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Rational Design of Stereoselectivity in the Class II Pyruvate Aldolase BphI

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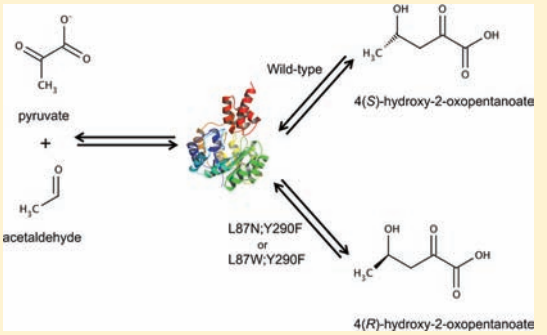
ABSTRACT: BphI, a pyruvate-specific class II aldolase, catalyzes the reversible carbon−carbon bond formation of 4-hydroxy-2-oxoacids up to eight carbons in length. During the aldol addition catalyzed by BphI, the *S*-configured stereogenic center at C4 is created via attack of a pyruvate enolate intermediate on the *si* face of the aldehyde carbonyl of acetaldehyde to form 4(*S*)-hydroxy-2-oxopentanoate. Replacement of a Leu-87 residue within the active site of the enzyme with polar asparagine and bulky tryptophan led to enzymes with no detectable aldolase activity. These variants retained decarboxylase activity for the smaller oxaloacetate substrate, which is not inhibited by excess 4-hydroxy-2-oxopentanoate, confirming the results from molecular modeling that Leu-87 interacts with the C4-methyl of 4(*S*)-hydroxy-2-oxoacids. Double variants L87N;Y290F and L87W;Y290F were constructed to enable the binding of 4(*R*)-hydroxy-2-oxoacids by relieving the steric hindrance between the 5-methyl group of these compounds and the hydroxyl substituent on the phenyl ring of Tyr-290. The resultant enzymes were shown to exclusively utilize only 4(*R*)- and not 4(*S*)-hydroxy-2-oxopentanoate as the substrate. Polarimetric analysis confirmed that the double variants are able to synthesize 4-hydroxy-2-oxoacids up to eight carbons in length, which were the opposite stereoisomer compared to those produced by the wild-type enzyme. Overall the k_{cat}/K_m values for pyruvate and aldehydes in the aldol addition reactions were affected ≤10-fold in the double variants relative to the wild-type enzyme. Thus, stereocomplementary class II pyruvate aldolases are now available to create chiral 4-hydroxy-2-oxoacid skeletons as synthons for organic reactions.



INTRODUCTION

Approximately 60% of all pharmaceutical agents currently on the market are chiral, and the development of enantiomerically pure formulations is often critical to ensure high specificity for the intended biological target.[1−4] Aldolases have the potential to catalyze the formation of C−C bonds with stereochemical control and are therefore powerful biocatalysts for the synthesis of pharmaceutical compounds, including enzyme inhibitors[5] and complex oligosaccharides.[6] Among aldolases, the dihydroxyacetone phosphate (DHAP) dependent aldolase, which possesses a strict requirement for DHAP as the nucleophilic component and can accept virtually any aldehyde as an electrophile, has been extensively investigated for organic synthesis.[7−9] However, since DHAP-dependent enzymes produce a 1,3,4-trihydroxy-2-butanone skeleton that is already highly functionalized and requires the expensive DHAP precursor, there has been emphasis in recent years on the development and discovery of other aldolases that have alternative substrate specificities. One such group is pyruvate aldolases that catalyze the aldol addition of cheaply available precursors, pyruvate and aldehyde, to produce a 4-hydroxy-2-oxoacid skeleton (Scheme 1) that can be further modified chemically or enzymatically to produce amino- or hydroxyl-substituted derivatives as chiral synthons for organic reactions.[10,11]

BphI is a metal-dependent, class II pyruvate aldolase found in the polychorinated biphenyl (PCB) degradation pathway of *Burkholderia xenovorans* that catalyzes the retro-aldol cleavage of 4-hydroxy-2-oxoacids to pyruvate and an aldehyde.[12,13] The enzyme can also be utilized in the reverse to catalyze the reverse aldol addition reaction. BphI exhibits strict stereochemical control as it can only utilize or produce the 4(*S*) enantiomer of 4-hydroxy-2-oxopentanoate.[14,15] The catalytic and kinetic mechanisms of the enzyme have recently been elucidated. In the aldol addition direction, proton abstraction from the C3 methyl generates a pyruvate enolate that is in turn stabilized by Arg-16 and the divalent metal cofactor.[15] The enolate attacks an aldehyde with *si*-facial selectivity, leading to carbon−carbon bond formation. His-20 serves as the acid catalyst that protonates the aldehydic oxygen during this attack to form 4-hydroxy-2-oxoacid. The enzyme has been demonstrated to exhibit broad substrate specificity for linear aldehyde moieties up to six carbons in length. Three amino acid residues, Leu-87, Leu-89, and Tyr-290, located in the active site have been implicated in substrate recognition.[15] Leu-87 interacts with the C4-methyl of the substrate as substitution to a shorter alanine side chain led to a >30-fold increase in K_m for acetaldehyde in the aldol addition reaction. Leu-89 governs chain length specificity of the aldehyde moiety of the substrate, and when substituted with an alanine, the enzyme can effectively utilize aldehydes up to five carbons in length with catalytic efficiency equal to that of the native acetaldehyde.[15] Tyr-290 functions to

