Induced Fit Movements and Metal Cofactor Selectivity of Class II Aldolases

STRUCTURE OF THERMUS AQUATICUS FRUCTOSE-1,6-BISPHOSPHATE ALDOLASE*

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Fructose-1,6-bisphosphate (FBP) aldolase is an essential glycolytic enzyme that reversibly cleaves its ketohexose substrate into triose phosphates. Here we report the crystal structure of a metallo-dependent or class II FBP aldolase from an extreme thermophile, Thermus aquaticus (Taq). The quaternary structure reveals a tetramer composed of two dimers related by a 2-fold axis. Taq FBP aldolase subunits exhibit two distinct conformational states corresponding to loop regions that are in either open or closed position with respect to the active site. Loop closure remodels the disposition of chelating active site histidine residues. In subunits corresponding to the open conformation, the metal cofactor, Co$^{2+}$, is sequestered in the active site, whereas for subunits in the closed conformation, the metal cation exchanges between two mutually exclusive binding loci, corresponding to a site at the active site surface and an interior site vicinal to the metal-binding site in the open conformation. Cofactor site exchange is mediated by rotations of the chelating histidine side chains that are coupled to the prior conformational change of loop closure. Sulfate anions are consistent with the location of the phosphate-binding sites of the FBP substrate and determine not only the previously unknown second phosphate-binding site but also provide a mechanism that regulates loop closure during catalysis. Modeling of FBP substrate into the active site is consistent with binding by the acyclic keto form, a minor solution species, and with the metal cofactor mediating keto bond polarization. The Taq FBP aldolase structure suggests a structural basis for different metal cofactor specificity than in Escherichia coli FBP aldolase structures, and we discuss its potential role during catalysis. Comparison with the E. coli structure also indicates a structural basis for thermostability by Taq FBP aldolase.

Aldolases are essential enzymes that catalyze carbon-carbon bond formation in living organisms. They are ubiquitous and highly abundant in pathways of intermediate cellular metabolism such as gluconeogenesis, the Calvin cycle, and glycolysis, where they reversibly cleave ketohexose sugars. In synthetic chemistry, the action of aldolases is precisely controlled by the stereochemistry of these reactions, and thus these enzymes are often used as an alternative to conventional chemical methods in biotransformations and synthetic organic chemistry (1, 2) and especially in the synthesis of novel antibiotics (3, 4).

Aldolases that cleave ketohexose substrates are among the most studied enzymes and, depending on their reaction mechanism, fall into two distinct groups. The class I enzymes utilize a lysine in Schiff base formation during catalysis and are mainly found in higher order organisms. Determination of the crystal structures of several class I enzymes (5–12) together with biochemical studies (13–19) have provided mechanistic details for ligand recognition and catalysis in class I aldolases. Structurally, these aldolases display an ($\alpha\beta$)$_4$ barrel in a homotetrameric arrangement. In contrast, class II enzymes, found in yeast, bacteria, fungi, and blue-green algae, are most often homodimeric ($\alpha\beta$)$_2$, barrels (20, 21) and require for catalysis a divalent metal cation, typically a transition metal such as Zn$^{2+}$. The divalent cation functions as a Lewis acid to polarize the carbonyl bond of the incoming ketoses, thereby promoting cleavage of the adjacent carbon-carbon bond as well as proton transfer during enamine formation. Class II aldolases are activated by monovalent cations, such as NH$_4^+$, are generally more stable than their class I counterparts, exhibit a wide range of substrate specificity, and are preferred for use in biotransformation chemistry (22, 23). Their reaction mechanisms are diverse. For instance, in one class II enzyme, 2-dehydro-3-deoxy-galactarate aldolase, a phosphate anion rather than an amino acid side chain mediates proton transfer during enamine formation (24). Chiral discrimination among class II aldolases is subtle, and in the case of the stereoisomers fructose-1,6-bisphosphate and tagatose-1,6-bisphosphate, recognition and turnover depend on fine details of active site interactions made with substrate (25). Class II aldolases also represent potential targets for the development of anti-bacterial and anti-fungal drugs because they almost exclusively belong to prokaryotes, yeasts, and lower order eukaryotes.

Among class II aldolases, FBP$^\dagger$ aldolase (E.C. 4.1.2.1) has been extensively characterized because of its important metabolic role in intermediate metabolism. The enzyme catalyzes the reversible aldol cleavage of FBP to the triose phosphates, dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate.

