie and Matsumura 2004; Chica et al. 2005; Parikh and Matsumura 2005). These data clearly demonstrate that changes within the substrate-binding pocket can have beneficial effects on substrate selectivity, an observation consistent with the suggestion that the preponderance of mutants far from the active site recovered in directed evolution processes has a probabilistic origin.

The efficient, stereospecific aldol cleavage of KHPB by the S184L mutant approaches values for the cleavage of the natural substrate, KDPG, by wild-type enzyme, making S184L a useful enzyme for chemical synthesis of nikkomycin analogs (Henderson et al. 1997). Additionally, this enhancement in activity is achieved while maintaining the high stereoselectivity of the wild-type enzyme. Other mutations in the phosphate-binding site may prove beneficial in efforts to engineer an enzyme with high levels of activity toward other unnatural substrates. We continue our efforts to redesign the catalytic activity of KDPG aldolase through both rational and combinatorial approaches and will report our results in a future article.

Materials and Methods

Construction of mutants

Mutations at serine 184 were made using the QuikChange (Stratagene) method with the following primers and their

reverse complements: S184A: 5'-GCTGTGCATCGGTGGTG CCTGGCTGGTTCCGGCAG-3'; S184L: 5'-GCTGTGCATCG GTGGTCTCTGGCTGGTTCCGGCAG-3'; and S184D: 5'-GC TGTGCATCGGTGGTGACTGGCTGGTTCCGGCAG-3'. The plasmid pET-ECEDA (Wymer et al. 2001) encoding the *E. coli eda* gene behind a T7 polymerase promoter was used as the template. After whole plasmid PCR amplification with PFU Turbo (Stratagene) and exhaustive digestion with DpnI (NEB), the product was transformed into Smartcells (GeneLantis). Following overnight growth on Luria-Bertani media agar plates containing 100 µg/mL kanamycin, a single colony was cultured and the plasmid DNA was purified using Wizard SV MiniPrep kit (Promega) and sequenced at the University of Michigan Sequencing Core, Ann Arbor.

Protein expression and purification

The pET-ECEDA plasmid encoding each mutant *eda* gene with a N-terminal His-tag was transformed into BL21(DE3) cells and grown on LB plates containing 100 µg/mL kanamycin. A single colony was used to inoculate an overnight starter culture that was subsequently diluted into a 1 L culture of Terrific Broth (Sambrook and Russell 2001) containing 100 µg/mL kanamycin. The cultures were incubated at 37°C until OD_{600 nm} = 0.6–1.0 and then 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added. The cultures were incubated for 4–8 h. The cells were pelleted by centrifugation, resuspended in 40 mL of HEPES (25 mM, pH 7.5), and lysed using a Sonic Dismembrator 550 (six 30-sec pulses followed by 4.5 min on ice). The crude cell extracts were clarified by centrifugation (30 min at 16,000g). The supernatant was loaded onto a Ni²⁺-charged Chelating Sepharose Fast Flow column (Amersham; 100 mL volume) for fractionation using a stepwise gradient of HEPES (100 mM, pH 7.5), NaCl (100 mM) containing 5 mM imidazole (1.5 column volumes), 155 mM imidazole (2 column volumes), and 500 mM imidazole (2 column volumes). Fractions containing the His-tagged KDPG aldolase eluted during the 500-mM imidazole wash. These fractions were combined and concentrated using Amicon Ultra (15 mL, 30,000 MWCO; Millipore) spin concentrators and extensively washed with HEPES (100 mM, pH 7.5), NaCl (100 mM) to remove the imidazole. Enzymes stored at 4°C in HEPES (100 mM, pH 7.5), NaCl (100 mM), 10% glycerol, at concentrations ranging from 1 to 16 mM, are stable for up to 1 yr.