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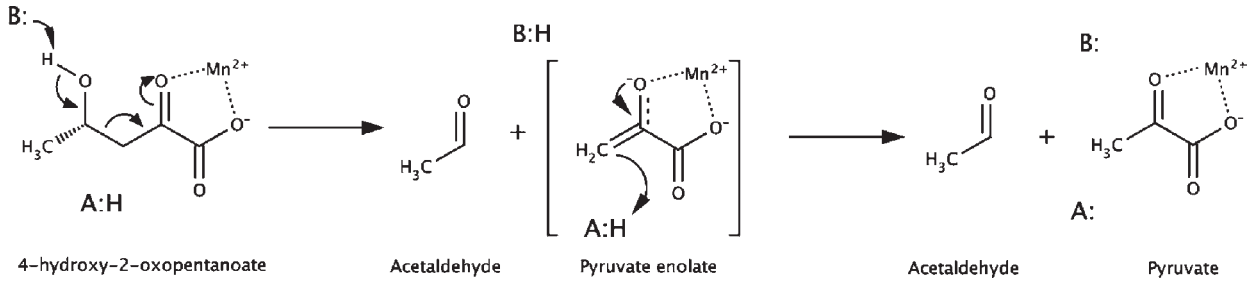
Scheme 1. General Mechanism of Pyruvate Class II Aldolases[a]



[a] The pyruvyl moiety of the substrate coordinates the divalent metal ion via its C1-carboxylate and C2-keto oxygens. A catalytic base, denoted as B, abstracts a proton from the C4-OH group of the substrate. This leads to C−C bond cleavage between C3 and C4, producing an aldehyde and a pyruvate enolate anion. A catalytic acid, denoted as AH, donates a proton to the pyruvate enolate to produce pyruvate.

position a water molecule that is proposed, on the basis of kinetic and solvent isotope experiments, to abstract a proton from pyruvate to form the pyruvate enolate intermediate.[15] Y290F and Y290S variants resulted in a 10-fold decrease in K_m and k_{cat} for the retro-aldol cleavage of 4-hydroxy-2-oxopentanoate. Surprisingly, the variants also lost stereochemical control and were able to catalyze aldol cleavage reactions of both 4(*S*) and 4(*R*) enantiomers of the 4-hydroxy-2-oxopentanoate with similar catalytic efficiencies.[15] This is consistent with the proposal that the *p*-hydroxyl of Tyr-290 in the wild-type enzyme also sterically restricts the binding of the 4(*R*) enantiomer and its removal in the variants enables the enzyme to utilize this enantiomer. While many class I and class II pyruvate aldolases exist, the class I 2-keto-3-deoxy-6-phosphogalactonate (KDPGal) aldolase is the only known member that appears to be stereospecific for the *R* enantiomer of its natural substrate. KDPGal aldolase can catalyze aldol addition using a broad range of electrophilic substrates, provided they incorporate polar functionality at C2, C3, or C4.[16] However, the enzyme is not efficient with longer aldehydes as electrophiles, and therefore, it is desirable to engineer other pyruvate aldolases, such as BphI, to create *R*-specific precursors of some compounds, such as 4-hydroxy-2-oxooctanoate for the antifungal syringomycin.[17,18]

Although the structure of BphI is not available, the structure of its orthologue, DmpG (56% sequence similarity to BphI), from the phenol degradation pathway of *Pseudomonas putida* CF600, has been solved by X-ray crystallography (PDB 1NVM).[19] This structure only contains bound oxalate, a pyruvate enolate analogue, but not the full substrate with the aldehyde moiety. Here we explore the use of molecular modeling and prior knowledge from the biochemical analysis of BphI[15] to rationally design the class II aldolase BphI to exclusively utilize or produce the opposing 4(*R*) enantiomer. Our results demonstrate that rational design to change the stereospecificity of an enzyme is possible without the availability of detailed experimentally determined enzyme−substrate structural information.

EXPERIMENTAL PROCEDURES

Chemicals. Sodium pyruvate, all aldehydes, L-lactate dehydrogenase (LDH; rabbit muscle), alcohol dehydrogenase (*Saccharomyces cerevisiae*), and Chelex 100 were from Sigma-Aldrich (Oakville, ON, Canada). Restriction enzymes, T4 DNA ligase, and *Pfu* polymerase were from Fermentas (Mississauga, ON, Canada) or New England Biolabs (Pickering, ON, Canada). Ni−nitrilotriacetic acid (NTA) Superflow resin was obtained from Qiagen (Mississauga, ON). Recombinant HpaI was purified according to a previously reported method.[20] All other chemicals were analytical grade and were obtained from Sigma-Aldrich and Fisher Scientific (Nepean, ON, Canada). 4(*S*)- and 4(*R*)-hydroxy-2-oxopentanoate and racemic 4-hydroxy-2-oxoacids with aldehyde substituents up to five carbons in length were synthesized and purified as previously described.[14]