These abbreviations used are: FBP, d-fructose 1,6-bisphosphate; SeMet, seleno-l-methionine; Taq, Thermus aquaticus.

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Substrate cleavage occurs during glycolysis, and the reverse reaction, aldol condensation, is used during gluconeogenesis or the Calvin cycle. Binding sites corresponding to both the catalytic divalent metal ion as well as the activation site of the monovalent cation were identified in the high resolution FBP aldolase crystal structure from *Escherichia coli* (21). More recently, the crystal structures of *E. coli* FBP aldolase crystal structure in complex with a triose phosphate transition state analogue suggested structural features involved in substrate recognition and processing (26). One important issue that remained unresolved was apparent induced fit movements that class II FBP aldolases undergo during the catalytic cycle and their relationship with active site binding. To address conformational changes during catalysis, the crystal structure of class II FBP aldolase was determined to 2.3 Å Bragg spacing from the extreme thermophile *Thermus aquaticus*, successfully crystallized in the presence of sulfate, a phosphate anion analogue. The resultant crystal structure not only defined the role of the induced conformational changes during the catalytic cycle but also provided an explanation as to the metal cofactor affinity by *Taq* FBP aldolase for Co$^{2+}$, rather than Zn$^{2+}$, which is preferred in mesophiles such as *E. coli*.

**MATERIALS AND METHODS**

**Structure Determination**—Native crystals of *Taq* FBP aldolase were grown as described (27) by vapor diffusion using sitting drops made up of 8.75 μl of protein at 1.75 mg ml$^{-1}$, 1 μl of 25% sucrose monolaurate, and 5 μl of the precipitant solution containing 1.7 M ammonium sulfate, 0.1 M Tris-HCl, pH 7.5, and 10 μl CaCl$_2$ that were then equilibrated against 1 ml of the precipitant solution at 295 K. The crystals harvested were cryoprotected in mother liquor supplemented with 0.6 M ammonium sulfate and 20 mM citric acid, pH 4, that was equilibrated against 1 ml of the precipitant solution at 295 K. The crystals were cryoprotected in mother liquor supplemented with 15% glycerol before freezing in liquid nitrogen. The crystals of the selenomethionine (SeMet) isoform of *Taq* FBP aldolase were obtained from hanging drops (27) that were made up of 5 μl of protein solution containing 0.25 mg ml$^{-1}$ of *Taq* FBP aldolase and 2 μl of the precipitant solution made of 0.6 M ammonium sulfate and 20 mM citric acid, pH 4, that was equilibrated against 1 ml of the precipitant solution at 295 K. The crystals were cryoprotected in mother liquor supplemented with 15% glycerol before freezing in liquid nitrogen.

X-ray data collection for the SeMet isoform of *Taq* FBP aldolase and the native protein in complex with cobalt has been described previously (27) and is summarized in Table I. The structure solution strategy consisted of determining the structure of the SeMet isoform, crystallized in tetragonal space group $I_4_1$ ($a = b = 88.6$ Å, $c = 164.1$ Å), and then using it to solve by molecular replacement the native structure, crystallized in monoclinic space group $P2_1$ ($a = 99.5$ Å, $b = 57.5$ Å, $c = 138.6$ Å, $\beta = 90.25$°). Multiple anomalous dispersion (MAD) data were scaled together with the CCP4 (28) program SCAL.EEFT. Twelve of the fourteen selenium sites expected in the asymmetric unit were determined using SOLVE (29). The N-terminal SeMet was not found, probably because of positional disorder. Initial phases were calculated with the program MLPHARE (30) and were improved by 200 cycles of solvent flattening and gradual phase extension from 3.65 to 2.8 Å resolution using the program DM (28). The final $R$ factor for the phase extension at 2.8 Å resolution was 0.347. The selenium positions allowed unambiguous matching of the electron density to the sequence and construction of an atomic model by using the program O (31). The modest resolution and apparent mobility of the two loop regions (residues 134–152 and 175–190), closing over the active site, made electron density tracing challenging.

