Crystal structures were determined to 1.8 Å resolution of the glycolytic enzyme fructose-1,6-bis(phosphate) aldolase trapped in complex with its substrate and a competitive inhibitor, mannitol-1,6-bis(phosphate). The enzyme substrate complex corresponded to the postulated Schiff base intermediate and has reaction geometry consistent with incipient C2–C3 bond cleavage catalyzed by Glu-187, which is adjacent to the Schiff base forming Lys-229. Atom arrangement about the cleaved bond in the reaction intermediate mimics a pericyclic transition state occurring in nonenzymatic aldol condensations. Lys-146 hydrogen-bonds the substrate C4 hydroxyl and assists substrate cleavage by stabilizing the developing negative charge on the C4 hydroxyl during proton abstraction. Mannitol-1,6-bis(phosphate) forms a noncovalent complex in the active site whose binding geometry mimics the covalent carbinolamine precursor. Glu-187 hydrogen-bonds the C3 hydroxyl of the inhibitor in the enzyme complex, substantiating a proton transfer role by Glu-187 in catalyzing the conversion of the carbinolamine intermediate to Schiff base. Modeling of the acyclic substrate configuration into the active site shows Glu-187, in acid form, hydrogen-bonding both substrate C2 carbonyl and C4 hydroxyl, thereby aligning the substrate ketose for nucleophilic attack by Lys-229. The multifunctional role of Glu-187 epitomizes a canonical mechanistic feature conserved in Schiff base-forming aldolases catalyzing carbohydrate metabolism. Trapping of tagatose-1,6-bis(phosphate), a diastereoisomer of fructose 1,6-bis(phosphate), displayed stereospecific discrimination and reduced ketohexose binding specificity. Each ligand induces homologous conformational changes in two adjacent α-helical regions that promote phosphate binding in the active site.

Aldolases are ubiquitous enzymes and have been a subject of continuous interest because of their ability to catalyze carbon bond formation in living organisms. Their role is best known in glycolysis, where fructose 1,6-bis(phosphate) (FBP) aldolases (EC 4.1.2.13) promote the reversible cleavage of FBP to triose phosphates, α-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (DHAP). The class I enzymes use covalent catalysis, implicating a Schiff base formed between a lysine residue on the enzyme and a ketose substrate. In vertebrates, there are three tissue-specific class I aldolases (aldolase A (found in skeletal muscle and red blood cells), aldolase B (found in liver, kidney, and small intestine), and aldolase C (found in neuronal tissues and smooth muscle)), and they are distinguishable on the basis of immunological and kinetic properties (1). The catalytic mechanism has been extensively studied using class I aldolase A from rabbit muscle, and key intermediates are depicted in Scheme I.

In the forward reaction, a reactive lysine residue in the active site attacks the ketose (2) of the acyclic FBP substrate (3, 4). Transient formation of a dipolar tetrahedral carbinolamine with the keto function yields a neutral carbinolamine species 1, which is then dehydrated to the protonated imine form of the trigonal Schiff base 2 (5, 6). Proton abstraction of the C4 hydroxyl initiates a rearrangement resulting in cleavage of the substrate C3–C4 bond and enamine formation, shown as species 3, in the active site (7). Following α-glyceraldehyde 3-phosphate release, the enamine upon stereospecific protonation (8) forms a Schiff base and is released as DHAP by the inverse reaction sequence shown in Scheme I.