The protein concentrations were determined by absorbance at OD₂₈₀ where the extinction coefficient was calculated to be $\epsilon_{280} = 15,510 \text{ M}^{-1} \text{ cm}^{-1}$ for both wild-type and mutant KDPG aldolases. The protein concentrations and purity were verified by 12% polyacrylamide gel electrophoresis followed by Coomassie staining and comparison of band intensity versus a known standard.

Overexpression of phosphogluconate dehydratase

The *edd* gene encoding phosphogluconate dehydratase (PGDH) was amplified using PCR from genomic DNA isolated from *E. coli* K-12 cells using PFU polymerase (Stratagene) and the following primers: PGDH-for 5'-GCTCTGACAACCTCAATTC AGGATCCCATATGAATCC-3', PGDH-rev 5'-CGCCTGATTA CAAATTTCTCGATTAAAAAGTGATACAGGTTGC-3'. The PCR was done using PFU turbo (2.5 U, Stratagene) and its accompanying buffer, dNTP mix (1 µL, 25 mM of each nucleotide), primers (125 ng each), *E. coli* genomic DNA (200 ng), and

water to make a final volume of 50 µL. The PCR thermocycling was done using Applied Biosystems GeneAmp 2400 programmed with the following cycles: 1 cycle of 95°C for 30 sec; 30 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1.5 min; 1 cycle of 72°C for 5 min.

The primers contained NdeI and XhoI restriction sites for incorporation into the plasmid pET20b (Novagen). The plasmid DNA and the PCR product were digested with both restriction enzymes and purified on a 1% agarose gel, and the two fragments were joined by incubation with T4 DNA ligase (NEB) to create an expression plasmid that encodes PGDH. The presence of the correct *edd* sequence was verified by DNA sequencing (UM Sequencing Core). This plasmid was named pET-PGDH. Calcium competent BL21(DE3) cells were transformed (Sambrook and Russell 2001) with pET-PGDH, and a single colony was diluted into LB media containing 100 µg/mL ampicillin and incubated at 37°C until OD₆₀₀ ~ 0.5 was achieved. To induce expression of the PGDH gene, 1 mM IPTG was added along with 100 µM MnCl₂ and 500 µM FeCl₂, and the culture was incubated at 34°C for 6 h. Cells were harvested and frozen for storage.

A small aliquot of cells was resuspended in degassed PDGH buffer (20 mM MES at pH 6.5, 30 mM NaCl, 5 mM MnCl₂, 0.5 mM FeCl₂, 10 mM 2-mercaptoethanol, and 10 mM cysteine). Cells were lysed by addition of 0.1% Triton X-100, 10 ng/mL DNase, 10 µg/mL phenylmethylsulfonyl fluoride (PMSF), 10 µg/mL Nα-(*p*-toluene sulfonyl)-L-arginine methyl ester (TAME), 100 µg/mL lysozyme and incubated for 2 h at 37°C under argon. After cell lysis, the mixture was clarified by centrifugation and the soluble fraction was retained. The residual KDPG aldolase activity in this crude lysate was inactivated by addition of 150 mM pyruvate and 10 mM sodium cyanoborohydride for 30 min at room temperature. This treatment has no effect on the PGDH activity. The lysate was then fractionated on a PD-10 column to remove excess pyruvate and sodium cyanoborohydride. The fractions containing protein were assayed for residual KDPG aldolase activity (KDPG cleavage assay; see below) and PGDH activity. (PGDH activity is measured by an enzyme coupled assay containing KDPG aldolase and lactate dehydrogenase [LDH]. PGDH activity is observed when 6-phosphogluconate is added to the reaction.) The fractions containing only PGDH activity were used to synthesize KDPG without further purification.

