Crystal Structure of Reaction Intermediates in Pyruvate Class II Aldolase

SUBSTRATE CLEAVAGE, ENOLATE STABILIZATION, AND SUBSTRATE SPECIFICITY

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Background: Hpai is a metal-dependent pyruvate aldolase bridging aromatic hydrocarbon degradation and central metabolism.

Results: Crystal structures of enzyme-substrate complexes reveal the catalytic mechanism.

Conclusion: Two water molecules, one of them Co2+-bound, are acid-base catalysts, whereas an arginine residue stabilizes transient negative charges.

Significance: Results provide a framework for engineering pyruvate aldolases with improved biocatalysis of C–C bond formation.

Crystal structures of divalent metal-dependent pyruvate aldolase, Hpai, in complex with substrate and cleavage products were determined to 1.8–2.0 Å resolution. The enzyme-substrate complex with 4-hydroxy-2-ketoheptane-1,7-dioate indicates that water molecule W2 bound to the divalent metal ion initiates C3–C4 bond cleavage. The binding mode of the aldehyde donor delineated a solvent-filled capacious binding locus lined with predominantly hydrophobic residues. The absence of direct interactions with the aldehyde aliphatic carbons accounts for the broad specificity and lack of stereospecific control by the enzyme. Enzymatic complex structures formed with keto acceptors, pyruvate, and 2-ketobutyrate revealed bidentate interactions with the divalent metal ion by C1-carboxyl and C2-carbonyl oxygens and water molecule W4 that is within close contact of the C3 carbon. Arg70 assumes a multivalent role through its guanidinium moiety interacting with all active site enzymatic species: C2 oxygen in substrate, pyruvate, and ketobutyrate; substrate C4 hydroxyl; aldehyde C1 oxygen; and W4. The multiple interactions made by Arg70 stabilize the negatively charged C4 oxygen following proton abstraction, the aldehyde alignment in aldol condensation, and the pyruvate enolate upon aldol cleavage as well as support proton exchange at C3. This role is corroborated by loss of aldol cleavage ability and pyruvate C3 proton exchange activity and by a 730-fold increase in the dissociation constant toward the pyruvate enolate analog oxaloacetate.

Aldolases catalyze reversible stereospecific carbon-carbon bond formation and play critical roles in carbon cycling in nature. They are also useful biocatalysts for synthesis of organic molecules for use as pharmaceuticals due to their carbon-carbon joining activity (1–3). They exhibit diversity in terms of their sequences, structures, substrate specificities, and nature of catalytic residues. For example, class I aldolases utilize a lysine residue that forms a Schiff base with the keto function of the substrate, whereas class II aldolases contain divalent metal ions that stabilize the carbaniolate intermediate. Within class II aldolases, the dihydroxyacetone phosphate-specific enzymes generally contain a tetrahedral coordinated Zn2+ ion as cofactor (4–7), whereas the pyruvate-specific enzymes contain an octahedral coordinated divalent metal cofactor, which is usually Mg2+, Mn2+, or Co2+ (8–12).

Hpai (also known as HpcH) (4-hydroxy-2-ketoheptane-1,7-dioate; EC 4.1.3) is a class II (metal-dependent) pyruvate aldolase involved in the last step of 3- and 4-hydroxyphenylacetate catabolism in Escherichia coli strains W and C (13). The physiological substrate for Hpai is 4-hydroxy-2-ketoheptane-1,7-dioate (HKHD),3 which it transforms through a retro-aldol reaction to pyruvate and succinic semialdehyde. The principal steps in the chemical mechanism comprise abstraction of a proton from C4-OH of the substrate (1) catalyzed by a base, aldol cleavage resulting in the formation of a pyruvate enolate intermediate (2) that is stabilized by the divalent metal ion, and proton donation at the C3 carbon of pyruvate enolate to form pyruvate (3) completing the reaction (Scheme 1).

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3 The abbreviations used are: HKHD, 4-hydroxy-2-ketoheptane-1,7-dioate; CHA, 4-carboxy-4-hydroxy-2-oxoadipate; HMG, 4-hydroxy-4-methyl-2-oxoglutarate; HOPA, 4-hydroxy-2-oxopentanoate; r.m.s.d., root mean square deviation.
Hpal displays broad substrate specificity as it is able to utilize other 4-hydroxy-2-oxoacid derivatives as substrates (11). For example, it can convert 4-hydroxy-2-oxopentanoate (HOPA) to pyruvate and acetaldehyde (14). It is also able to utilize both pyruvate and 2-ketobutyrate as carbonyl donors in the aldol addition reaction, although the enzyme lacks stereospecific control, producing equal mixtures of 4R and 4S enantiomers (15).

The three-dimensional structures of Hpal in its apo form and containing the pyruvate structural analog oxamate have been determined (9). The enzyme adopts a triose-phosphate isomerase barrel fold with helix 8 of the ($\beta_9$)$_8$ barrel in each subunit, forming a domain-swapped dimer with a neighboring subunit in an overall hexameric structure. This structure resembles that of its homologs, 2-dehydro-3-deoxyglyceraldehyde 3-phosphate dehydrogenase (8) and 2-dehydro-3-deoxy-1,7-dihydroxy-2-ketoheptane-1,7-dioate aldolase (YfaU) (16), which are involved in sugar metabolism in microorganisms. An enzyme-bound phosphate molecule was found in the structure of apo-2-dehydro-3-deoxyglyceraldehyde aldolase and has been proposed to be the acid that donates a proton to the pyruvate enolate (8). However, in phosphate-free buffer, Hpal is able to catalyze the half-reaction of pyruvate C3 proton exchange (11), thus discounting the role of phosphate in the catalytic mechanism of the enzyme. Instead, an arginine residue (Arg70) in the active site appears to be important for catalysis because substitution of this residue in Hpal with alanine led to inactive enzyme that was unable to catalyze pyruvate C3 proton exchange (11). Replacements of histidine 45 in Hpal to alanine and glutamine on the other hand led to a decrease in $K_{cat}$ of the enzyme by 78- and 2059-fold, respectively, and the dependence of the enzyme activity on the concentration of hydroxide ions (17). Lacking among currently available structures of Hpal and its homologs are structures showing substrate binding as well as that of the aldehyde product. How the enzyme recognizes its substrates and products provides the requisite structural information that delineates the roles of pertinent active site residues and the reaction mechanism by which the enzyme promotes catalysis.