From crystallographic structure determination (9), the active site in rabbit muscle aldolase, shown in Fig. 1, contains a number of charged residues, vicinal to the Schiff base-forming Lys-229 (10), that can potentially participate in catalysis. These residues can mediate proton transfers as general acid/base catalysts and stabilize or destabilize charges, and because of their proximity to each other, they are susceptible to electrostatic modification of their pKₐ values, making role assignment of active site residues exceedingly complicated. Residues such as Glu-187, adjacent to Lys-229, have, on the basis of mutagenic, kinetic, and structural data, more than one mechanistic role that includes substrate cleavage, charge stabilization, and mediating proton transfers at the level of the ketimine intermediate (11). Other residues, such as Asp-33 and Lys-146, also have consequential roles in catalysis, since their catalytic activity is significantly compromised upon mutagenesis (12, 13, 14); the mutation Lys-146→Arg was shown to perturb substrate cleavage and Schiff base formation (13, 15). Asp-33 and Glu-187 are within hydrogen bonding distance of Lys-146 in...
ends of the may potentially perturb each other's p
participate in proton transfers and because of their mutual close proximity
The active site has a number of acidic and basic amino acids that can
...
within a tetramer was analyzed by the program Polypose (31) and tions to discover regions in the liganded structures that underwent structural elements were then used in all subsequent structure superposi-
differences, and placed at least 89% of nonglycine and nonproline residues of the four (0.190), 0.167 (0.204), and 0.167 (0.210), respectively. The correspond-
structure of the covalent complex formed by archaeal Thermoproteus aldolase. Superposition consisted of matching the atomic positions of Lys-229 N\textsubscript{e} and FBP carbon atoms C\textsubscript{3} and C\textsubscript{5} codes IZAH, IZAI, IZAJ, and IZAL, respectively. The final structure models of native aldolase and covalently bound FBP, MBP, and TBP noncovalent complexes have an R\textsubscript{cryst} (R\textsubscript{free}) of 0.167 (0.205), 0.155 (0.190), 0.167 (0.204), and 0.167 (0.210), respectively. The corresponding Luzzati atomic coordinate error was estimated at 0.18, 0.16, 0.19, and 0.19 Å, respectively. Ramachandran analysis with PROCHECK placed at least 89% of nonglycine and nonproline residues of the four structures in the most favorable region and with the remainder found in allowed and generously allowed regions, attesting to good model geometry in the structures. Errors in hydrogen bond distances, positional differences, and B-factors are reported as S.D. values and were estimated based on their value in each aldolase subunit.

**Structure Comparisons**—Superpositions were performed with the program PyMOL (22) using C\textsubscript{α} atom coordinates of identical blocks of amino acid sequences and comparing native with liganded aldolase tetramers. Due to conformational heterogeneity among subunits in N-terminal and C-terminal regions, comparisons were performed using residues 10–343. Root mean square (r.m.s.) deviations based on superposition of identical blocks of atom coordinates, that were less atom coordinates of identical blocks of amino acid sequences and comparing native with liganded aldolase tetramers. Due to conformational heterogeneity among subunits in N-terminal and C-terminal regions, comparisons were performed using residues 10–343. Root mean square (r.m.s.) deviations based on superposition of identical blocks of atom coordinates, that were less

| Data collection and refinement statistics | Native | FBP | MBP | TBP |
|-----------------------------------------|-------|-----|-----|-----|
| Data collection                          |       |     |     |     |
| Resolution (Å)                          | 50–1.80 | 50–1.76 | 20–1.89 | 50–1.89 |
| Wavelength (Å)                          | 0.9795 | 0.9795 | 0.9795 | 1.0000 |
| Reflections                              |       |     |     |     |
| Observed                                | 1,681,473 | 955,932 | 2,002,015 | 1,461,622 |
| Unique                                  | 127,047 | 134,970 | 104,042 | 110,492 |
| Completeness (%)                        | 97.0 (87.1) | 96.7 (78.9) | 96.9 (77.2) | 97.8 (85.8) |
| Average I/\(I_{free}\)                  | 22.5 (22.8) | 17.9 (20.2) | 12.6 (2.5) | 15.0 (2.1) |
| \(R_{cryst}\) (%)                       | 0.049 (0.25) | 0.069 (0.41) | 0.172 (0.78) | 0.070 (0.44) |
| \(R_{free}\) (%)                        | 22.5 (2.8) | 17.9 (2.0) | 12.6 (2.5) | 15.0 (2.1) |
| Space group                             | P\_2\_1 | P\_2\_1 | P\_2\_1 | P\_2\_1 |
| Unit cell parameters                     |       |     |     |     |
| \(a\ (Å)\)                               | 83.1 | 102.9 | 84.7 | 98.4 |
| \(b\ (Å)\)                               | 83.0 | 103.2 | 84.3 | 98.8 |
| \(c\ (Å)\)                               | 103.2 | 84.3 | 98.4 | 98.9 |
| \(\beta^\circ\)                          | 83.1 | 102.9 | 84.7 | 98.4 |

**Table I**

Conformational changes were identified on basis of differences in equivalent atomic positions by comparing liganded aldolases with the native enzyme. The comparison consisted of superimposing liganded tetramers onto the native tetramer using the same selected residues in each subunit. Values tabulated are r.m.s. differences between equivalent C\textsubscript{α} atoms and are given in Å.