Structure determination was substantially aided by the higher diffracting native enzyme x-ray data. The preliminary SeMet model was successfully used as a search model to determine the native *Taq* FBP aldolase monoclinic crystal structure by molecular replacement using the program AMoRe (28). Structure solution revealed two half-tetramers in the asymmetric unit cell of the native enzyme, and except for the modest resolution and apparent mobility of the two loop regions (residues 134–152 and 175–190), closing over the active site, made electron density tracing challenging.

**Crystalllographic data statistics**

| Data collection | Wavelength (Å) | Resolution (Å) | Total data | Space group | Unique data | Redundancy | Overall completeness | Completeness (last shell) | F' > 3σ(F) | Rmerge (overall) | Rmerge (last shell) |
|-----------------|----------------|----------------|------------|-------------|-------------|------------|---------------------|-------------------------|------------|----------------|-----------------|
|                 | 0.9791         | 2.8            | 387,740    | $I_4_1$     | 14,208      | 27.3       | 0.912               | 0.962                   | 0.859      | 0.092          | 0.408           |
|                 | 0.9783         | 2.8            | 390,048    | $I_4_1$     | 14,078      | 27.7       | 0.911               | 0.960                   | 0.860      | 0.088          | 0.367           |
|                 | 2.83           | 2.8            | 396,141    | $I_4_1$     | 14,065      | 28.2       | 0.908               | 0.960                   | 0.851      | 0.088          | 0.367           |
|                 | 2.33           | 2.3            | 389,324    | $P2_1$      | 14,105      | 28.2       | 0.977               | 0.980                   | 0.851      | 0.075          | 0.195           |
|                 | 2.00           | 2.0            | 391,215    | $P2_1$      | 14,105      | 28.2       | 0.928               | 0.928                   | 0.851      | 0.067          | 0.195           |

| Crystallographic refinement statistics | Co$^{2+}$ | Native |
|---------------------------------------|-----------|--------|
| No. of reflections                    | 68,519    | 98,502 |
| Final model parameters                | 1.225     | 1.226  |
| No. of protein atoms                  | 9,192     | 9,272  |
| No. of solvent molecules              | 933       | 974    |
| Resolution range (Å)                  | 40.4–2.3  | 40.6–2.3 |
| Last shell (Å)                        | 2.3–2.44  | 2.13   |
| R factor (overall)$^a$                 | 0.211     | 0.252  |
| R factor (last shell)$^a$              | 0.265     | 0.358  |
| Rmerge (overall)$^a$                   | 0.252     | 0.259  |
| Rmerge (last shell)$^a$                | 0.303     | 0.373  |
| Average main chain B factor (Å$^2$)   | 33.25     | 30.45  |
| Average side chain B factor (Å$^2$)   | 33.77     | 31.31  |
| Average water molecule B factor (Å$^2$)| 42.73     | 39.43  |
| Root mean square deviation from ideal geometry |
| Covalent bond lengths (Å)             | 0.006     | 0.006  |
| Bond angles (°)                       | 1.2       | 1.2    |

$^a$ R = Σ|Fobs| - |Fcalc|/Σ|Fcalc| where N represents the number of equivalent reflections, and hkl represents the total number of unique Bragg reflections.

$^b$ Rmerge represents the $R$ factor calculated for a test data set randomly selected from the observed reflections prior to refinement. The test data set contained 5% of the total observed data and was not used throughout refinement.
residues 134–151, 180–187, and 230–231 in the open conformer. The half-tetramer having all subunits in their open conformation density was weakest for residues 146–148, 182–187, and 229–231 in one subunit and 183 and 230 in the other subunit. The temperature factors of these residues refined to ~40–50 Å² only with the occupancy set to 0.5. The electron density was also weak for one of the two sulfate anions bound to the active site of all three protomers in their open conformation, and their occupancy was set to 0.5. Water molecules were initially identified in the Fo–Fc maps and screened for reasonable geometry and refined thermal factor <80 Å². The tables show the overall crystallographic R factor and the free R factor for all observed reflections within the indicated resolution range. A Ramachandran plot analysis by the program PROCHECK (28) indicates that 90.4% of all residues lie in most favorable regions, and 9.6% lie in additional allowed regions. The structure analysis also showed that all stereochemical parameters are better than expected at the given resolution. A Luzzati plot indicated a 0.33 Å error in the atomic coordinates.