A crystallographic study of Hpal in complex with reaction products pyruvate, 2-ketobutyrate, and succinic semialdehyde was undertaken. Hpal crystals were crystallized with pyruvate and then soaked with excess succinic semialdehyde trapped the physiological substrate 4-hydroxy-2-ketopentanate-1,7-dioate. We also present crystal structures of R70A and D42A variants of Hpal aldolase. The availability of the first structures of a class II pyruvate aldolase containing the full substrate and the cleavage products significantly advances our understanding of how substrate binding and catalysis occur in this important group of enzymes.

### MATERIALS AND METHODS

**Chemicals** — l-Lactate dehydrogenase (rabbit muscle) and Chelex 100 were from Sigma-Aldrich. Restriction enzymes and Pfu Ultra DNA polymerase were from Invitrogen or New England Biolabs (Pickering, Ontario, Canada). (S)-4-Hydroxy-2-ketopentanoate used in kinetic analysis was generated enzymatically from catechol using catechol dioxygenase, 2-hydroxy-6-oxohepta-2,4-dienoate hydrolyase, and 2-hydroxypent-2,4-dienoate hydratase (11). 4-Hydroxy-3-methyl-2-ketopentanoate on the other hand was synthesized using Mn$^{2+}$-Hpal according to a previous method (15) that generated a racemic mixture. All other chemicals were of analytical grade and were obtained from Sigma-Aldrich and Fisher Scientific.

**DNA Manipulation** — The R70A variant of Hpal has been constructed previously (11). The QuikChange method (Stratagene) for site-specific mutagenesis was used to create the R70K and D42A variants of Hpal. The sequences of the sense oligonucleotides used in the mutagenesis procedure are CCGGTGGTGATCCGCAGACCG and GACTGGTTATTGATGCACGGTGAGACG, respectively (the altered codons are underlined). The reactions contained 30 ng of wild-type hpal in the vector pT7-7, 115 ng of each mutagenic oligonucleotide, 0.2 mM dNTPs, and 2.5 units of Pfu Ultra DNA polymerase (Stratagene). Mutants were screened by direct DNA sequencing.

**Expression and Purification of Recombinant Wild-type and Mutant Enzymes** — The overexpression and purification of wild-type and mutant Hpal were performed according to previous methods (11).

**Enzymatic Assay** — Kinetics assays were performed in 100 mM sodium HEPES, pH 8.0 with 0.5 mM CoCl$_2$ as described previously (11). Solvent isotope effects were determined using the normal enzyme activity assays except that assay solutions and substrate were dissolved in D$_2$O, evaporated to dryness two times, and then redissolved in the appropriate amount of D$_2$O. The assay buffer was adjusted to pH 8.0 using NaOD according to the equation pD = pH$_{meter reading}$ + 0.4 (18).

**Protein Fluorescence Measurements** — Protein fluorescence titration was used to determine the equilibrium dissociation constant of substrate analogs in wild-type and mutant enzymes as described previously (11). Tryptophan quenching of about 50% was observed when the enzymes were saturated with ligands. The concentration of enzymes in all measurements was fixed at 90 $\mu$g/ml. Metal-free apoenzyme was prepared using Chelex 100 (Sigma) as described previously (11).
**Crystallization and Data Collection** — The wild-type enzyme and D42A and R70A mutants were crystallized by the hanging drop vapor diffusion technique. Each drop consisted of 2 mg of protein solution (15 and 10 mg/ml, respectively) and 2 μl of reservoir solution (0.5 M ammonium dihydrogen phosphate, 50 mM acetic acid, 50 mM MES, and 1.0 mM CoCl₂ in deuterated buffer by 1H NMR (15). The effects of pD on the proton exchange rate of pyruvate, ketobutyrate, pyruvate and succinate semialdehyde, and HKHD as well as the R70A mutant in complex with pyruvate and D42A mutant have been deposited with the Protein Data Bank (see Table 2 for Protein Data Bank accession codes). The final structure models have R cryst (R free) values of 15.2% (17.9%), 15.5% (18.5%), 13.0% (15.4%), 15.3% (17.9%), 14.4% (18.4%), and 16.5% (19.3%), respectively. All figures were prepared using the program PyMOL (27).

For all structures, electron density was observed that was consistent with cobalt metal binding. However, the use of a fully occupied cobalt binding site during structure refinement resulted in a negative peak in the F o − F c electron density difference map for the cobalt atom. Analysis of the anomalous scattering data sets collected near the cobalt K edge (1.6 Å) revealed an electron density peak in the anomalous difference map that was coincident with the metal binding site and confirmed the presence of bound cobalt (supplemental Fig. S1). Refinement of the anomalous data sets indicated partial occupancy by the cobalt cofactor; however, full occupancy was indicated for the ligands. Refinement was continued with a mixture of cobalt and magnesium ions whose combined popu-
TABLE 2
Refinement statistics

