The Structural Basis for Substrate Promiscuity in 2-Keto-3-deoxygluconate Aldolase from the Entner-Doudoroff Pathway in Sulfolobus solfataricus*

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The hyperthermophilic Archaea Sulfolobus solfataricus grows optimally above 80 °C and metabolizes glucose by a non-phosphorylative variant of the Entner-Doudoroff pathway. In this pathway glucose dehydrogenase and gluconate dehydrase catalyze the oxidation of glucose to gluconate and the subsequent dehydration of gluconate to 2-keto-3-deoxygluconate (KDG). KDG aldolase (KDGA) then catalyzes the cleavage of KDG to D-glyceraldehyde and pyruvate. It has recently been shown that all of the enzymes of this pathway exhibit a catalytic promiscuity that also enables them to be used for the metabolism of galactose. This phenomenon, known as metabolic pathway promiscuity, depends crucially on the ability of KDGA to cleave KDG and 2-keto-3-deoxygalactonate (KDGal), in both cases producing pyruvate and D-glyceraldehyde. In turn, the aldolase exhibits a remarkable lack of stereoselectivity in the condensation reaction of pyruvate and D-glyceraldehyde, forming a mixture of KDGA and KDGal. We now report the structure of KDGA, determined by multi-wavelength anomalous diffraction phasing, and confirm that it is a member of the tetrameric N-acetylneuraminate lyase superfamily of Schiff base-forming aldolases. Furthermore, by soaking crystals of the aldolase at more than 80 °C below its temperature activity optimum, we have been able to trap Schiff base complexes of the natural substrates pyruvate, KDGA, and pyruvate plus D-glyceraldehyde, which have allowed rationalization of the structural basis of promiscuous substrate recognition and catalysis. It is proposed that the active site of the enzyme is rigid to keep its thermostability but incorporates extra functionality to be promiscuous.

Sulfolobus solfataricus is a hyperthermophilic Archaea that grows optimally at 80–85 °C and pH 2–4, utilizing a wide range of carbon and energy sources (1). Central metabolism in this organism involves a non-phosphorylative variant of the Entner-Doudoroff pathway (2), and similar metabolic routes have been detected in Sulfolobus acidocaldarius (3), Thermoplasma acidophilum (4), Thermoproteus tenax (5), and Aspergillus fungi (6).

In S. solfataricus, the enzymes of this non-phosphorylative Entner-Doudoroff pathway have been shown to possess a substrate promiscuity that enables them to catalyze the metabolism of both glucose and galactose (7). The first enzyme in the pathway, glucose dehydrogenase, catalyzes the oxidation of glucose to gluconate and galactone. Subsequently, gluconate dehydrase is known to catalyze the dehydration of gluconate to 2-keto-3-deoxygluconate (KDG), and galactone to 2-keto-3-deoxygalactonate (KDGal). The third enzyme, KDGA aldolase (KDGA), catalyzes the aldol cleavage of KDGA and KDGal, with both substrates yielding pyruvate and D-glyceraldehyde. Moreover, in the aldol condensation reaction, KDGA exhibits no facial selectivity, condensing pyruvate with D-glyceraldehyde to produce a mixture of KDGA and KDGal (7). KDGA is thus an extremely unusual aldolase in not exhibiting stereoselectivity with its natural substrates, and the whole metabolic pathway in S. solfataricus contrasts with the situation observed in other microorganisms where separate enzymes exist for the catabolism of the two sugars.

Aldolases are divided into two main groups, Type I and Type II. Type I aldolases proceed through a Schiff base intermediate formed between an active site lysine and the α-keto acid moiety of the substrate, whereas Type II aldolases are non-Schiff base forming and require a metal cofactor. S. solfataricus KDGA is a homotetrameric enzyme and has a monomeric molecular mass of 33 kDa with 293 residues per monomer (8). It is a Type I aldolase and a member of the N-acetylneuraminate lyase (NAL) subfamily (9) for which a number of crystal structures have been determined (10). All of these enzymes are tetramers, with each monomer having a TIM barrel fold decorated with additional helices at the C terminus. These enzymes are thought to utilize a common mechanism, in which a strictly conserved lysine serves to form the Schiff base intermediate; other strictly conserved residues are involved in stabilizing the intermediate enzyme-substrate complex (11). Each reaction is reversible, with pyruvate being the common substrate, and they are each specific for non-phosphorylated substrates.