DNA Manipulation. DNA was purified, digested, and ligated using standard protocols.[21] *bphI* was previously cloned into plasmid pBTL4-T7.[12] Mutations were incorporated according to a modified Quikchange (Stratagene) method that uses nonoverlapping primers.[22] The sequences of the sense oligonucleotides used to construct the L87N and L87W variants were GTCAGTGCCAATCTCTTGCCCGGCATCGGCACC and GTCAGTGCCTGGCTCTTGCCCGGCATCGGCACC, respectively, with altered codons underlined. Site-directed mutagenesis to replace Tyr-290 was carried out according to the megaprimer method using previously generated primers.[15,23] PCR reactions contained 10 ng of plasmid DNA, 0.5 unit of *Pfu* polymerase, a 20 nM concentration of each dNTP, 1× *Pfu* buffer, 1 mM $MgSO_4$, and a 2 μM concentration of each primer in a total volume of 50 μL. The following amplification profile was followed: 95 °C for 1 min, followed by 15 cycles of 95 °C for 1 min, 45 °C for 1 min, and 72 °C for 6 min with a final extension of 72 °C for 10 min. Genes from positive clones were sequenced at the Guelph Molecular Supercenter, University of Guelph.

Expression and Purification of BphI. Variants of BphI were expressed in recombinant *Escherichia coli* BL21 (λDE3) cells and were expressed and purified as previously described.[12] The concentration of purified protein was determined by the Bradford assay using bovine serum albumin as the standard.[24]

Enzyme Assays. Assays were performed in both the cleavage (retro-aldol) direction and the aldol addition reaction. All assays were performed at 25 °C, in a total volume of 1 mL of 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, pH 8.0, containing 1 mM $MnCl_2$. The retro-aldol and oxaloacetate decarboxylase assays were performed by coupling pyruvate formation with NADH oxidation using lactate dehydrogenase as previously described.[12] One unit of enzyme represents the amount of protein that produces 1 μmol of pyruvate from the substrate in 1 min.

Aldol addition assays contained varying concentrations of pyruvate or aldehydes and were initiated by the addition of 100 μg of enzyme. Following incubation for 60 min at 25 °C, the reaction was quenched with 200 μL of concentrated HCl and incubated overnight at 25 °C to lactonize the product. A 500 μL aliquot from each reaction was subjected to HPLC using an ÄKTA Explorer 100 apparatus (Amersham Pharmacia Biotech, Baie d'Urfé, QC, Canada) equipped with an Aminex fast acid analysis ion exchange column (100 mm × 7.8 mm) with a mobile phase of 0.1% formic acid at a flow rate of 0.6 mL/min. Products were detected at a wavelength of 215 nm and quantified using a standard curve of pure compounds of known concentrations. Data were fitted by nonlinear

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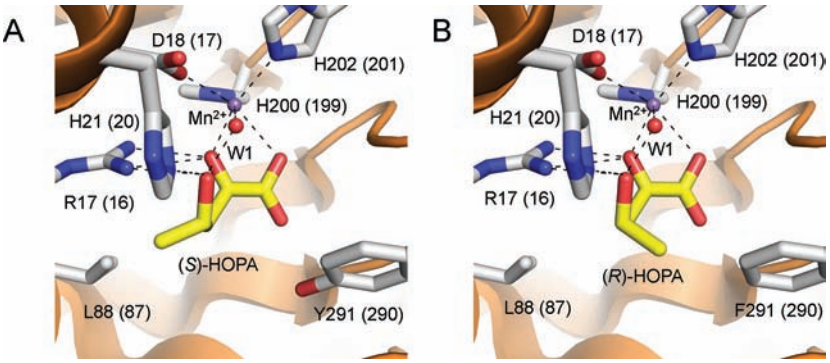

Figure 1. Active site of DmpG with modeled substrates. (A) The 4-methyl substituent of 4(*S*)-HOPA is directed toward Leu-87 at the active site of the wild-type enzyme. (B) 4(*R*)-HOPA can be accommodated in the active site if Tyr-290 is replaced with phenylalanine. Numbers in parentheses correspond to the residue numbers in BphI. Images were generated using PyMOL.[43]

Table 1. Steady-State Kinetic Parameters of BphI and Its Variants for the Aldol Cleavage of the Stereoisomers of 4-Hydroxy-2-oxopentanoate[a]

	4(*S*)-HOPA			4(*R*)-HOPA		
enzyme	$K_{m,app}$ (μM)	k_{cat} (10^{-2} s^{-1})	$k_{cat}/K_{m,app}$ (10^2 $M^{-1}\cdot s^{-1}$)	$K_{m,app}$ (μM)	k_{cat} (10^{-2} s^{-1})	$k_{cat}/K_{m,app}$ (10^2 $M^{-1}\cdot s^{-1}$)
WT	89 ± 8	400 ± 0.7	447 ± 40	NA	NA	NA
Y290F[b]	13 ± 1	13.2 ± 0.2	126 ± 9	13 ± 1	13.1 ± 0.3	104 ± 6
L87N;Y290F	NA	NA[c]	NA	757 ± 89	9.8 ± 0.5	1.3 ± 0.06
L87W;Y290F	NA	NA[c]	NA	435 ± 42	4.3 ± 0.2	1.0 ± 0.1