| Residues selected | FBP | MBP | TBP |
|-------------------|-----|-----|-----|
| 10–343            | 0.44 | 0.46 | 0.28 |
| 158–259           | 0.12 | 0.14 | 0.13 |
| 34–65             | 1.01 | 0.95 | 0.61 |
| 302–329           | 0.80 | 0.91 | 0.49 |
| 35°               | 1.25 | 0.97 | 0.72 |
| 38°               | 0.64 | 0.50 | 0.21 |
| 303°              | 0.75 | 0.79 | 0.47 |

*Calculation of r.m.s. differences for these residues was based on superpositions of liganded complexes onto native enzyme that used exclusively residues 158–259, as described under ‘Materials and Methods.’

in the Schiff base structure of mammalian FBP aldolase against equivalent atoms in the target aldolase structures. In *E. coli* d-2-deoxyribose-5-phosphate aldolase (DERA) mutant structure (Protein Data Bank entry 1JCJ) (20), Lys-167 N\textsubscript{e} atom and DERA substrate C\textsubscript{1} and C\textsubscript{2} carbon atoms were formally equivalent to Lys-229 N\textsubscript{e}, FBP C\textsubscript{2}, and FBP C\textsubscript{3} atoms, respectively. In *E. coli* transaldolase B, equivalent atoms in the reduced Schiff base dihydroxyacetone analogue (Protein Data Bank entry 1UCW) (32) were Lys-133 N\textsubscript{e} and carbon atoms C\textsubscript{1}, C\textsubscript{2}, and C\textsubscript{3} of the dihydroxyacetone ligand, respectively. For the carbinolamine precursor formed with pyruvate in *E. coli* KDPG aldolase (Protein Data Bank entry 1EU4) (19), atoms equivalent to Lys-229 N\textsubscript{e}, FBP C\textsubscript{1}, and FBP C\textsubscript{2} were Lys-133 N\textsubscript{e} and pyruvate carbon atoms C\textsubscript{1} and C\textsubscript{2}. In the structure of the covalent complex formed by archaeal *Thermoproteus tenax* FBP class I aldolase with DHAP (Protein Data Bank entry 10K4)
(33), equivalent atoms were Lys-177 Nζ and carbon atoms C1, C2, and C3 of the DHAP ligand. Superposition of the structure of S. pyogenes TBP class I aldolase used 166 residues homologous with rabbit muscle FBP aldolase and yielded an r.m.s. deviation of 1.64 Å based on Cα atoms, as determined by the program DeepView/Swiss-PdbViewer (34). Moreover, the carboxylamine intermediate of the native DER (PDB entry 1JCL) (20) was structurally identical to the Schiff base intermediate in the DER mutant structure, (r.m.s. deviation = 0.14 Å using all Cα atoms), allowing comparison to be made solely among native liganded structures.

**RESULTS AND DISCUSSION**

**Schiff Base**—Flash freezing of rabbit muscle aldolase crystals in the presence of the substrate trapped a covalent complex in the active site under equilibrium conditions. Continuous electron density, extending beyond Lys-229 Nζ in each subunit, shown in Fig. 2A, indicates formation of a stable covalent adduct with FBP. The planar shape of the electron density observed about the FBP C1 carbon indicates trigonal hybridization and is consistent with trapping of a Schiff base intermediate in each aldolase subunit. Comparison of average B-factors between bound FBP and interacting side chains, 25.0 ± 2.8 and 20.0 ± 6.0 Å², respectively, suggests nearly full active site occupancy by FBP. Furthermore, the conformation of the crystallized enzyme is not inconsistent with that of a catalytically active conformer. Within measurement errors, kinetic parameters of soluble rabbit muscle aldolase (not shown) were unaffected by crystallization buffer, precipitant concentration used for crystallization, or glycerol cryoprotectant, and full activity was recovered upon dissolution of the crystalline enzyme.