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1 The abbreviations used are: KDGA, 2-keto-3-deoxygluconate; KDGA, KDG aldolase; KDGal, 2-keto-3-deoxygalactonate; NAL, N-acetylneuraminate lyase; EcNAL, Escherichia coli NAL; HiNAL, Haemophilus influenzae NAL; EcDHDPS, Escherichia coli dihydrodipicolinate synthase.
In the current study we present the native structure of KDGA at 2.0-Å resolution, derived by multil wavelength anomalous diffraction. By virtue of the hyperthermophilic nature of the enzyme, we have been able to trap pyruvate, KDG, or KDGal in crystals of KDGA at low temperatures and so visualize Schiff base formation at resolutions of between 1.7 and 2.1 Å. From these structures we are able to rationalize the substrate and mechanism of promiscuity of this novel aldolase and thereby understand the enzymatic basis of the unique metabolic promiscuity we have discovered in archaeal sugar metabolism.

EXPERIMENTAL PROCEDURES

Crystallization of Recombinant KDGA—Several crystal forms of KDGA were previously obtained, the best diffraction data being to 2.15 Å from an orthorhombic crystal form (a = 135.1 Å, b = 135.9 Å, c = 188.7 Å) grown at pH 4.0 (12). Solvent content estimations indicated the presence of two tetramers in the asymmetric unit related by a pseudo-centering. Numerous attempts at molecular replacement with Escherichia coli NAL (EcNAL), Hemophilus influenzae NAL (HiNAL), or E. coli dihydricarpinolactone synthase (EcDHDDS) failed to provide a solution, and therefore selenomethionine labeling of the protein was carried out.

Preparation of Selenomethionine Derivative—JM109 cells containing the pREC7-KDGA expression vector were prepared as described previously (8). The gene was then inserted into a pET3a expression plasmid, which confers resistance to ampicillin, contains the lacZ promoter, and is compatible with the methionine auxotrophic E. coli strain, B834. Transformed B834(DE3) cells were grown in basic medium containing l-selenomethionine and 20 mg/liter ampicillin at 37 °C. Once the optical density of the cells had reached 0.5 at 600 nm, they were induced with 1 mM isopropyl-β-D-thiogalactopyranoside and grown for a further 24 h at 250 rpm, 37 °C. The purification procedure for the Se-Met derivative was as for the native protein (12) except that all buffers contained 5 mM dithiothreitol. Screening for crystallization conditions had to be repeated as no crystals were obtained under the conditions described for the native protein (8). Reproducible thin triangular crystals were obtained at 20 °C in 0.1 M HEPES, pH 6.0, 8% (v/v) isopropanol, 13% (v/v) polyethylene glycol 4000 (buffer A). The crystals belonged to space group P2_{1}2_{1}2_{1}, with cell dimensions a = 84.1 Å, b = 131.5 Å, c = 132.5 Å. The pH optimum for the enzyme is pH 6.0, and therefore this crystal form is at a more physiologically relevant pH value compared with the acidic conditions under which the original crystals were obtained. Crystals of the native protein were also obtained at these pH 6.0 conditions and used for collection of the native data to 2.0 Å and for substrate soaks.

Data Collection and Reduction—The multil wavelength anomalous diffraction data were collected on a MAR-CCD detector at three wavelengths around the Se-K edge on station ID14-4 at the European Synchrotron Radiation Facility, Grenoble. Cryoprotection of the crystals was achieved through equilibration against mother liquor containing 25% (v/v) 25% (v/v) glycerol, and data were collected at 100 K from a single crystal.

Structure Solution—The selenium substructure was determined using version 1.15 of SOLVE (14). Estimates of the solvent content suggested that there was one tetramer present in the asymmetric unit, and all 16 expected selenium atoms were identified by Patterson analysis, resulting in a figure of merit of 0.27. The selenium sites had peak height of ~15σ. The phase estimates from SOLVE were used with DM (16) to calculate a solvent-flattened electron density map, which had distinct solvent boundaries and evident regions of secondary structure. A mask was generated from the experimental bones of the solvent-flattened map, and full noncrystallographic symmetry averaging was performed in DM. The initial correlation between the monomers was 0.70, and this increased to 0.9 after 50 cycles of averaging, solvent flattening, and full noncrystallographic symmetry averaging in DM. The initial correlation between the monomers was 0.70, and this increased to 0.9 after 50 cycles of averaging, solvent flattening, and full noncrystallographic symmetry averaging in DM. The final correlation was 0.27. The figure of merit in the top resolution shell was 0.79.