[a] Aldol cleavage activity was completed with 4(*S*)- and 4(*R*)-HOPA. Assays were performed at 25 °C and contained 0.4 mM NADH, 1 mM $MnCl_2$, and 19.2 units of LDH in 100 mM HEPES (pH 8.0) in a total volume of 1 mL. NA denotes no detectable activity. [b] Data from ref 15. [c] Activity is <0.0001 s^{-1}.

regression to the Michaelis−Menten equation using the program Leonora.[25] In situations where the enzyme was unable to be saturated because of high K_m values, the apparent second-order rate constants at low substrate concentrations were estimated from the linear gradient of velocity versus substrate concentration graphs.[26,27]

Determination of Stereospecificity. The stereoselectivity of the wild type and variants were assessed through the degradation of racemic mixtures of 4-hydroxy-2-oxoacids with aldehyde substituents up to five carbons in length. Assays contained 100 μM racemic 4-hydroxy-2-oxoacid, 10 μg for the wild-type BphI or 500 μg for the BphI variants, 0.4 mM NADH, 1 mM $MnCl_2$, and 19.2 units of LDH in 100 mM HEPES buffer, pH 8.0. Reactions were measured continuously at 340 nm until completion (≤120 min). Both the wild type and variants were also used to synthesize the 4-hydroxy-2-oxoacids in assays containing 2 M aldehyde, 100 mM pyruvate, and 4 mg of the wild-type BphI or variants in 100 mM HEPES, pH 8.0. After 3 h, reactions were quenched by the addition of Chelex to remove the Mn^{2+} cofactor and subsequently filtered through a YM10 filter (Millipore) to remove the enzyme. Samples were lyophilized and resuspended in 4 mL of water. The pH was adjusted to 2.0 using HCl, allowing 4-hydroxy-2-oxoacids to lactonize overnight. Samples were loaded on an Aminex HPX-87H column (300 × 7.8 mm) with a mobile phase of 0.1% formic acid at a flow rate of 0.6 mL/min. Products were detected at 215 mm, and retention times were consistent with products previously confirmed by LC−MS,[14] collected, and lypholized. Synthesized substrates were resuspended in 100 mM HEPES, pH 8.0, and the optical rotation of purified substrates was determined using a Rudolph Autopol IV automatic polarimeter at 25 °C using a wavelength of 589 nm.

RESULTS

Molecular Modeling and Creation of Enzyme Variants. 4-Hydroxy-2-oxopentanoate (HOPA) was previously modeled into the crystal structure of DmpG, an orthologue of BphI, by superimposing the pyruvyl moiety to the position of the experimentally determined oxalate within the active site of the enzyme.[15] In this position the C1-carboxyl and C2-carbonyl oxygens of HOPA coordinate the divalent metal cofactor.[15] The van der Waals radius of the hydroxyl oxygen of the Tyr-291 residue (corresponds to Tyr-290 in BphI) overlaps with the C4 carbon of HOPA, necessitating a clockwise rotation of the phenyl ring of Tyr-291 by 14.5° to relieve this steric clash. Even in the alternative confirmation of Tyr-291, there is insufficient space proximal to this residue to accommodate the 4-methyl substituent of the *R* enantiomer of HOPA. The C4-methyl of 4(*S*)-HOPA, on the other hand, orients toward Leu-88 (Leu-87 in BphI) in a space opposite Tyr-291 (Figure 1A).

To engineer the enzyme to utilize the 4(*R*) enantiomer, the phenolic oxygen was removed in BphI by substitution of Tyr-290 with phenylalanine, thus creating more space for the 4(*R*)-methyl substituent of the substrate (Figure 1B). Replacements of Leu-87 with the polar asparagine (L87N) and the bulky tryptophan (L87W) residue were then attempted to create unfavorable polar−hydrophobic interactions or to introduce steric constraints to prevent binding of the 4(*S*) enantiomer.

Single variants (L87N, L87W) and double variants (L87N; Y290F and L87W;Y290F) were successfully created by site-specific mutagenesis, and they could be purified to homogeneity with yields similar to those of the wild-type enzyme.

Stereospecificity for HOPA in the Retro-Aldol Reaction. Variants were initially tested for their ability to catalyze the retro-aldol cleavage of 4(*S*)- or 4(*R*)-HOPA. The double variants L87N;Y290F and L87W;Y290F were inactive toward 4(*S*)-HOPA but were able to catalyze the aldol cleavage of the opposing

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Table 2. Steady-State Kinetic Parameters of BphI and Its Variants for Oxaloacetate Decarboxylation[a]

enzyme	$K_{m,app}$ (mM)	k_{cat} (10^{-1} s^{-1})	$k_{cat}/K_{m,app}$ ($M^{-1}\cdot s^{-1}$)
WT	7.4 ± 0.4	163 ± 4	2200 ± 100
L87N	0.21 ± 0.02	0.5 ± 0.01	254 ± 30
L87W			5.8 ± 0.3[b]
Y290F	0.92 ± 0.07	2.7 ± 0.1	530 ± 40
L87N;Y290F	0.84 ± 0.06	0.8 ± 0.02	98 ± 7
L87W;Y290F	6.2 ± 0.5	4 ± 0.1	64 ± 5

[a] Assays were performed with 50 μg of enzyme, 0.4 mM NADH, 19.2 units of LDH, and oxaloacetate varying between at least $0.1K_m$ and $10K_m$ in 100 mM HEPES buffer, pH 8.0, in a total volume of 1 mL. [b] Due to the high apparent K_m for the substrate, the specificity constant can be estimated from the linear gradient of specific activity vs substrate concentration graph.