The Schiff base intermediate, shown in Fig. 2B, engages in numerous hydrogen bonding and electrostatic interactions with active site residues. The binding mode by the P1 phosphate was isomorphous with that reported for the NaBH₄ reduced covalent complex with DHAP (Protein Data Bank entry 1J4E) (18) (r.m.s. deviation = 0.14 Å based on equivalent Cα atoms) wherein Arg-303 curls around and interacts electrostatically with the oxyanion, creating a phosphate oxyanion binding pocket. In addition to the electrostatic interactions, five hydrogen bonding interactions were made with the P1 phosphate oxyanion in the binding pocket including an unusually short hydrogen bond between Ser-271 side chain and the oxyanion (2.45 ± 0.02 Å) and indicating strong active site attachment by the FBP P1 phosphate oxyanion. The P6 phosphate binding site includes active site residue Lys-107 whose participation in P6 phosphate binding is corroborated from affinity labeling of Lys-107 by pyridoxal-P that abrogated FBP binding but not DHAP (35). Although both P1 and P6 phosphate oxyanions make two electrostatic interactions with respective residues, binding by the P6 phosphate oxyanion is slightly weaker, since it participates in only three additional hydrogen bonding interactions.

Hydrogen bonding by C2 and C4 hydroxyls is very strong involving interactions with cationic or anionic active site residues (36). Notable among ketohexose interactions are hydrogen bonds made between the FBP C3 hydroxyl and residues Lys-146 and Glu-187 shown in Fig. 2A and suggests incipient C2-C4 bond cleavage. The hydrogen-bonding pattern is consistent with Glu-187 abstracting the C4 hydroxyl proton as general base, thereby initiating cleavage of the C3–C4 bond in FBP and corroboration of the interpretation of enzymological data (11). Interestingly, the atoms of Lys-229 Nζ, FBP C2, C3, C4, and O4 as well as a Glu-187 carboxylate oxygen forms a near chair-like structure in Fig. 2A that mimics the spatial arrangement of reactants in the pericyclic transition state of nonenzymatic aldol condensations with preformed enolates (37, 38, 39). A salient feature is the observed noncoplanarity of the FBP C4 atom with respect to the Schiff base demanded in the transition state with respect to atoms about the cleaved bond.

The possibility of Lys-146 acting as general base is unlikely, since aldolase is active at pH 8.5 but not at pH 6.5 (40) suggests an alkaline pKₐ for Lys-146. Rather, Lys-146 in its ammonium form stabilizes by electrostatic interaction the resultant negative charge created on the C4 hydroxyl ion in the transition state. Loss of the positive charge on Lys-146 would thus be critical and severely compromise activity and is consistent with acidic and neutral Lys-146 mutations that abrogate catalytic activity (13, 14). Asp-33 interacts with both FBP C3 hydroxyl and Lys-146 stabilizes FBP binding and the positive charge on Lys-146 in the active site. The negative charge on Asp-33 is reciprocally stabilized by Lys-107 at the active site periphery. Charge stabilization by Asp-33 appears to be important for aldolase catalysis, since neutral mutations of Asp-33 result in a significant loss of activity (12, 14).

**Carboxylamine Intermediate**—The electron density in Fig. 3A shows formation of a stable noncovalent hexose-P₂ adduct in
the rabbit muscle aldolase active site. The nonplanar shape of the electron density about the C_2 atom of the hexose-P_2 indicates tetrahedral hybridization and is consistent with trapping of (2R)-MBP in each aldolase subunit. Binding by the (2R)-HBP stereoisomer implies stereospecific active site recognition. The (2S)-GBP stereoisomer is discriminated against, since other binding modes were not observed in electron density maps. The quite similar B-factors for MBP and interacting active site residues, 23.1 ± 4.8 and 16.1 ± 5.9 Å^2, respectively, are consistent with full active site occupancy by MBP. Different from the substrate-bound enzyme, Glu-187 forms a hydrogen bond with the MBP C_2 hydroxyl and lengthens its previous hydrogen bond to make a close contact with the C_4 hydroxyl (3.4 Å). In the MBP complex, Lys-229 N_z atom is positioned perpendicular to the plane defined by MBP atoms C_1, O_2, C_3 and is 3.3 Å from C_2, consistent with face si nucleophilic attack on the substrate ketose and predicts formation of the (2R)-carbinolamine intermediate obtained upon NaBH_4 reduction of the Schiff base (41). The potent inhibition observed for HBP (K_i = 0.45 μM (42)) consequently arises from the bound (2R)-MBP configuration mimicking the transient carbinolamine precursor 1 in FBP aldolase and acts as a transition state analogue by maximizing its active site interactions. The presence of a hydrogen bond between the MBP O_2 and Glu-187, shown in Fig. 3A, suggests a role by Glu-187 in mediating proton transfers at the level of the carbinolamine precursor, enabling stabilization of the carbinolamine transition state.