Model Building and Refinement—A polyalanine model was constructed manually into the averaged map using the bones as a guide. Sequence assignment started at the clearest part of the model, which was the stretch around the three tyrosines at residues 102, 103, and 105. Manual model building was followed by refinement by simulated annealing, as implemented in crystallography NMR software (17), against the 2.5-Å reflection data with a 10% subset of reflections being omitted from the refinement calculations. 200 cycles of simulated annealing resulted in the R-factor dropping from 48.0 to 34.0%, and the R-free went from 47.6 to 35.9%. Subsequent refinement was through manual adjustment of side chains followed by energy minimization and individual B-factor refinement with no noncrystallographic symmetry restraints. Water molecules were then added. The final model is complete in all monomers, with every residue clearly seen in the density from Pro-2 to Glu-294. The final R-factor was 19.7% and R-free factor 24.1%. This model was then refined against the 2.0-Å data using REFMAC to give statistics shown in Table I.

Schiff Base Formation—Crystals of KDGA were soaked with 50 mM pyruvate in buffer A for 30 min at 20 °C. The KDGA Schiff based complex was formed by soaking crystals with 30 mM KDG in buffer A for 1 min at 4 °C. The KDGA Schiff based complex was formed by soaking crystals with 50 mM KDGA in buffer A for 1 min at 4 °C. In all cases, soaked crystals were then cryoprotected by rapid stepwise equilibration against buffer A with substrate containing 10% (v/v), followed by 20% (v/v) glycerol. Crystals were then flash-frozen in a nitrogen stream at 100 K, and data were collected on beamline ID14-1 at the European Synchrotron Radiation Facility (λ = 0.993 Å). The complexes were refined using REFMAC to give statistics shown in Table I. KDGA and KDGal were produced as described earlier (7), with diastereomeric purity confirmed by high pressure liquid chromatography and 1H NMR.

RESULTS

Overall Structure—Each subunit of KDGA displays the same fold as the other members of the NAL subfamily, with the (α/β)_{8} TIM barrel structure decorated with three additional helices at the C terminus. KDGA has 26, 28, and 27% sequence identity with EcNAL, HiNAL, and EcDHDDS, and their monomeric structures are all very similar, with the β-strands being highly superimposable (Fig. 1a).

The KDGA tetramer is also similar to the other members of the NAL subfamily, with root mean square deviation values ranging from 2.1 to 2.3 Å with any of the other enzymes. The tetramer can be thought of as a dimer of dimers (18) having a more extensive subunit interface between the AD (or BC) dimer with 1640 Å^{2} of surface buried compared to 1100 Å^{2} buried at the AC (or BD) interface (Fig. 1b). In all members of the family the AC (or BD) interface is polar, whereas the AD (or BC) interface is hydrophobic. The AD (or BC) interface in KDGA, however, is larger than in the other enzymes, and this is reflected in the greater number of interactions between the KDGA subunits. KDGA also has more intersubunit ion pairs than the others. In the AC (or BD) interface, Arg-169 of one subunit forms ion pairs with each of Glu-234 and Asp-230 of the other subunit. Similar pairs are seen in the AD (or BC) interface between Lys-110 of one subunit and the Glu-271 or Asp-268 of the other. The localization of ion pairs across the subunit interfaces differs among the other members of the family; EcDHDDS has no intersubunit ion pairs across either of the interfaces; HiNAL and EcNAL both have intersubunit pairs across the AC interface, but only EcNAL has ion pairs across the AD interface.

Enzyme-Substrate Complexes—All complexes were obtained by soaking protein crystals with substrate prior to flash freezing in liquid nitrogen. The crystals belong to space group P2_{1}2_{1}2_{1}, with a KDGA tetramer in the asymmetric unit, and the same structural features were observed in each monomer unless otherwise stated.

Pryuvate Schiff Base Complex—A Schiff base complex with pyruvate was obtained by soaking a crystal in 50 mM pyruvate at pH 6.0 for 30 min at 21 °C, more than 60 °C below the physiological temperature of the enzyme. The difference Fourier maps identified a covalent bond between the Nε of Lys-155 and C-2 of pyruvate, the planar trigonal geometry about the C-2 position being consistent with the Schiff base intermediate form (Fig. 2a). The carboxylate group of the pyruvate Schiff base forms hydrogen bonds with the backbone amides of Thr-43 and Thr-44 and the Oy of Thr-44. Thr-43 is stabilized through hydrogen bonding of its Oy to the hydroxyl of Tyr-103 from