4(*R*) enantiomer (Table 1). The catalytic efficiencies of the retro-aldol activities for these variants were however compromised due to a ~40-fold decrease in k_{cat} and 30−60-fold higher K_m values compared to those of the wild-type enzyme. The decrease in k_{cat} is possibly associated with the loss of the phenolic oxygen of Tyr-290 that hydrogen bonds with a catalytic water molecule.[15] No detectable activity (activity of <0.0001 s^{-1}) was observed in single variants L87N and L87W for either enantiomer of HOPA.

Oxaloacetate (OAA) Decarboxylase Activity. To determine if the inactivity of L87N and L87W single variants in the retro-aldol reaction above is due to catalytic inactivation of the variant enzymes or the inability of the enzymes to bind to HOPA, we tested the enzymes for OAA decarboxylase activity. Oxaloacetate decarboxylation is a secondary reaction of pyruvate aldolases[12,20,28] that proceeds through C−C bond cleavage between C3 and C4, followed by proton donation to a pyruvate enolate intermediate, analogous to the retro-aldol reaction. Oxaloacetate is however one carbon shorter than HOPA and more highly oxidized so that C−C bond cleavage leads to production of carbon dioxide rather than an aldehyde. Both L87N and L87W were found to possess OAA decarboxylase activity, confirming that they are catalytically active. The catalytic efficiencies of OAA decarboxylase in the L87N and L87W variants were however lower than those of the wild-type enzyme by 10- and 380-fold, respectively (Table 2). The L87N variant exhibited a 35-fold decrease in K_m relative to the wild type, while the K_m for the L87W variant was much higher than that of the wild type as the enzyme could not be saturated with the substrate under the assay conditions. The k_{cat} values for OAA decarboxylase activity were also lower than those of the wild type, possibly due to the absence of the phenolic oxygen of Tyr-290 that is important in positioning the catalytic water that protonates the pyruvate enolate. Both enantiomers of HOPA at a concentration of 10 mM did not inhibit the OAA decarboxylase activities of the two single leucine variants, confirming that the lack of aldolase activity is due to the inability of the enzymes to bind HOPA.

OAA decarboxylase activity was not dramatically affected in the L87N;Y290F variant relative to the single L87N variant. In comparison, the K_m for the L87W;Y290F variant is lower than that of the L87W variant, providing evidence that Trp-87 also sterically impedes the proper binding of the carboxylate of oxaloacetate in the single L87W variant, and this is alleviated upon the removal of the phenolic oxygen of Tyr-290 in the double variant.

Table 3. Degradation of Racemic Mixtures of 4-Hydroxy-2-oxoacids[a]

	degradation of racemic mixtures of 4-hydroxy-2-oxoacids (%)			
enzyme	4-hydroxy-2-oxopentanoate	4-hydroxy-2-oxohexanoate	4-hydroxy-2-oxoheptanoate	4-hydroxy-2-oxooctanoate
WT	51.6 ± 1.0	50.6 ± 0.8	51.2 ± 0.7	48.6 ± 0.8
Y290F	99.0 ± 3.3	98.6 ± 3.2	97.9 ± 2.2	98.2 ± 2.7
L87N;Y290F	48.5 ± 1.3	49.6 ± 1.2	50.6 ± 1.9	49.3 ± 1.2
L87W;Y290F	46.2 ± 1.6	48.8 ± 1.8	51.2 ± 2.1	50.6 ± 1.9

[a] Assays contained 20 μM 4-hydroxy-2-oxoacid, 0.4 mM NADH, 1 mM $MnCl_2$, and 19.2 units of LDH in 100 mM HEPES buffer, pH 8.0. The amount of enzyme added was 10 μg for the wild type and 500 μg for the variants.

Table 4. Specific Rotation of 4-Hydroxy-2-oxoacids Synthesized by BphI and Its Variants[a]

	specific rotation of synthesized 4-hydroxy-2-oxoacids $[\alpha]_D^{25}$			
enzyme	4-hydroxy-2-oxopentanoate	4-hydroxy-2-oxohexanoate	4-hydroxy-2-oxoheptanoate	4-hydroxy-2-oxooctanoate
WT	+15.4	+6.7	+14.0	+33.3
Y290F	0	−0.8	−0.2	0
L87N;Y290F	+13.6	−7.9	−14.7	−33.1
L87W;Y290F	+14.0	−7.9	−13.7	−34.0

[a] Samples were synthesized by each respective enzyme, lactonized and purified with an Aminex HPX-87H column with a mobile phase of 0.1% formic acid, and lyophilized. The optical rotation of purified substrates was determined using a Rudolph Autopol IV automatic polarimeter at 25 °C at a wavelength of 589 nm.