Superposition of FBP and MBP structures (r.m.s. deviation = 0.13 Å for C atoms of residues 10–343) indicates identical interactions made by the ligands with active site residues and is shown in Fig. 3B. Although binding by the P_1 phosphate oxyanion is virtually indistinguishable in both structures (0.3 ± 0.1 Å) including the same strong hydroxyl bond made by Ser-271 with the oxyanion (2.50 ± 0.05 Å in MBP), positioning of the atoms from the C_3 hydroxyl to the P_6 phosphate oxyanion is not. The MBP C_2 carbon, because of its sp^3 hybridization geometry, expands the distance between the P_1 and P_6 phosphates to 9.6 Å from 8.9 Å in the Schiff base. As a result, the positions of equivalent atoms in MBP from the C_3 hydroxyl to the P_6 phosphate oxyanion are each shifted by 0.9 ± 0.1 Å with respect to FBP in the direction of the P_6 phosphate binding locus. Active site residues in contact with FBP and MBP exhibit only slight conformational differences (r.m.s. deviation = 0.3 Å, including side chains).

Binding to a rigid active site affords a structural basis for stereoisomer selectivity in rabbit muscle aldolase. Attachment by each stereoisomer to the same binding site would not be identical and would entail differences in binding affinity that are reflected by preferential binding of MBP instead of GBP. Indeed, the reduced affinity for GBP by aldolase (K_i = 12 μM (43)) originates from a binding mode different from MBP, since isostructural binding by GBP would result in steric conflict between its C_2 hydroxyl and Glu-187 and Lys-229 side chains.

Substrate Binding—To further probe the role of Glu-187 in mediating carbinolamine formation, the acyclic form of FBP was modeled into the aldolase active site using the MBP noncovalent complex as template. To mimic incipient Schiff base formation, Lys-229 was modeled as its nucleophilic form. To maximize hydrogen-bonding potential with the attacked carbonyl, the acid form of Glu-187 was used. Slight torsional oscillation of the Glu-187 side chain in the native structure would place the carboxylate within hydrogen bonding distance of Lys-229, allowing it to accept a proton from Lys-229 and thereby making it acidic. Equally, protonation of Glu-187 can occur by a proton relay implicating the adjacent Glu-189 at the active site periphery (11).

![Fig. 3. A, electron density showing competitive inhibitor MBP (2R)-HBP) bound in the rabbit muscle aldolase active site. MBP mimics the carbinolamine precursor of the Schiff base. Glu-187 hydrogen-bonds MBP O_2 (green dash). Difference electron density calculated from a 1.9-Å annealed F_o - F_c omit map encompassing MBP and contoured at 3σ. The view is as in Fig. 1. B, detailed comparison of the FBP Schiff base and MBP-bound aldolase structures. Superposition was done with PyMOL using averaged subunits as described under “Materials and Methods.” Yellow and cyan colors were used to depict the structures corresponding to the FBP Schiff base and MBP, respectively. The P_1 phosphate of each ligand occupies the same binding locus. Differences were observed at the C_2 atom due to its different hybridization states and from atoms of the C_3 carbon to the P_6 oxyanion. Except for a small difference in P_6 phosphate bonding loci, reflected by a relative shift in the α-helix containing residues Ser-35 and Ser-38, positional differences by all other active site residues contacting FBP and MBP were nominal.](http://www.jbc.org/)

![Fig. 4. Acyclic form of FBP docked in the active site and superposition with MBP bound structure.](http://www.jbc.org/)
as well as to the C2 carbonyl, not possible as conjugate base, and corroborates Glu-187 participation in proton transfers at the level of carbinolamine precursor formation as well as in aligning the substrate ketose for nucleophilic attack.

**Ligand Recognition**—Surprisingly, TBP was not recognized as a substrate by rabbit muscle aldolase. $K_i$ determined on the basis of competitive inhibition of aldolase by TBP (125 ± 16 μM) is comparatively weaker than $K_i$ for FBP (5 ± 1 μM) and $K_i$ of HBP (0.45 μM), determined previously (42). Weaker yet similar TBP binding is reflected in the structural analysis, where partial occupancy of the P1 and P6 phosphate binding loci was observed at saturating TBP levels in the electron density maps (see Fig. 5). Partial occupancy of both phosphate binding loci nevertheless suggests that the enzyme can interact with the acyclic form of TBP but not apparently in a unique manner with the intervening ketohexose. Controls showed no evidence for binding by P1 at 500 μM in native crystals, a concentration greater than 180 μM that was present in the TBP soaking solution (data not shown).