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Table I

| Data collection and refinement statistics | Pyruvate | KDG Schiff Base Complex | KDG | KDGal Schiff Base Complex |
|--------------------------------------|----------|-------------------------|-----|--------------------------|
| Wavelength (Å) | 0.980 | 0.980 | 0.980 | 0.980 |
| Resolution (Å) | 2.5 | 2.0 | 2.0 | 2.0 |
| No. of observations | 269,334 | 268,882 | 273,640 | 660,907 |
| No. of unique reflections | 52,193 | 52,011 | 52,196 | 98,295 |
| Completeness (%) | 98.0 (89.4) | 97.7 (89.1) | 98.7 (96.0) | 99.1 (99.1) |
| Rmerge | 15.3 | 17.6 | 15.1 | 15.0 |
| Refinement | No. of working/test set reflections | 88,470/9,825 | 142,220/15,718 | 74,888/8,347 | 77,251/8,678 |
| No. of protein/water/ligand atoms | 9,570/901/49 | 9,542/878/80 | 9,438/812/62 | 9,389/819/112 |
| B-factor (Å²) | 19.1 | 18.9 | 22.2 | 18.9 |
| r.m.s.d. bond lengths (Å) | 0.023 | 0.018 | 0.025 | 0.025 |
| r.m.s.d. bond angles (°) | 1.73 | 1.59 | 1.87 | 1.59 |

Values in parentheses refer to the highest resolution shell.

The only significant changes in the protein upon pyruvate binding are the positions of Tyr-130 and Phe-39 (Fig. 2b), which is also the case for complexes with KDG and KDGal. In the native structure the hydroxyl of Tyr-130 forms hydrogen bond interactions with the amide group and Oγ of Thr-43, the Nε of Lys-155, and two water molecules, one of which (W1) hydrogen-bonds to the Oγ of Thr-157. In the pyruvate complex one of these water molecules is displaced by the carboxylate group of the pyruvate, and Tyr-130 rotates around χ1 and χ2, leading to a shift of ~1 Å in its hydroxyl position. In its new conformation, this tyrosyl hydroxyl is in close proximity (3.1–3.2 Å) to one of the carboxylate oxygens of the pyruvate, while still hydrogen-bonding to the Oγ of Thr-43 and the Oγ of Thr-157 via W1. This shift in Tyr-130 brings the plane of the tyrosine ring parallel to the plane of the pyruvate, which is held on its opposite face by Pro-7, forcing the methyl group (C-3) of pyruvate into close proximity with W1 (~3.1 Å).

**KDG Schiff Base Complex**—A Schiff base complex with KDG was obtained by soaking a crystal in 30 mM KDG at pH 6.0 for 1 min at 4 °C, more than 80 °C below the physiological temperature of the enzyme. The difference Fourier map reveals clear electron density for the trapped KDG forming a Schiff base with Lys-155 (Fig. 2c). The carboxyl group of KDG lies in the same position as the carboxyl group of pyruvate, making the same hydrogen bond interactions with the enzyme. The hydroxyl (O-4) connected to C-4 of KDG takes the place of water W1 of the pyruvate complex and forms a close interaction with the OH group of Tyr-130, ranging from 2.3 to 2.5 Å. O-4 also makes a hydrogen bond interaction with Oγ of Thr-157. In contrast to these groups, the C-5 hydroxyl (O-5) shows significant deviations in its position across the tetramer. Despite this, in three of the monomers it is seen interacting with a water molecule (W2) that hydrogen-bonds to the main chain carbonyl oxygen of Gly-179 and the amide of Ala-198. O-6 also displays some flexibility and in addition to the hydrogen bond it forms makes a hydrogen bond to the Oγ of Thr-132, directly (2.8–3.3 Å) or via a water molecule, a water-mediated interaction with the Oγ of Thr-44 is observed in two of the monomers.