Kinetic assays

KDPG cleavage activity was assayed using a lactate dehydrogenase (LDH) coupled assay where the production of pyruvate was measured by the decrease in NADH absorbance at 340 nm. All assays were done in quartz microcuvettes (70 µL) using a CARY 100 Bio UV/Vis spectrometer fitted with a peltier temperature controller set at 25°C. The reactions contained the following final concentrations: HEPES (100 mM, pH 7.5), NADH (250 µM), LDH (0.023 U/µL), 50 µM–100 mM substrate, depending on the *K_M*. The enzyme concentration used varied with the substrate: 25 nM–1 µM for KDPG, 10 µM for KDGL, 1 µM for KHO, 0.5 µM for KHPB, and 10–100 µM for KDPGal. The slow background decrease in NADH concentration observed upon addition of LDH at high KHO concentrations was subtracted from the observed rate. The Michaelis–Menten equation was fit to the data using the curve fitting program GraphPad PRISM 4, and the reported errors are the standard errors determined from these fits (Michaelis and Menten 1913).

Synthesis of D-2-keto-3-deoxy-6-phosphogluconate (KDPG)

The synthesis of KDPG was done according to the procedure developed by O'Connell (O'Connell and Meloche 1982) beginning with the conversion of glucose-6-phosphate to 6-phosphogluconate by bromine oxidation. One hundred milligrams of 6-phosphogluconate were dissolved in 1 mL PGDH buffer and incubated with ~ 1 U⁴ of PGDH, (described above) in an anaerobic container for 12 h at room temperature. The reaction mixture was purified over a Dowex 1 column (Cl-form, 1 cm $\times$ 5 cm) in 600 mL of 0–0.1 M HCL gradient. Fractions that contained KDPG were combined and neutralized with lithium hydroxide and lyophilized.

Synthesis of D-2-keto-3-deoxygluconate (KDG)

KDPG (200 mg) was dissolved in 1.3 mL of HEPES (pH 6). Four units of sweet potato alkaline phosphatase (Sigma) were added, and the reaction was incubated at 37°C overnight. The white insoluble precipitate, Li₂PO₄, was separated by filtration and the mixture was fractionated on a PD-10 column to remove the phosphatase. The resulting solution was lyophilized. The presence of unreacted KDPG was assayed using the lactate dehydrogenase/KDPG aldolase coupled assay. At millimolar concentrations of KDG, no rapid formation of pyruvate was observed, indicating that KDPG was completely reacted, but a reaction that is >100 times slower was measured, indicative of KDG cleavage (Ingram and Wood 1966).

Synthesis of (RS)-2-keto-4-hydroxyoctonate (KHO)

KHO was synthesized using the non-stereoselective method described by Griffiths et al. (2004). The crude extract was purified by resuspension into a minimal amount of water followed by addition of 10 times the volume of methanol to precipitate the KHO salt. This procedure yielded pure KHO with peaks identical to the reported values (Griffiths et al. 2004). Enzymatic assay for the presence of pyruvate indicated that there is <0.3% pyruvate contaminant.

Synthesis of (4S)-2-keto-4-hydroxy-4-(2'-pyridyl)butyrate (KHPB)

Preparative scale enzymatic synthesis of the aldol addition product between pyruvate and 2'-pyridine carboxaldehyde for kinetic studies was done according to the previously reported enzymatic method using wild-type *E. coli* KDPG aldolase (Griffiths et al. 2002). Synthetic scale preparation to determine enantioselectivity was done using wild-type *E. coli* KDPG aldolase or the S184L mutant. The products catalyzed by the wild-type and mutant aldolases were derivatized to 2,2-dithioethyl-4-(2'-pyridyl)-4-butyro- γ -lactone and run over a chiral CG using a Chrompack Chirasil-L-Val column using the protocol that was reported previously (Griffiths et al. 2002). The appearance of a single peak and identical elution times indicates that wild-type stereoselectivity is retained.

Synthesis of D-2-keto-3-deoxy-6-phosphogalactonate (KDPGal)

Enzymatically synthesized KDPGal was generously provided by Matthew J. Walters at Duke University.

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⁴One unit is able to convert 1 μ M of 6-phosphogluconate to KDPG per minute.

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