Modeling of (4S)-TBP bound in the active site using either the noncovalent or covalent FBP complex as template resulted in identical docking by TBP except for the enantiomeric C4 hydroxyl of TBP that did not interact with any active site residue. The dissimilar binding mode observed for the TBP diastereoisomer in the crystal structure implies stereospecific discrimination by the enzyme at the level of the substrate C4 hydroxyl. The C4 hydroxyl makes strong hydrogen bonding interactions with Lys-146 and Glu-187 in case of FBP that are not possible for TBP if TBP was to bind isostructurally. Loss of these strong interactions thus induces a different binding mode.

TBP binding suggests a hierarchical mode of attachment by aldolase ligands, which arises from the constraints imposed by a rigid binding site. Partial binding by TBP phosphates suggests promiscuous attachment by oxyanions of bisphosphorylated structural analogues whenever the interatomic distance between the analogue phosphates matches the distance between the enzyme’s P1 and P6 binding sites and without remaining analogue atoms introducing structural clashes. Strong oxyanion binding, particularly charged interactions made with Lys-107 and Arg-303, would assure phosphate binding independent of whether weaker interactions such as hydrogen bonding with hydroxyls have been satisfied. Such reduced ligand binding specificity rationalizes the similar micromolar inhibition constants of diverse bisphosphorylated compounds, many of which are structural analogues of FBP (43), yet on basis of their structures cannot interact identically with the active site.

**Induced Fit**—Active site binding induces identical conformational changes in each aldolase subunit with respect to native enzyme. Backbone displacement due to ligand interaction is localized to two adjacent α-helical structures (residues 34–65 and residues 302–329) not involved in subunit contacts and comprising the wall of the active site cleft distant from subunit contacts, shown in Fig. 6. From Table II, conformational displacements of these helical regions are small yet significant when compared with residues 158–259 of the β-barrel, which make up part of the active site and intersubunit contacts and results in the asymmetric narrowing of the active site cleft.

Superposition of the native and the FBP bound structures are shown in Fig. 7 and delineates a conserved network of interacting active site residues and water molecules, present in all structures, that is used to bind ligand phosphate moieties as well as C3, C4, and C5 hydroxyls. Only positions of five residues (Ser-35, Ser-38, Arg-42, Gly-302, Arg-303), each participating in phosphate binding, are displaced by the conformational change that narrows the active site cleft. Arg-303, whose side chain projects out of the active site in the native enzyme, reorganizes and curls binding the FBP P1 phosphate. The Arg-303 side chain conformation is stabilized by a hydrogen bond to a water molecule, shown in Fig. 7, in each subunit, which in turn interacts with the Glu-189 side chain on the opposite wall of the active site cleft. The water molecule-mediated interaction is only possible due to the backbone displacement of Arg-
303 (–0.8 Å in Table II) with respect to the native enzyme. This water molecule is invariant and is part of three water molecules shown in Fig. 7, conserved in native and liganded structures that comprise the P1 phosphate binding locus. The change in Arg-303 side chain conformation induces a flip in the backbone carbonyl of Gly-272 to avoid steric collision that further stabilizes P1 phosphate attachment through hydrogen bonding with the Gly-272 backbone nitrogen, shown in Fig. 2B. The same backbone displacement further enhances P1 phosphate attachment by promoting oxyanion hydrogen bonding with Gly-302.

P6 phosphate binding induces a slightly larger conformational change that involves the α-helix containing residues Ser-35 and Ser-36 (~1 Å in Table II) and enabling these residues to hydrogen bond the P6 phosphate oxyanion. Arg-42 undergoes a likewise displacement allowing its side chain to interact with a water molecule bound by both C6 hydroxyl and P1 phosphate. Homologous conformational changes are observed upon MBP binding, shown in Table II, indicating that substrate binding and not covalent complex formation that induces the conformational changes. Furthermore, TBP binding induces similar conformational changes. From Table II, their extents are smaller and consistent with partial occupancy of the P1 and P6 phosphate binding sites by TBP. The absence of significant conformational changes by all other active site residues (r.m.s. change in position of active site atoms contacting FBP or MBP = 0.4 ± 0.1 Å) corroborates the interpretation of a rigid active site that does not adapt to binding events except by large scale movement of secondary structures.