**KDGal Schiff Base Complex**—To trap KDGal in the enzyme, a crystal was soaked in 25 mM KDGal at pH 6.0 for 1 min at 4 °C. The Fo – Fe difference electron density map reveals clear electron density for Schiff base formation with Lys-155 (Fig. 2d); however, in all four monomers there is both density corresponding to the methyl C-3 of pyruvate and connecting density corresponding to the C-3–C-4 bond of KDGal. During refinement a number of models were tested to account for the electron density: pyruvate and D-glyceraldehyde, KDGal, and a mixture of all three ligands. The most consistent interpretation, measured by lack of unexplained electron density in the final Fo – Fe electron density maps, was a mixture of ligands with occupancies of 80% for pyruvate and D-glyceraldehyde and 20% for KDGal. Comparing the KDGal complex with the KDG complex, the major difference is the conformation of the sugar from C-3 to C-6 and the interactions made by its hydroxyl oxygens. KDGal is the C-4 epimer of KDG, and in the KDGal complex the C-3 and C-4 atoms shift relative to their positions in the KDG complex, thereby still allowing O-4 to hydrogen bond to the OH of Tyr-130 and Oγ of Thr-157 (Fig. 2e). In the KDGal complex, however, the hydrogen bond distance between O-4 and Tyr-130 increases by an average of 0.2 Å, and the interaction with Thr-157 becomes closer (2.5–2.8 Å). As in the case of KDG, the C-5 and C-6 hydroxyls also display flexibility in the KDGal complex. However, O-5 now hydrogen-bonds directly with the hydroxyl of Tyr-132, whereas O-6 is seen inter-
acting differently with the enzyme in the four monomers. In monomers A and B, O-6 hydrogen-bonds to O$_\gamma$ of Thr-44 and makes a water-mediated interaction with O$_\gamma$ of Ser-241 and N$_\delta$2 of Asn-245. In monomers C and D, O-6 is shifted and makes the same interactions as in monomers A and B via a water molecule that occupies its previous position.

The complex with pyruvate and D-glyceraldehyde has the pyruvate in the same position as observed in the pyruvate Schiff base complex discussed above, whereas D-glyceraldehyde takes up a slightly shifted position relative to the C-4 to C-6 moiety of KDGaL. The glyceraldehyde O-3 (equivalent to O-4 in KDGaL) hydrogen-bonds to O$_\gamma$ of Thr-157 and the OH of Tyr-130 but with longer hydrogen bonds (2.7 Å and 3 Å, respectively) than seen in the KDGaL complex (Fig. 2f). Glyceraldehyde O-2 (equivalent to O-5 in KDGaL) is observed either hydrogen bonding to Tyr-132 (monomer A) or making a water-
mediated interaction with the main chain carbonyl oxygen of Gly-179 and the amide of Ala-198 (monomers C and D). Glyceraldehyde O-3 is involved in the same interactions as its O-6 equivalent in KDGal.

**DISCUSSION**

Previous studies on the NAL superfamily have trapped pyruvate Schiff base complexes either by borohydride reduction in the case of EcNAL (9), at high pH in the case of EcDHDPS (19), or by adding pyruvate to the crystallization buffer in the case of *Nicotiana sylvestris* DHDPS (20). Substrate analogues have also been used to understand substrate recognition and the catalytic mechanism (9, 11, 19) as well as acid quenching to trap Schiff base intermediates in Type I aldolases (21). In this study we have been able to trap the natural substrates pyruvate, KDG, and KDGal by soaking crystals of KDGA for a short time at 80 °C below the physiological temperature of the enzyme. In the case of the KDGal complex, we have also observed a complex with the cleavage products pyruvate and D-glyceraldehyde from turnover of the substrate in the crystal.

KDGA was predicted to belong to the NAL superfamily (11), members of which are proposed to have similar active sites but are modulated to accommodate varying substrates. Our structural studies largely confirm this prediction, and a scheme for the catalytic mechanism of KDGA, based on that proposed for NAL (11), is shown in Fig. 3. However, there are differences in the details of how KDGA and KDGal are recognized by KDGA. A primary group of active site residues, conserved across the NAL superfamily, was defined as being associated with the binding of the α-keto acid moiety of the substrate and the enzyme reaction. In KDGA these include: (i) Lys-155, located at the C-terminal end of strand β6, which forms the Schiff base; (ii) Tyr-130 at the C-terminal end of strand β5; and (iii) a GTTG motif, including Thr-43 and Thr-44, in the loop between strand β2 and helix α2, involved in binding the carboxylate group of the substrate. It was previously suggested that the carbonyl of Gly-179 would be involved in binding the hydroxyl at C-4 of the substrate in KDGA (11). However, our structural studies show that this is not the case; O-4 interacts with Oγ of Thr-157 and with the OH of Tyr-130. In all four KDGA structures presented here, the native and three Schiff base complexes, Tyr-130 interacts with the Oγ of Thr-43, which in turn interacts with the OH of Tyr-103 from another monomer. The structures of EcNAL, HiNAL, and EcDHDPS also have equivalent relays of hydrogen bonds involving a tyrosine from another monomer stabilizing the position of their equivalents of Thr-43. It is interesting to note that in both native and complex structures, Tyr-130, Tyr-103, and Tyr-132 occupy disfavored regions of the Ramachandran plot.