| Crystal structure                  | WT pyruvate complex | WT ketobutyrate complex | WT pyruvate + succinic semialdehyde complex | WT 4-hydroxyl-2-ketoheptane-1,7-dioate complex | R70A pyruvate complex | D42A pyruvate complex |
|-----------------------------------|---------------------|-------------------------|--------------------------------------------|-----------------------------------------------|----------------------|----------------------|
| Number of atoms                   |                     |                         |                                            |                                               |                      |                      |
| Protein                           | 3.828               | 3.800                   | 3.806                                      | 3.828                                        | 11.473               | 3.808                |
| Water                             | 1.091               | 815                     | 808                                        | 803                                          | 1.858               | 896                  |
| Hetero                            | 62                  | 60                      | 100                                        | 132                                          | 65                  | 68                   |
| $R_{	ext{cryst}}$ (%)             | 15.2                | 15.5                    | 13.0                                       | 15.3                                         | 14.4                | 16.5                 |
| $R_{	ext{free}}$ (%)             | 17.9                | 18.5                    | 15.4                                       | 17.9                                         | 18.4                | 19.3                 |
| Root mean square deviation        |                     |                         |                                            |                                               |                      |                      |
| Bond length (Å)                   | 0.018               | 0.011                   | 0.014                                      | 0.004                                        | 0.010               | 0.017                |
| Bond angle (°)                    | 1.65                | 1.32                    | 1.39                                       | 0.868                                        | 1.25                | 1.46                 |
| Average B-factor (Å²)             | 24.2                | 32.4                    | 23.9                                       | 35.9                                         | 21.2                | 24.6                 |
| Ramachandran analysis (%)         |                     |                         |                                            |                                               |                      |                      |
| Favored regions                   | 99.6                | 99.5                    | 99                                         | 99                                           | 98.8                | 98.6                 |
| Allowed regions                   | 0.4                 | 0.5                     | 1                                         | 1                                            | 1.2                 | 1.4                  |
| Atomic coordinate error (Å)       | 0.16                | 0.17                    | 0.13                                       | 0.24                                         | 0.16                | 0.19                 |
| Protein Data Bank accession codes| 4BS5                | 4BST                    | 4BSU                                       | 4BSV                                         | 4BSW                | 4BSX                 |

* $R_{	ext{cryst}} = \frac{\Sigma_{|hkl|}[F_{o}(hkl)] - |F_{c}(hkl)|}{\Sigma_{|hkl|}[F_{o}(hkl)]}$, where $T$ is a test data set randomly selected from the observed reflections prior to refinement. The test data set was not used throughout refinement and contained 7.5–10% of the total unique reflections.  
* Analyzed by Molprobity.

RESULTS

Intermediate Trapping and General Structure—Initial electron density maps of HpaI aldolase crystal soaked with ligands, especially aldehydes, did not always show full occupancies in the active site. To ensure highest occupancy of intermediates, the cryoprotection buffer consisting of mother liquor (pH 5.0), 15% (v/v) glycerol, and 1.0 mM CoCl$_2$ was adjusted to include a 10 mM concentration of the appropriate ligands wherever feasible.

A modified trapping strategy used for active site binding of HKHD as HpaI crystals soaked with an excess of pyruvate and succinic semialdehyde did not reveal electron density consistent with aldol condensation. Although loss of catalytic activity under crystallization conditions would explain the absence of trapped substrate in the electron density map, the kinetic parameters toward HOPA, a surrogate HpaI aldolase substrate, indicated turnover under crystallization conditions even though $k_{\text{cat}}$ was reduced 500-fold to 0.69 ± 0.04 s$^{-1}$ compared with HpaI activity in 100 mM sodium HEPES buffer, pH 8.0. For HpaI crystals soaked in the presence of 10 mM substrate prior to cryotrapping, this would result in a number of turnovers on a minute time scale. Although substrate binding occurs at 10 mM concentrations under crystallization conditions as $K_m$ for HOPA increased only 10-fold to 3.4 ± 0.6 mM from the reference condition, turnover would result in not only HOPA but also pyruvate and acetaldehyde trapped in the active site and make interpretation of the composite electron density map challenging. To minimize the number of potential intermediates trapped in the active site, crystals of HpaI aldolase grown in the presence of pyruvate were soaked with excess succinic semialdehyde (10 mM) to chase all 2-keto acid into substrate. Under these soaking conditions, electron density was observed in the active site of HpaI crystals whose shape was consistent with HKHD binding.

Ligands and metal were modeled into the electron density map in the last stages of refinement, and the quality of the fit to the electron density was validated by inspection of simulated annealing and kicked omit maps (32). The high quality of the electron density map enabled interpretation of all bound ligands and metal ions in the active site.
Each HpaI aldolase subunit exhibits a triose-phosphate isomerase barrel fold containing a repeated 8-fold (β/α) motif with the active site located on the C-terminal side of the β barrel, which is consistent with the apo- and oxamate-bound HpaI structures determined previously (9). The asymmetric unit contains one HpaI aldolase dimer, and the 3-fold crystallographic symmetry generates a hexameric assembly with 32 symmetry, which is the physiologically relevant quaternary structure, as corroborated by size exclusion chromatography (9). The crystal structure of the R70A variant possessed a hexamer exhibiting 32 non-crystallographic symmetry in the asymmetric unit cell, confirming that the HpaI quaternary structure is indeed a trimer of dimers (supplemental Fig. S2).

**Metal and 2-Keto Acid Binding**—The active site of HpaI aldolase recognizes pyruvate and 2-ketobutyrate identically (Fig. 1, A and B), which is consistent with the similar dissociation constants ($K_a$) observed for the two compounds (0.32 ± 0.02 and 0.47 ± 0.02 mM, respectively). Backbone amides of conserved residues Ala^{174} (3.00 Å) and Asp^{175} (3.04 Å) form hydrogen bonds and direct the carboxylate oxygens of the pyruvate molecule, and Gln^{147} and Arg^{160} interact with the keto oxygen; these interactions are shown by black dashes. These interactions are used to specifically orient pyruvate in the active site. Hydrogen bonding interactions not directly contacting the metal ion or pyruvate (gray dots) organize remaining active site residues and water molecules. Interactions from Val^{118} and Asp^{84} of an adjacent contacting subunit (blue) complete the active site binding locus. The consistency in hydrogen bonding interactions made with W3 implies that Glu^{149} and Glu^{172} are hydrogen bond acceptors, whereas His^{159} is a hydrogen bond donor. Electron density encompassing the ligands was calculated from a simulated annealing $F_o - F_c$ omit map and contoured at 3.5σ.