Water Molecules—MBP binding and Schiff base formation both displace 14 water molecules, shown in Fig. 7. The average B-factor for the water molecules ejected from the native structure upon FBP and MBP binding was 37 ± 10 Å², and was not significantly different from B-factors averaged over all water molecules, 42 ± 15 Å². The absence of significant difference in positional disorder of ejected water molecules precludes entropic gain due to solvent displacement into the nearest solvation shell as a significant factor in promoting ligand binding. Furthermore, no water molecule was observed within a van der Waals distance of the FBP C6 carbon in the Schiff base. The water molecule closest is 4.3 Å removed (orange dash shown in Fig. 7) and forms part of the network of water molecules conserved in all structures. This water molecule, which hydrogen-bonds Glu-189 and the P1 oxyanion in Fig. 7, also makes a hydrogen bond to the C2 hydroxyl in the MBP structure and suggests a candidate binding site for the water molecule expelled upon carbinolamine dehydration. The absence of a water molecule binding site within close contact of the C2 carbon in the Schiff base is not inconsistent with the stability of the covalent FBP enzyme intermediate in solution (3). Reduction in solvent translational mobility upon flash freezing (44) could promote further accumulation of the Schiff base population, not inconsistent with the apparent full occupancy by the FBP Schiff base intermediate in aldolase crystals.

Substrate Cleavage—A multiple catalytic role for Glu-187 in FBP aldolase catalysis was advanced from enzymological data that implicated the residue in C3–C4 bond cleavage as well as in proton transfers at the level of the ketimine intermediate (11). The structural analysis herein reveals Glu-187 participation in hydrogen bond formation with key reaction intermediates (7, 45). A catalytic mechanism by which to promote cleavage of the C–C bond in class I FBP aldolases integrating enzymology and structure data is outlined in Scheme II.

The catalytic cycle begins by FBP binding in the active site cleft. Substrate attachment induces a conformational adjustment in two α-helical regions that serves to stabilize attachment of the P1 and P6 phosphates and is accompanied by expulsion of water molecules. Glu-187, in its acid form, hydrogens-bonds both FBP C2 carbonyl and C5 hydroxyl, thereby aligning the electrophilic C6 carbon for face si attack by Lys-229, outlined in the first panel. As general acid, Glu-187 transiently stabilizes the resultant dipolar carbinolamine shown in the second panel (reaction a), and mediates stereoselective proton transfers (reaction b), that yield the neutral carbinolamine precursor 1. A second series of proton transfers catalyzed by Glu-187 as general acid dehydrates carbinolamine intermediate 1 and promotes Schiff base formation 2, reaction c. The Schiff base formed is particularly stable due to the absence of a water molecule-binding site within van der Waals distance of the FBP C6 carbon. Proton abstraction from the C4 hydroxyl is catalyzed by Glu-187, as conjugate base, which induces a rearrangement cleaving the C3–C4 bond, reaction d. The remaining active site residues are critical for substrate alignment in the active site and afford electrostatic stabilization of charges during proton transfers. The mechanism is consistent with a trajectory of least atomic motion requiring...
only slight torsional librations by Glu-187 glutamate side chain.

**Conserved Reaction Divergent Mechanism**—The reaction chemistry underlying substrate turnover has mechanistic features that are common to diverse class I aldolases. Substrate cleaved by mammalian FBP aldolase, DERA, transaldolase B, archaeal FBP aldolase, KDPG aldolase, and TBP aldolase implicates the same C–C bond relative to the ketose moiety, and Schiff base formation necessitates a lysine residue in each enzyme. In addition, substrates recognized by each aldolase have chemical structures similar if not identical to FBP. To examine potential homology among active site residues catalyzing Schiff base formation and substrate cleavage, structures of Schiff base intermediates from bacterial and archaeal aldolases were compared by superposition with mammalian FBP aldolase. In aldolases lacking structures of Schiff base intermediates derived from substrate, the Schiff base formed in mammalian FBP aldolase was used as surrogate substrate for analysis. The structure elements common to all aldolase substrates are FBP atoms starting at C2 and extending to the P6 oxyanion. Shown in Fig. 8 are superpositions with DERA, transaldolase B, archaeal FBP aldolase, and KDPG aldolase. In all structures, oxygen and nitrogen atoms are shown in red and blue, respectively. Superposition with TBP aldolase, not shown for visual clarity, has Glu-163 and Lys-205, coinciding respectively with Glu-187 and Lys-229 in mammalian FBP aldolase. The view is as in Fig. 1.