Stereochemical Control for Longer Aldehyde Substrates. To determine if the stereospecificity for the opposing enantiomer to the wild-type enzyme is preserved in the double variants for aldehydes longer than acetaldehyde, variants were tested for activity toward racemic mixtures of 4-hydroxy-2-oxoacids containing alkyl chains eight or more carbons in length (pyruvate addition products with propionaldehyde, butyraldehyde, and pentaldehyde). These racemic compounds were synthesized using HpaI, an enzyme that does not exhibit stereochemical control.[14] The wild type and L87N;Y290F and L87W;Y290F variants were only able to degrade ~50% of racemic 4-hydroxy-2-oxoacids (Table 3). In addition, the ~50% 4-hydroxy-2-oxoacids remaining after treatment of the wild-type enzyme could be further degraded to completion by the L87N;Y290F or L87W;Y290F variants, showing that the variants utilize the opposite enantiomer that the wild-type enzyme cannot utilize. Similarly, when racemic 4-hydroxy-2-oxoacids were treated with Y290F;L87N or Y290F;L87W variants first, the wild-type enzyme was able to degrade the remaining substrate. In contrast, the Y290F variant was able to cleave ~98% of these racemic mixtures, confirming that this single variant is not stereospecific.

The aldol addition products of pyruvate and various aldehydes synthesized by the wild-type enzyme and each variant enzyme were also subjected to polarimetric analysis. The specific rotations of 4-hydroxy-2-oxoacids produced by L87N;Y290F and L87W;Y290F variants were opposite those of 4-hydroxy-2-oxoacids synthesized by the wild-type enzyme (Table 4). The

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Y290F single variant produced substrates with an optical rotation of approximately 0, further confirming that the enzyme exhibits poor facial selectivity. Together, these results demonstrate that constructed variants exhibit the stereoselectivity opposite that of the wild type and that the alkyl chain length of the aldehyde does not affect stereochemical control.

Kinetic Analysis of the Aldol Addition Reaction. Steady-state kinetic parameters were determined in the aldol addition reaction using pyruvate and various aldehydes. The $K_{m,app}$ values for pyruvate were approximately 2−4-fold higher in the variants than in the wild-type enzyme (Table 5), suggesting that pyruvate binding is largely unaffected. All variants exhibited similar k_{cat} values that were 3−10-fold lower than that of the wild type. In addition, the variants exhibited specificity constants ($k_{cat}/K_{m,app}$) toward acetaldehyde and propionaldehyde similar to those of the wild-type enzyme, suggesting that the C2 of the aldehyde forms favorable hydrophobic interactions with the phenyl ring of Phe-290 (Table 6). The Y290F single variant exhibited similar specificities for all aldehydes and had notably higher specificity for pentaldehyde compared to the wild type and double variants. Similar to the wild-type enzyme, the double variants exhibited a reduction in catalytic efficiencies as the aldehyde chain length increased to more than three carbons. This is primarily due to an increase in $K_{m,app}$ for longer aldehydes. Overall the k_{cat}/K_m for the double variants in the aldol addition reaction was affected ≤10-fold relative to that of the wild-type enzyme.

Table 5. Steady-State Kinetic Parameters for the Utilization of Pyruvate in the BphI Wild Type (WT) and L87N;Y290F and L87W;Y290F Variants in the Aldol Addition Reaction[a]

enzyme	$K_{m,app}$ (mM)	$k_{cat,app}$ (s^{-1})	$(k_{cat}/K_m)_{app}$ ($M^{-1}\cdot s^{-1}$)
WT	13.0 ± 0.6	1.20 ± 0.02	91.5 ± 4.7
Y290F	20.0 ± 1.9	0.19 ± 0.01	8.4 ± 0.8
L87N;Y290F	30.2 ± 1.6	0.11 ± 0.01	3.6 ± 0.2
L87W;Y290F	38.2 ± 3.1	0.16 ± 0.01	4.3 ± 0.4

[a] Assays were performed at 25 °C and contained 120 μg of BphI, 1 mM $MnCl_2$, and 240 mM propionaldehyde with varying concentrations of pyruvate in 100 mM HEPES buffer, pH 8.0. The 4-hydroxy-2-oxohexanoate product formed was lactonized and detected at 215 nm.

DISCUSSION

Pyruvate aldolases are a diverse group of enzymes that differ in their substrate specificities, stereospecificities, and catalytic mechanisms. For example, some pyruvate aldolases that utilize catalytic lysine residues in their reaction mechanisms (class I aldolases), such as *N*-acetylneuraminic acid (NAL) aldolase[29] and 2-keto-3-deoxygluconate (KDG) aldolase, lack stereochemical control.[30,31] Other class I aldolases such as 2-keto-3-deoxy-6-phosphogluconate-6-phosphate (KDPG) aldolase and 2-keto-3-deoxy-6-phosphogalactonate (KDPGal) aldolase catalyze the identical reaction, but with *si*- and *re*-facial selectivity at C4, respectively, providing access to both stereochemical sequences.[10] However, they only utilize aldehydes with polar groups at C2, C3, or C4 as substrates.[16] Pyruvate aldolases that are divalent metal dependent (class II aldolases) include HpaI and 4-hydroxy-4-methyl-2-oxoglutarate/4-carboxy-4-hydroxy-2-oxoadipate (HMG/CHA) aldolases that, unlike BphI, lack stereospecificity.[14,28,32] HpaI and BphI exhibit broad specificity for the aldol reaction of pyruvate with nonphosphorylated nonsubsituted aldehydes and are therefore useful for synthesis of 4-hydroxy-2-ketoacid precursors for organic synthesis.