**FIGURE 1. Trapped 2-keto acids in active site of HpaI pyruvate aldolase.** A, electron density of pyruvate bound in the active site of the native enzyme. B, electron density of ketobutyrate trapped in the active site of the native enzyme. C, stereoview showing active site interactions when pyruvate binds to HpaI aldolase. In A and B, water molecules occupying the aldehyde binding site (presented in Fig. 3) are shown in green. Magenta dashes depict coordination of Co^{2+} made by the two conserved water molecules (W1 and W2), pyruvate (blue), and residues Glu^{149} and Asp^{175}. The backbone nitrogens of Ala^{174} and Glu^{175} direct the carboxylate oxygens of the pyruvate molecule, and Gln^{147} and Arg^{160} interact with the keto oxygen; these interactions are shown by black dashes. These interactions are used to specifically orient pyruvate in the active site. Hydrogen bonding interactions not directly contacting the metal ion or pyruvate (gray dots) organize remaining active site residues and water molecules. Interactions from Val^{118} and Asp^{84} of an adjacent contacting subunit (blue) complete the active site binding locus. The consistency in hydrogen bonding interactions made with W3 implies that Glu^{149} and Glu^{172} are hydrogen bond acceptors, whereas His^{159} is a hydrogen bond donor. Electron density encompassing the ligands was calculated from a simulated annealing $F_o - F_c$ omit map and contoured at 3.5σ.
The structure resulting from HpaI aldolase crystals in complex with pyruvate and oxalate, as shown in Fig. 1A. Occupancies were determined such that HKHD B-factors did not differ significantly with respect to B-factors of interacting active site residues. The occupancy determined for HKHD is reasonable for occupancy for the surrogate substrate HOPA at 10 mM is 0.75, which would be expected under the crystallization conditions given a Michaelis constant of 3.4 ± 0.6 mM for HOPA.

Active site interactions made by HKHD are shown in Fig. 3B. The substrate coordinates the cobalt ion through its keto (2.69 Å) and carboxylate oxygen atoms (2.76 Å). The 2-carboxyl oxygen forms a weak hydrogen bond with the η1 guanidinimum nitrogen of Arg70 (3.13 Å), which in turn makes a salt bridge with Asp42. This electrostatic interaction will raise the pK_a of Arg70 to ~17 and maintain its positive charge at physiological pH based on computational pK_a calculations (34). The η2 guanidininium nitrogen of Arg70 contacts the C4-OH of HKHD (3.28 Å), and its positive charge would facilitate C4-OH deprotonation by stabilizing the resultant negative charge. Moreover, water molecule W2, coordinated to the Co^2+ metal ion, is also proximal to the substrate C4-OH (2.12 Å) and interacts with the backbone oxygen of Val118 (2.13 Å) and His45 (3.07 Å). His45 further forms an ion pair interaction with Asp^44 of the symmetry-related neighboring subunit. Small torsional rotations of the His^45 imidazole side chain could readily position it within hydrogen bonding distance of W2 and without breaking its interaction with Asp^38 would make W2 the likely candidate mediating proton abstraction at the C4 hydroxyl. The W2-cobalt ion distance of 2.12 Å in this structure and 2.22 Å in the structure bound by the cleavage products described below is consistent with W2 coordination by Co(II) metal ion (35).

Aldehyde Binding and Recognition—The structure derived from the HpaI crystals soaked with 10 mM pyruvate and 10 mM succinic semialdehyde showed electron density that was modeled based on the structures of the two cleavage products and is shown in Fig. 3C. Pyruvate was refined as full occupancy and yielded B-factors similar to those refined for the 2-keto acid in the pyruvate-bound HpaI structure, whereas occupancy of the aldehyde binding site was set to 0.66 with water molecules (shown in Fig. 1A) used to fill the residual electron density at an occupancy of 0.33. Refinement of succinic semialdehyde yielded B-factors similar to those for the nascent aldehyde moiety of HKHD.

The interpretation of the electron density map of HpaI in complex with both pyruvate and succinic semialdehyde resulted in two distinct aldehyde configurations as shown in Fig. 3C. These two prochiral binding modes of each aldehyde (shown in Fig. 3D), positioned si or re face to the attacking pyruvate enolate, are consistent with the absence of stereospecificity in this enzyme (15). In each binding mode, the plane of the aldehyde moiety was oriented vicinal and roughly perpendicular to the putative trajectory of the attacking pyruvate enolate.

Anchoring of the aldehyde donor molecule in the active site occurs through hydrogen bonding of its aldehyde oxygen with the η2 guanidininium nitrogen of Arg^70 (2.66 Å) and water molecule W2 (2.86 Å) for both prochiral orientations of succinic

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**TABLE 3**

Dissociation constants of pyruvate and oxalate in HpaI aldolase determined by protein fluorescence quenching titration

| Enzymes       | Pyruvate | Pyruvate and 0.5 mM Co^{2+} | Oxalate and 0.5 mM Co^{2+} |
|---------------|----------|----------------------------|----------------------------|
| ApoHpaI       | 21.2 ± 2.4 | 0.32 ± 0.022               | 10.4 ± 0.75                |
| ApoR70K       | 18.8 ± 1.3 | 0.32 ± 0.004               | 8.2 ± 0.3                  |
| ApoR70A       | 26.9 ± 3.1 | 0.47 ± 0.001               | 7601 ± 552                 |
| ApoD42A       | 32.5 ± 1.7 | 31.2 ± 1.6                 |                           |

* No fluorescence quenching was observed for sodium oxalate at concentrations up to 40 mM.

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semialdehyde (Fig. 3D). Additional interactions with the semialdehyde molecule occur between its carboxylate moiety and backbone amide dipoles of the enzyme (not shown) that form a collar of positive charge at the active site entrance (Fig. 2, B and C).

Hpai exhibits broad specificity toward a variety of aldehyde donors with diverse lengths and substituents (15). In the presence of the divalent metal ion and pyruvate, the aldehyde binding site is effectively neutral except for the collar of positive charge at the active site cavity entrance (Fig. 2B). Hydrophobic residues line this binding locus: Trp19, Ala174, Leu212, and Val234 as well as Val118, Ala121, and Leu122 from the neighboring subunit at 5–6-Å distance from the aliphatic atoms of the aldehyde molecule, forming a large cavity. As none of these residues makes close contact with the ligand, water molecules would solvate the aldehyde substrates. Succinic semialdehyde binding entails displacement of at least six well ordered water molecules with respect to the pyruvate-liganded structure, whereas acetaldehyde would displace fewer such water molecules. The facile displacement of water molecules in the structures indicates an aldehyde donor binding locus that readily accommodates aldehyde molecules of different lengths.