**Scheme 2.** A catalytic mechanism promoting cleavage of the C–C bond in class I FBP aldolases.

**Fig. 8.** Superposition of Schiff base intermediates from diverse class I aldolases. Comparison among class I aldolases was made at the level of the Schiff base intermediate by superposing equivalent atoms as described under “Materials and Methods.” The Schiff base intermediate of rabbit muscle aldolase is depicted in yellow, and shown are active site residues: Asp-33, Glu-187, Lys-229, and FBP. In cyan is illustrated the DERA carbinolamine intermediate formed with Lys-167 and D-2-deoxyribose-5-phosphate substrate (Protein Data Bank entry 1JCJ) as well as Lys-201 and a water molecule that has a central role in the catalytic mechanism of DERA. The DERA Schiff base mutant structure (Protein Data Bank entry 1JCL), corresponding to Lys-201 → Leu, is isostructural with carbinolamine intermediate including the DERA substrate, allowing interchangeable use of these structures in the superposition. The cyan dash illustrates the hydrogen bond observed between the water molecule and Lys-201 and the putative hydrogen bond made by the water molecule with FBP O4, which is coincident with the equivalent O hydrogen in the DERA substrate. Green depicts the covalent intermediate observed between Lys-132 of transaldolase B and its dihydroxyacetone substrate (Protein Data Bank entry 1UCW). Glu-96 hydrogen-bonds (green dash) a water molecule that is also within hydrogen bonding distance of FBP O. Also shown is Asp-17 of transaldolase B, homologous to Asp-33 in mammalian class I aldolase. In violet is shown the covalent adduct between DHAP and Lys-177 of KDPG aldolase (Protein Data Bank entry 1EUA). Tyr-146 hydrogen-bonds directly FBP O (violet dash), as observed for Glu-187 in the Schiff base complex of mammalian FBP aldolase. Asp-24 in the archaeal aldolase is nearly coincident with Asp-33 in mammalian FBP aldolase. In rose is shown the pyruvate carbinolamine adduct formed with Lys-133 in KDPG aldolase (Protein Data Bank entry 1KCI). Glu-45 hydrogen-bonds directly FBP O (rose dash). Phe-135 in KDPG aldolase is nearly coincident with Asp-17 of transaldolase B. In all structures, oxygen and nitrogen atoms are shown in red and blue, respectively. Superposition with TBP aldolase, not shown for visual clarity, has Glu-163 and Lys-205, coinciding respectively with Glu-187 and Lys-229 in mammalian FBP aldolase. The view is as in Fig. 1.
strate in superposition of the Schiff base intermediates.

Superposition aligned the C4 hydroxyl of each aldolase substrate facing a possible proton acceptor, namely a glutamate, a tyrosine, or a water molecule that in turn could be activated by a glutamate or lysine residue, shown in Fig. 8. In KDPG aldolase, the putative substrate O4 is predicted to make a hydrogen bonding interaction with Glu-45. For transaldolase B, C4 hydroxyl hydrogen bonding occurs with a water molecule, which in turn hydrogen-bonds Glu-96 and suggests in both instances that a glutamate residue is ultimately responsible for proton abstraction, hence substrate cleavage. In archaeal FBP aldolase, superposition is consistent with Tyr-146 as the residue mediating proton abstraction at the C4 hydroxyl, whereas in DERA, it is a water molecule activated by Lys-201.

Available data support the analysis and suggest additional participation in Schiff base formation by the catalytic entity facing the C4 hydroxyl. In KDPG aldolase, since Glu-45 is the only residue in the active site that is capable of acting as general acid/base catalyst (19), the superposition is entirely consistent with a multifunctional catalytic role by Glu-45 that includes Schiff base formation as well as C–C bond cleavage. In DERA, the water molecule that hydrogen-bonds both its substrate C4 hydroxyl and Lys-201 has a central role in all aspects of the reaction mechanism, including substrate cleavage and Schiff base formation (20). In transaldolase B, site-directed mutagenesis of Glu-45 perturbed cleavage activity (46), and Glu-96 was proposed on structural grounds to activate the Schiff base-forming aldolase in carbohydrate metabolism. A multifunctional catalytic role by Glu-45 that only residue in the active site that is capable of acting as general acid/base catalyst (21), p. 48, Springer-Verlag, New York

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Covalent Schiff Base Intermediate in Rabbit Muscle Aldolase
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