The complexes with KDG and KDGal provide the basis for understanding the substrate promiscuity displayed by KDGA and give an insight into its lack of facial selectivity in the condensation reaction. The active site allows sufficient flexibility in the sugar substrate such that the O-4 hydroxyl of both substrates is in a position to interact with the tyrosyl oxygen of Tyr-130, which mediates proton extraction by the substrate carboxylate (22). Indeed, the close interaction observed between O-4 and this tyrosyl oxygen in the KDGA and KDGal
complexes (typically 2.4–2.6 Å), as well as the short distance between the Tyr-130 hydroxyl and the substrate carboxylate observed in all three complexes, supports this mechanism. In this substrate-assisted model of catalysis, Thr-157, Tyr-103, and Thr-43 may be playing a supporting role, helping to stabilize the positions of the Tyr-130 hydroxyl and key substrate groups. They may, however, also be more catalytically relevant and take an active part in proton shuffling. Similarly, W1 is in an ideal position to assist in proton relay during the condensation reaction, although it may simply be displaced by the carbonyl oxygen of δ-glyceraldehyde prior to catalysis.

Superposition of the KDG and KDGal complexes shows that the active site is quite rigid, yet it has sufficient functionality built in, as well as a certain degree of redundancy, to allow recognition of the O-5 and O-6 hydroxyls even when they are in very different positions (Fig. 4). In the case of KDG, O-5 makes a water-mediated interaction with the main chain groups of Gly-179 and Ala-198, whereas in KDGal O-5 interacts with the...
tyrosyl oxygen of Tyr-132. In KDG, O-6 can make a water-mediated interaction with the side chain of Thr-44 or interact directly and via a conserved water with Tyr-132. On the other hand, in KDGal O-6 is able to interact either directly or via a water molecule with the side chain of Thr-44, as well as to make water-mediated interactions with the side chains of Asn-245 and Ser-241. These alternative hydrogen-bonding opportunities and the relative disorder observed for O-5 and O-6 point to a lack of specificity by the enzyme for its substrates at their C-5–C-6 positions. KDG and KDGal have $K_m$ values of 26 and 10 mM, respectively, with $S$. solfataricus KDGA (7). The lower $K_m$ for KDGal could be explained by the interactions it makes with the enzyme at its C-4 and C-5 hydroxyl positions, which lead to greater stabilization of this diastereomer compared with KDG.

The lower $K_m$ for KDGal could also explain why we appear to have caught KDGA in flagrante delicto, as the electron density seems consistent with a mixture of aldol cleavage states in the crystal. At 80 °C below its normal operating temperature, the catalytic activity of KDGA should be slowed by a factor of 256. It appears that within the 1-min time scale of the ligand soak prior to flash freezing KDGal has partially turned over in the crystal, whereas KDGA has not.

The functionality of the active site would also explain the lack of stereospecificity observed in the condensation reaction in KDGA. D-Glyceraldehyde can interact with pyruvate to produce KDG and KDGal, as glyceraldehyde could bind in the two positions we observed for C-4 through C-6 of KDG and KDGal. Indeed, for the KDGal condensation we can assume that d-glyceraldehyde takes up a similar position to that observed in the complex in which KDGal has been cleaved.

In summary, by exploiting the hyperthermophilic properties of KDGA we have been able to trap Schiff base complexes with natural substrates at physiological pH by rapid soaking at low temperature followed by flash freezing prior to x-ray data collection. The complexes explain the substrate promiscuity of KDGA, with a rigid ligand-binding site able to accommodate both KDG and KDGal (Fig. 4). KDGA may have solved the problem of maintaining promiscuity at high temperature by retaining its rigidity (to keep its stability) and incorporating extra functionality (to be promiscuous). The alternative option of achieving promiscuity with a flexible binding site may not be an option for a hyperthermophilic enzyme. The complexes also offer compelling evidence in support of the previously proposed mechanism of substrate-assisted catalysis mediated by Tyr-130. In addition to metabolic and mechanistic insights, these studies provide a unique opportunity for directed evolution or rational mutagenesis of KDGA toward stereoselective aldolases with a preference for each substrate configuration. Given the importance of aldolases in synthetic chemistry to catalyze carbon–carbon bond formation (24–26) and the ability of the KDGA to operate with non-phosphorylated substrates, such a mutagenesis strategy would be a valuable objective.

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