To further investigate the potential substrate diversity of Hpai aldolase, compounds cleaved by YfaU, such as 2-keto-3-deoxy-L-lyxonate, 2-keto-3-deoxy-L-mannonate, 2-keto-3-deoxy-L-rhamnonate (16), and HOPA, were modeled into the active site using the HKHD binding locus as a template and then further refined on the basis of their fit to the electron density omit map shown in Fig. 3A. These substrates were readily threaded onto the HKHD template (supplemental Fig. S3) and resulted in no close contacts or steric clashes in the active site, indicating that they are potential substrates for Hpai aldolase.

Active Site Binding Geometries of Reaction Intermediates—Superposition of the HKHD structure onto the cleavage product structure yielded an overall r.m.s.d. value of 0.12 Å with no significant conformational differences between the two structures. The resultant superposition of the active site geometries of the reaction intermediates is shown in Fig. 4. Comparison between the two structures on the basis of interatomic dis-
distances reveals the 2-keto moiety of the HKHD substrate not as strongly bound in the active site as is the respective cleavage product, pyruvate. Although Arg70 makes potentially charged interactions in both structures, its interactions with the respective nascent pyruvate and aldehyde atoms in the HKHD structure are slightly longer and are no longer considered hydrogen bonds. The weaker active site affinity for HKHD explains the inability to trap significant concentrations of substrate in the presence of 10 mM pyruvate and 10 mM succinic semialdehyde in HpaI crystals. Only when pyruvate concentrations were low (stoichiometric with respect to enzyme concentration in HpaI crystals such that most pyruvate could be chased into HKHD with excess aldehyde) was it possible to trap HKHD in the active site.

Conformation Changes following Ligand Binding—Similar conformational responses were observed in all structures containing 2-keto acid moieties. Superimpositions between the structure of the apoenzyme (8) and each of the liganded complexes yielded an overall r.m.s.d. value of 0.73 ± 0.03 Å, which is slightly greater than the calculated r.m.s.d. value based on superimposition of only ligand complexes (0.20 ± 0.05 Å). The

FIGURE 3. Substrate and cleavage products trapped in HpaI pyruvate aldolase active site. A, electron density of substrate HKHD trapped in the native enzyme. HKHD orientation corresponding to R chirality at C4 is shown in yellow, and that of S chirality is shown in red. The inset shows the same electron density looking down and corresponds to a counterclockwise rotation in the vertical plane of −90°. B, active site interactions implicating the metal cofactor, HKHD, Arg70, and W2. C, electron density of cleavage products pyruvate and succinic semialdehyde (re face orientation in yellow; si face in pink). D, interactions highlighting succinic semialdehyde binding and recognition. Hydrogen bonds are shown as short dashes, whereas long dashes connect atoms that are chemically bound upon aldol condensation. Water molecules in alternate conformation with the aldehyde binding site are shown in green. Upon pyruvate binding, Arg70 and the conserved water W2 (red) orient the carbonyl moiety of the aldehyde substrate. In B and D, the angle subtended by either C4 hydroxyl or aldehyde oxygen, W2, and Val118 corresponds to tetrahedral hybridization geometry. Electron density encompassing the ligands was calculated from a simulated annealing Fo − Fc omit map and contoured at 3.5σ. The divalent cobalt ion is depicted by a salmon sphere.

FIGURE 4. Superposition of the active site binding geometries of HKHD and resultant cleavage products trapped in the active site of HpaI pyruvate aldolase. Substrate HKHD (orange) is depicted for both R and S conformations at its C4 hydroxyl, whereas the aldehyde (dark gray) is shown in re and si face orientations. Hydrogen bonds are shown as short dashes. Close van der Waals contacts made by Arg70 with HKHD O3 and O4 atoms are shown by long dashes (yellow). The divalent cobalt ion (Co) is depicted by a salmon sphere. The composite image corresponds to superposition of one HpaI subunit bound with HKHD and the equivalent subunit in complex with its cleavage products. The interactions by HKHD with Co(II) and Arg70 are weaker, indicated by longer interatomic distances, compared with the equivalent interactions made with Co(II) and Arg70 by the oxygen atoms of the 2-keto moiety, O2 and O3, as well as aldehyde C1 oxygen in the structure binding the cleavage products. Ald, aldehyde; Pyr, pyruvate.
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difference can be attributed largely to conformational changes from the loop region defined by residues 116–124. Indeed, the overall r.m.s.d. value computed using only these residues increased to 2.94 ± 0.10 Å between apoenzyme and holoenzyme complexes and was reduced to 0.47 ± 0.05 Å for all structures if residues 116–124 were excluded from the calculation. Interestingly, the movement of this polypeptide region (116–124) permits the interaction of the backbone oxygen of Val118, with water molecule W2, which is coordinated to the active site metal ion (Figs. 1C, 3B and 3D). Binding by the aldehyde acceptor molecule on the other hand does not require a conformational change in the presence of the bound 2-keto acid moiety.

Arg70 and Asp42 Mutant Structures—We successfully trapped pyruvate in the crystal structure of the R70A variant of Hpal (supplemental Fig. S4A); however, trapping of the aldehyde or the condensation products was unsuccessful under the experimental conditions. The loss of the charged interaction between the pyruvate C2 carbonyl and the guanidinium group of Arg70 only slightly perturbed the 2-keto acid binding locus, resulting in a slight displacement of pyruvate in its plane with respect to its position in the native structures (0.79 ± 0.23 Å). However, the C2 carbonyl retains its hydrogen bonding interaction with Gln147 (supplemental Fig. S4B). This residue also interacted with Arg70 in the wild-type enzyme, indicating that Arg70 is not mandatory for ensuring alignment of the 2-keto acid moiety in the active site. Further support comes from similar $K_a$ values for pyruvate binding in the wild-type and mutant proteins (R70K and R70A) in the presence or absence of Co$^{2+}$ (Table 3). However, the loss of the charged interaction destabilized binding with water molecule W4, consistent with its absence in the mutant structure.