During the aldol addition catalyzed by BphI, the *S*-configured stereogenic center at C4 is created via attack of a pyruvate enolate intermediate onto the *si* face of the aldehyde carbonyl of acetaldehyde. The availability of an X-ray crystal structure of the orthologous DmpG (56% amino acid identity),[19] containing the pyruvate enolate analogue oxalate, previously enabled us to propose a model for the binding of 4(*S*)-hydroxy-2-oxopentanoate in the active site.[15] A critical residue is Tyr-290, which prevents the binding of the 4(*R*) isomer due to steric clash between the C4-methyl substituent of the substrate and the hydroxyl oxygen of the tyrosyl residue. Hence, in the Y290F variant, steric constraint at the stereogenic carbon is alleviated, resulting in a loss of stereochemical control. Since Tyr-290 functions to position a catalytic water in the reaction mechanism, the activity of the Y290F variant is, however, also slightly compromised. Interestingly, a T161V substitution in KDPG aldolase also eliminated the stereochemical control in the enzyme, allowing the enzyme to accept either KDPG or KDPGal as the substrate with roughly equal efficiencies.[11] However, unlike

Table 6. Steady-State Kinetic Parameters for the Utilization of Various Aldehydes of the BphI Wild Type and Variants in the Aldol Addition Reaction[a]

enzyme	kinetic parameter	acetaldehyde, two carbons	propionaldehyde, three carbons	butyraldehyde, four carbons	pentaldehyde, five carbons
WT	$K_{m,app}$ (mM)	64.2 ± 3.7	136 ± 9.6		
	$k_{cat,app}$ (s^{-1})	0.860 ± 0.020	1.79 ± 0.07		
	$k_{cat}/K_{m,app}$ ($M^{-1}\cdot s^{-1}$)	13.4 ± 0.80	13.2 ± 1.1	2.19 ± 0.12[b]	0.470 ± 0.01[b]
Y290F	$K_{m,app}$ (mM)	81.0 ± 6.8	81.2 ± 7.0	104 ± 8.3	80.5 ± 7.6
	$k_{cat,app}$ (s^{-1})	0.20 ± 0.005	0.16 ± 0.006	0.16 ± 0.005	0.14 ± 0.003
	$k_{cat}/K_{m,app}$ ($M^{-1}\cdot s^{-1}$)	2.5 ± 0.2	2.0 ± 0.2	1.5 ± 0.1	1.7 ± 0.1
L87N;Y290F	$K_{m,app}$ (mM)	70.7 ± 3.7	54.9 ± 3.8		
	$k_{cat,app}$ (s^{-1})	0.30 ± 0.003	0.10 ± 0.003		
	$k_{cat}/K_{m,app}$ ($M^{-1}\cdot s^{-1}$)	4.3 ± 0.2	1.8 ± 0.1	0.16 ± 0.008[b]	0.040 ± 0.002 [b]
L87W;Y290F	$K_{m,app}$ (mM)	86.3 ± 8.5	24.4 ± 1.9		
	$k_{cat,app}$ (s^{-1})	0.59 ± 0.003	0.14 ± 0.003		
	$k_{cat}/K_{m,app}$ ($M^{-1}\cdot s^{-1}$)	6.9 ± 0.7	5.7 ± 0.5	0.31 ± 0.004[b]	0.051 ± 0.003 [b]

[a] Assays were performed at 25 °C and contained 120 μg of BphI, 1 mM $MnCl_2$, and 100 mM pyruvate with varying concentrations of aldehyde in 100 mM HEPES buffer, pH 8.0. The 4-hydroxy-2-oxoacid products formed were lactonized and detected at 215 nm via HPLC. [b] Due to the high apparent K_m for the substrate, the specificity constant can be estimated from the linear gradient of specific activity vs substrate concentration graph.

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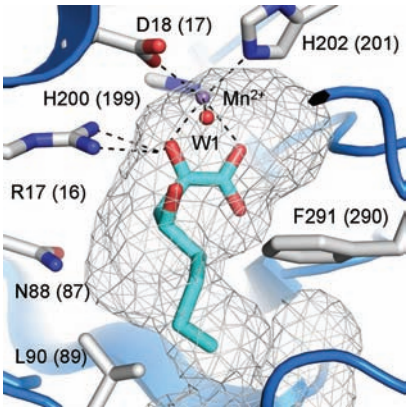

Figure 2. Model of 4(*R*)-hydroxy-2-oxooctanoate in the substrate binding site of DmpG. Tyr-291 and Leu-88 of the active site are replaced with phenylalanine and asparagine, respectively, in the model. The distal end of the substrate can adopt different conformations to fit within the solvent-accessible areas of the active site (mesh). Carbon atoms of the substrates are colored cyan, and the residues in the substrate binding sites are colored gray. Corresponding residue numbers in BphI are given in parentheses.

the BphI Y290F variant, this substitution was accompanied by a significant loss of k_{cat} (10^4) against both the natural and epimeric substrates. The reason why the T161V variant loses stereospecificity is not clear, but it suggests catalytic involvement in stereochemical control.