Superimposition of the D42A mutant structure onto the pyruvate-bound native structure (r.m.s.d. 1.20 ± 0.02 Å) shows two loop regions, residues 42–56 and 116–124, that are displaced by 3.09 ± 0.04 and 3.85 ± 0.02 Å, respectively, and whose movement disrupts the structural integrity of the active site (supplemental Fig. S5). The active site disruption results in a concomitant loss of the metal ion in the D42A mutant structure. In the liganded native enzyme, residues Glu44 and His45 in one loop interact with water molecules W1 and W3, whereas Val118 in the second loop of the symmetry-related subunit interacts through its backbone carbonyl with water molecule W2. The repositioning of these active site residues in the D42A mutant induces loss of the three active site water molecules, two of which, W1 and W2, directly coordinated the metal ion cofactor, and underscores the essential structural role of Asp42 in maintaining active site integrity in the native enzyme.

Kinetic Analysis—The $k_{cat}$ value for HOPA cleavage in deuterated buffer was reduced by 30% with a solvent isotope effect of $k_{cat}^D$ and $(k_{cat}/K_m)^D$ of ~1.5, implying an involvement of water in catalysis (Table 4). A similar solvent isotope effect was shown for another divalent metal ion-dependent C–C bond lyase, α-isopropylmalate synthase (1.8–2.2), which also implicated a catalytic water molecule (36). The pD profile for the pyruvate C3 proton exchange reaction in the native enzyme showed deprotonation with a $pK_a$ of 6.5 ± 0.1 (Fig. 5), which could correspond to the base that abstracts a proton from the C3 methyl of pyruvate. Although a slight shift in p$K_a$ value can occur in deuterated buffer (~0.4 pH units) (37), this p$K_a$ value is not significantly different from the p$K_a$ of 6.5 associated with a catalytic base that abstracts the proton from C4 hydroxyl in the HOPA cleavage reaction (17), suggesting two chemical steps involving the same base/acid. Hpal aldolase is also able to efficiently catalyze the aldol cleavage of 2-keto-3-methyl-4-hydroxypentanoate to form 2-ketobutyrate and acetaldehyde with a $K_m$ value of 0.05 ± 0.01 mM and $k_{cat}$ of 14.0 ± 1.1 s$^{-1}$. Thus, it is not surprising that the aldolase can catalyze C3 proton exchange of 2-ketobutyrate (15). A single exponential decay is observed for $^1$H NMR signal loss at C3 of 2-ketobutyrate (15). A single exponential decay rate (Table 5), indicating that pyruvate binding in D42A mutant induces loss of the three active site water molecules, two of which, W1 and W2, directly coordinated the metal ion cofactor, and underscores the essential structural role of Asp42 in maintaining active site integrity in the native enzyme.

Pyruvate binding was similar for mutants and native enzyme in the metal-free apoenzymes (Table 3); however, in the presence of 0.5 mM Co$^{2+}$, the $K_a$ of pyruvate binding in D42A remains unchanged, being 100-fold weaker compared with wild-type enzyme and Arg70 mutants. In the presence of oxalate, a pyruvate enolate analog, the measured $K_a$ is significantly

| Table 4 |
| Solvent | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
|---------|----------|-------|-------------|
| H$_2$O  | 361.5 ± 24.7 | 0.35 ± 0.06 | $(1.0 ± 0.1) \times 10^6$ |
| D$_2$O  | 259.0 ± 19.1 | 0.39 ± 0.04 | $(6.6 ± 0.8) \times 10^5$ |
| Ratio (H$_2$O/D$_2$O) | 1.40 | 0.90 | 1.52 |

FIGURE 5. pD profile of pyruvate C3 proton exchange reaction in Hpal aldolase. Assays were performed in deuterated constant ionic strength buffer containing 100 mM Tris, 50 mM acetic acid, 50 mM MES, and 1.0 mM CoCl$_2$ with pD ranging from 5.4 to 9.4. The data were fitted to Equation 1 by non-linear regression using LeNoona and showed a p$K_a$ of 6.5 ± 0.1.
The diversity of catalytically competent aldehyde donors arises from the nature of its large binding pocket that is lined by non-directional hydrophobic residues and is filled with more than 15 water molecules that can be readily displaced by an incoming aldehyde. Aldol condensation of diverse electrophiles with pyruvate is supported by the different cleavable substrates that can be modeled into the Hpai aldolase active site without steric clash (supplemental Fig. S3). From the same modeling, small chemical substituents on the nascent aldehyde moiety would also be tolerated in the active site cavity.

The C1 carbon of succinic semialdehyde is positioned within close contact of the pyruvate enolate and with the plane containing the C1 carbon of each aldehyde oriented nearly co-planar with respect to the pyruvate molecule (Fig. 3D). Anchoring of the aldehyde within the active site is minimal, entailing two interactions by its C1 oxygen with the guanidinium nitrogen of Arg70 and W2. Additional weak interactions can occur, depending on the chemical structure of the aldehyde, with primarily backbone amide dipoles as was observed with the carboxylate tail of succinic semialdehyde. The orientation of the aldehyde moiety perpendicular to the plane containing the nucleophile is consistent with an attack trajectory for incipient C–C bond formation with the enolate (shown as long dashed lines in Fig. 3D). The capacious aldehyde binding locus further allows the plane containing the electrophile to rotate without steric hindrance, enabling the pyruvate enolate to attack *si* or *re* face to yield racemic products with *R* and *S* configurations at C4 (15).

**Catalytic Mechanism**—A catalytic mechanism to promote cleavage of the C–C bond in pyruvate aldolases integrating enzymology and structure data is outlined in Scheme 2. The reaction chemistry catalyzed by Hpai aldolase entails a water molecule that is bound to the divalent metal ion, which ionizes the water molecule, thereby enabling the metal-bound hydroxide to act as a base catalyst in mediating proton transfers. A key feature is an arginine residue that directs and stabilizes formation of reaction intermediates to facilitate proton transfers.

In the C3–C4 bond cleavage of HKHD, a catalytic base is required to deprotonate C4–OH, and a catalytic acid is required to donate a proton to pyruvate enolate. His45 was proposed previously to be the catalytic base (9); however, this is unlikely due to the following considerations. The identical hydrogen bonding pattern by the His45 Ne nitrogen in pyruvate aldolase structures crystallized over the pH range 5–8 indicates a basic pKₐ for His45 Ne nitrogen that places it outside the pH-dependent range attributed to C3 proton exchange and HKHD substrate cleavage. Basic pKₐ values have been observed from NMR studies in proteins where raised pKₐ values of histidines (7.6–7.9) have been attributed to their interaction with an aspartate or glutamate residue (38–40) as compared with lower pKₐ values of histidines in comparable environments and that are not engaged in charged interactions (40). Indeed, in Hpai aldolase, Asp115 of the adjacent subunit forms a hydrogen bond with His15 N8 nitrogen (2.74 Å) that would argue in favor of a basic pKₐ shift by the His45 Ne nitrogen. Furthermore, slight repositioning of the His45 imidazole side chain without breaking its hydrogen bond to Asp115 would allow it to donate a hydrogen bond to W2 and promote geometry where W2 donates a hydro-
gen bond to the backbone carbonyl of the neighboring Val\textsuperscript{118}, generating the geometry conducive for in-line proton transfer between W2 and W4 or C4 hydroxyl of the substrate. Although the guanidinium nitrogen of Arg\textsuperscript{70} interacts with C4-OH of HKHD (3.28 Å), the possibility of Arg\textsuperscript{70} acting as general base/acid in catalysis is also unlikely by virtue of its salt bridge with Asp\textsuperscript{42} that ensures structural integrity of the active site. Here, we propose a well ordered and conserved water, W2, as the catalytic base. The pK\textsubscript{a} of a water molecule coordinated by a divalent cobalt metal ion ranges from 6.2 to 9.8 for metal complexes in an aqueous medium (41) and decreases to ~5.8 in cobalt-substituted carboxypeptidase A (42). In HpaI aldolase, W2 is bound to the cobalt metal ion and donates a hydrogen bond to the Val\textsuperscript{118} backbone carbonyl. It can also be further stabilized by accepting a hydrogen bond from His\textsuperscript{45}, resulting in an orientation that would allow W2 as a metal-bound hydroxide ion to catalyze in-line proton transfer with W4, which hydrogen bonds with W2. The analogous water in the structure of YfaU, a homolog of HpaI, has also been proposed to be a catalytic base (16). Base catalysis by a metal-bound hydroxide ion represents an efficient mechanism to generate a transient nucleophile in a solvated environment. The proposed

### TABLE 5

Steady-state kinetic parameters for HOPA cleavage and pyruvate α-proton exchange by wild-type and mutant HpaI enzymes

| Enzymes | $K_{\text{m}}$ | $K_{\text{cat}}$ | $k_{\text{cat}}/K_{\text{m}}$ | $V_{\text{exchange}}$ |
|---------|---------------|-----------------|-----------------------------|----------------------|
| HpaI    | 0.38 ± 0.01   | 4.56 ± 0.14     | (9.41 ± 0.28) × 10\textsuperscript{2} | 359.8 ± 10.2         |
| R70K    | 3.32 ± 0.04   | 11.8 ± 0.08     | (3.55 ± 0.05) × 10\textsuperscript{2} | 191.2 ± 7.2          |
| R70A    | ND            | 0.04            | ND                          | ND                   |
| D42A    | ND            | ND              | ND                          | ND                   |

*a The substrate inhibition constant derived from analysis of the kinetic data at high substrate concentrations.

*b No substrate inhibition was observed.

`ND, no activity was detectable.

### SCHEME 2

**Proposed catalytic mechanism (reactions a–d and intermediates 1–4) of C–C bond cleavage in pyruvate aldolase.**

R = CH\textsubscript{2}CH\textsubscript{2}COOH.
Crystal Structure of Reaction Intermediates in HpaI

The decarboxylation of oxaloacetate observed with HpaI aldolase (11) would use a mechanism identical to the proposed cleavage mechanism. As oxaloacetate and HKHD are isostERIC, differing at the C5 position of HKHD, which in oxaloacetate becomes the carboxylic oxygen atom, superimposition of oxaloacetate in acid form onto the HKHD binding site in HpaI aldolase results in an intermediate isostERIC with 1. As the pK_a of the 4-carboxylate group of oxaloacetate is 4–5, the conjugate base would be the dominant species at physiological pH. In the decarboxylation of β-keto acids, the bond undergoing cleavage by Schiff base catalysis must be out of the plane of the imine π bond (43). Similarly, in HpaI aldolase, the close contact between the W2 hydroxyl and C4 carboxyl oxygen would force the C4 carboxyl out of the O2-C2-C3-C4-O4 plane and favor the expected geometry for decarboxylation.

Evolutionary Relationships with Other Enzymes—Using the Dali server, the HpaI structure was shown to be closely related to that of 2-dehydro-3-deoxygalactarate aldolase, YfaU, macromolecular synthase, citrate lyase, and pyruvate kinase as reported previously (9). Intriguingly, the enzyme was also structurally related to the P-enolpyruvate binding domain of enzyme I (residues 261–573) in bacterial P-enolpyruvate-dependent carbohydrate:phosphotransferase systems (Protein Data Bank code 2HWG) (44) and of pyruvate, phosphate dikinase (residues 534–874) (Protein Data Bank code 1DIK) (45) with both structures yielding identical r.m.s.d. values of 2.7 Å (Fig. 7A). The residues used in metal binding (Glu149 and Asp179 for HpaI, Glu313 and Asp455 for enzyme I, and Glu745 and Asp769 for PPDK) and the residue equivalent to Arg70 of HpaI (Arg332 in enzyme I and Arg617 in PPDK) were spatially superimposable in the enzyme structures (Fig. 7A). Although these enzymes catalyze different overall reactions, they all share a common partial chemical reaction step of pyruvate enolate formation and protonation of the enolate to form pyruvate. In enzyme I and PPDK, a cysteine was proposed as the residue that donates a proton to pyruvate enolate from its γ C (Cys831 for PPDK and Cys502 for enzyme I). In HpaI, this residue would correspond to Leu212, which cannot function as a proton donor, implying the use of a different functional entity in HpaI aldolase. A binding role was proposed for Arg617 in terms of charge interactions with the phosphoryl group in PPDK (46). The structural analogy with Arg70 in HpaI suggests an additional role of Arg617 in PPDK and Arg332 in enzyme I in pyruvate enolate stabilization.