Aromatic residues within the active sites of other enzymes have been implicated in stereoselectivity. Steric occlusion by Phe-297 within the active site of acetylcholinesterase is the primary determinant of both enantiomeric preference and substrate selectivity.[33] Similarly, Phe-77 and Phe-138 in aryl sulfotransferase IV, which catalyzes the sulfation of many drugs, carcinogens, and xenobiotics, was shown by alanine substitutions to provide significant steric interactions that both impart stereospecificity and enhance catalytic efficiency.[34] Among aldolases, fructose-1,6-bisphosphate aldolase was engineered to catalyze the formation of the opposite enanatiomer, tagatose, by replacing a histidine residue (His-26) with an aromatic tyrosine (H26Y). The subtle change in conformation of the active site in this variant affects the position of the bound glyceraldehyde 3-phosphate (G3P) plane relative to the dihydroxyacetonephosphate (DHAP) enediolate, permitting the attack of DHAP on the opposite face of the incoming G3P molecule.[35]

While Tyr-290 in BphI prevents binding of the 4(*R*) isomer of the substrate, Leu-87, positioned opposite Tyr-290 in the active site of BphI, appeared to be important for interaction with the 4-methyl of the *S* enantiomer of the substrate. This is supported by previous studies where replacement of Leu-87 with a shorter alanine reduced the specificity constants of the enzyme toward acetaldehyde 32-fold while maintaining a similar specificity constant for pyruvate in the aldol addition reaction.[15] As demonstrated through inhibition studies, Leu-87 variants lost the ability to bind and catalyze the cleavage of HOPA, although they were still catalytically active as they retained OAA decarboxylase activities. OAA differs from HOPA in that it lacks the C5-alkyl group. Therefore, in the L87N variant, the incompatibility between the hydrophobic C5-alkyl of HOPA and the polar side chain of Asn-87 is a possible factor that has a negative effect on HOPA binding and catalysis. In the L87W variant, an unfavorable steric clash is thought to occur between the C5-alkyl and the bulky side chain of Trp-87. At the same time, these Leu-87 single variants are unable to accommodate the 4(*R*) enantiomer due to the steric blockage by Tyr-290. Thus, combining mutations at two positions in the double variants (L87N;Y290F and L87W;Y290F) led to enzymes with stereoselectivity for the 4(*R*) enantiomer. $K_{m,app}$ values for pyruvate binding were unaffected in these variants, consistent with our model indicating that these amino acid substitutions are sufficiently removed from the carbonyl donor. The L87W;Y290F variant had a marginally higher k_{cat}/K_m in both the retro-aldol and aldol addition reactions relative to the L87N;Y290F variant possibly due to the more favorable accommodation of the hydrophobic tail of 4-hydroxy-2-oxoacids if the active site is also hydrophobic. The stereoselectivity is retained for longer aldehyde substrates, suggesting that Phe-290 does not sterically hinder their binding. Inspection of the crystal structure of DmpG suggests that there is sufficient space at the distal end of the active site pocket to accommodate these aldehydes (Figure 2).[13]

There are few examples of successful rational design of stereoselectivity among aldolases. In fact, directed evolution based on random mutagenesis has been the preferred method to alter the stereoselective profiles of aldolases and other biocatalysts.[36−40] Although no prior structural information on the enzyme is necessary for directed evolution, its main limitation is the requirement for robust screening methods to select for desired variants from a pool of thousands of mutants.[41] An alternative method to modify the stereoselectivity of aldolases is by rational design, an approach that has been attempted with 2-keto-3-deoxygluconate aldolase.[42] The natural enzyme produces the diasteroisomers D-2-keto-3-deoxygluconate (D-KDGlu) and D-2-keto-3-deoxygalactonate (D-KDGal) in almost equal mixtures using pyruvate and D-glyceraldehyde as substrates. Detailed structures of the enzyme containing bound D-KDGlu or D-KDGal were required to modify the enzyme by site-specific mutagenesis to preferentially produce one or the other enantiomer, exploiting the subtle differences in hydrogen-bonding interactions between the enzyme and the C4 (the stereogenic center), C5, and C6 oxygens/hydroxyls of the D-glyceraldehyde moiety of the respective enantiomer.

CONCLUSION

For many aldolases, the mechanism for stereochemical control is not fully understood. We have shown how structure modeling can be used to rationally design a stereocomplementary variant of BphI. The results presented herein showed that both Leu-87 and Tyr-290 govern stereochemical control and that relatively few amino acid substitutions within the active site can result in large changes in the stereoselectivity of an enzyme. This approach compares favorably and can complement directed evolution approaches to create biocatalysts of desired stereospecificity.

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