Arginine residues are also found in spatially similar positions in the active sites of other evolutionarily unrelated yet analogous pyruvate lyases, including DmpG (10), HMG/CHA aldolase (12), and oxaloacetate decarboxylase (30) (Fig. 7B). The arginine residue in DmpG was directed through its electrostatic interaction with a glutamate residue (Glu48) whose carboxylate function efficiently in the direction of aldol addition with the tighter binding pyruvate displacing HKHD upon synthesis.

The catalytic mechanism is consistent with a trajectory of least atomic motion requiring minimal atomic librations and imposes no stereochemical constraint at C4 to select either the R or S isomer of HKHD. In the condensation direction, the catalytic mechanism allows for a random binding order with respect to pyruvate and aldehyde donor.

As for the catalytic acid, W2 was suggested previously to serve as the proton donor to protonate pyruvate enolate (11, 16). But a direct role of W2 mediating pyruvate α-proton exchange is unlikely as W2 is unfavorably positioned to promote direct proton transfer to the pyruvate C3 carbon. Rather, W4, positioned by Arg70 perpendicular to the plane of the pyruvate molecule, is within close contact of the C3 carbon of pyruvate, allowing catalysis of proton exchange with the pyruvate enolate. In the 2-ketobutyrate-ligated structure, the W4 binding site is also present in a position similar to that in the pyruvate-bound structure, and Arg70 interacts with W4 (seen in Fig. 1C), consistent with their roles in promoting C3 proton exchange in 2-ketobutyrate. In the R70K mutant, only a slight shift by W4 (~1 Å) would be required for the Lys70 side chain to orient W4 for nucleophilic attack by the enolate.

Through its guanidinium moiety interactions with substrate C2 and C4 oxygens, pyruvate and ketobutyrate C2 oxygens, aldehyde C1 oxygen, and W4, Arg70 plays multiple roles in HpaI catalysis by facilitating deprotonation of substrate C4 hydroxyl, stabilizing the enolate, and mediating W4 protonation of the enolate. This is supported by kinetic evidence showing the loss of pyruvate α-proton exchange and aldehyde cleavage activities in the R70A mutant (11) and the significant decrease in catalytic efficiency for the R70K mutant (Table 5) that would result from reduced stabilization of reaction intermediates by the shorter lysine side chain. A significant K_d increase for binding by the pyruvate enolate analog oxalate in the R70A mutant compared with the wild type reinforces its critical role in pyruvate enolate stabilization in HpaI (Table 5). The primary role of Arg70 is thus to stabilize in the transition state the resultant negative charge created at the C4 hydroxyl and indirectly at the enolate by virtue of its electrostatic interaction.

Therefore, the catalytic cycle (Scheme 2) begins by HKHD binding in the active site cavity whereby its coordination with the metal ion creates an electron sink at the C2 keto carbonyl in intermediate 1 and simultaneously induces a small conformational adjustment in loop region (116–124) to position W2, enabling it to hydrogen bond with the HKHD C4 hydroxyl. The positive charged side chain of Arg70 interacts with both the 2-keto oxygen and the C4 hydroxyl of HKHD. Proton abstraction from the C4 hydroxyl by the W2 hydroxide ion induces C3–C4 bond cleavage to yield intermediate 2 (reaction a). Reaction a is reversible as the pyruvate enolate and succinic semialdehyde are bound proximally to Arg70 and can recombine by aldol addition. Exchange with W4 releases the aldehyde in intermediate 2 and initiates a proton relay from W2 to W4 to the C3 methyl of the pyruvate enolate intermediate 3 (reaction b). Protonation of pyruvate enolate by W4 yields the keto product 4 and regenerates the hydroxide ion at W2 (reaction c). Finally, exchange in intermediate 4 with HKHD releases the keto product, pyruvate, and recommences the catalytic cycle (reaction d). At low reactant concentrations, the enzyme would

The mechanism of rate-limiting base catalysis mediated by the W2 hydroxide molecule would explain the pH profile observed for proton exchange (Fig. 5). The involvement of water molecules W2 and W4 in the proposed mechanism is further supported by the solvent kinetic isotope effect observed.
The guanidinium moieties of adjacent arginine residues cluster tightly (identiﬁed as W2/W2, equivalent to W2 and W4; however, Arg159 orientation is not directed by an interaction with a proximal carboxylate group. Replacement of the respective arginine residues by a neutral residue in HMG/CHA aldolase, BphI aldolase, and 3-hydroxy-3-methylglutaryl-CoA lyase also severely compromised catalytic activities (12, 29, 31).

The structural comparison of the active sites of evolutionarily distant and non-homologous enzymes (shown in Fig. 7, A and B) has highlighted a highly conserved constituent in their active sites, an arginine residue, which in HpaI aldolase is central to proton exchange with the pyruvate enolate and to cleavage of the C–C bond. The conservation of this single catalytic entity indicates signiﬁcant commonality in the reaction chemistry catalyzed by these enzymes and is reinforced by similar spatial orientation of putative catalytic water molecules in the pyruvate aldolases. However, as the aldehyde or keto acid acceptor speciﬁcities of these pyruvate aldolases are different, extensive sequence divergence appears to have occurred to accommodate recognition of diverse substrates.

Thus, HpaI pyruvate aldolase epitomizes an exceedingly simple yet highly conserved catalytic mechanism to promote C–C bond cleavage of non-phosphorylated compounds. Our results provide an important framework for future utilization and engineering of class II pyruvate aldolases as improved biocatalysts. Moreover, the substrate/product trapping methods described here applied to other aldolases should shed further insight into the catalytic mechanism of these enzymes.

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