**Substrate Modeling**—The acyclic conformation of FBP (atomic coordinates from Protein Data Bank entry 1FDJ) was modeled into the active site of the refined structure by superposing the phosphate residues located on the sulfate-binding sites. The substrate was oriented in the active site such that its C1-phosphate coincided with the fully bound sulfate anion in the closed conformation and keto oxygen oriented toward the metal cation. In this orientation, the C2-phosphate could be readily superimposed onto the second sulfate-binding site. 200 cycles of coordinate energy minimization were then performed at 300 K using CNS (version 1.1) without the x-ray term to relax possible bad contacts introduced by substrate modeling into the active site. The coordinates of the protein and the substrate did not deviate significantly after minimization compared with the starting coordinates.

**RESULTS**

**Structure of the Taq FBP Aldolase Protomer**—The structure of the Taq FBP aldolase protomer (subunit molecular mass of 33 kDa) adopts an (α/β)8 barrel fold (Fig. 1). The dimensions of the protomer are ~70 Å in height and 45 Å in width, with a depth of 38 Å. The barrel is closed on its N-terminal end by an α-helix (α8) comprising residues 5–14. The core of the structure consists of an eight-stranded parallel β-strand assembly (strands labeled β1–β8) and each β-strand is accompanied by an α-helix. In addition to the eight times repeated β-strand-loop-α-helix-loop motif, the structure also contains a α-helix directly following a β-strand, in the case of β2 (labeled α-helix α2a), β3 (labeled α-helix α3a), and β8 (labeled α-helix α8a). Furthermore, the two C-terminal α-helices, αm and αn, are anti-parallel to each other and create an arm from the barrel that mediates oligomerization. Within each protomer, 27 pairs of residues are involved in electrostatic interactions.

**Quaternary Structure**—Class II Taq FBP aldolase behaves as a homotetramer in solution with a molecular mass of 139 kDa (35) consistent with point group 222 (Fig. 2). The mutually perpendicular molecular dyads are defined as a right-handed set of axes P, Q, and R, where the P dyad is the crystallographic 2-fold axis, whereas the Q and R dyads are the local 2-fold axes. The dimensions of the tetramer are ~103 Å in height (along the R axis), 91 Å in width (along the P axis), and 83 Å in depth (along the Q axis). Each protomer is in contact with the other three subunits within the tetramer. The intersubunit interactions across the Q dyad are more extensive than those across the R dyad (Fig. 2A). As a consequence, the former interface buries about three times as large a surface area (1844 Å² buried per subunit, representing 27% of total surface area) as the latter (582 Å² buried per subunit, or 9% of total surface area) upon tetramerization, whereas the interactions between the P axis-related subunits are minor (386 Å² per subunit, 6% of total surface; Fig. 2B). This arrangement results in a dimer of dimers within the homotetramer.

The interdimer interactions across the R dyad involve residues located on α-helices α2, α3, and α4, as well as those in the loop regions following α-helices α3 and α4. Dimer-dimer interactions implicate seven residues (Leu, Arg, Arg, Gly, and others).
across the Q the /H9251 11828, while in P the three 2-fold axes of lactate dehydrogenase (46). In A and R looking down the molecular dyad (\( R \)). The dyads (\( R \) and Q in A and P and Q in B) are indicated by solid lines. Each protomer is shown in a different color.

Fig. 2. A cartoon drawing of the FBP aldolase oligomer with point group 222. The three different molecular dyads comprise a right-handed orthogonal set of axes P, Q, and R as originally defined for the three 2-fold axes of lactate dehydrogenase (46). In A, the view is looking down the crystallographic dyad (\( P \)), while in B the orientation is looking down the molecular dyad (\( R \)). The dyads (\( R \) and Q in A and P and Q in B) are indicated by solid lines. Each protomer is shown in a different color.

Ala\(^{121}\), Val\(^{125}\), and Val\(^{127}\) that participate in hydrophobic interactions and four residues (Tyr\(^{26}\), Leu\(^{92}\), Arg\(^{86}\), and Phe\(^{89}\)) that promote interdimer hydrogen bonds.

More extensive and predominantly hydrophobic interactions are formed between Q axis-related subunits. Several secondary structural elements contribute up to 40 residues that participate in intersubunit (intradimer) van der Waals’ contacts across the Q dyad. These contacts involve residues located on the \( \alpha \)-helices \( \alpha_1, \alpha_2, \alpha_{2a}, \alpha_7, \alpha_{8a}, \) and \( \alpha_8 \), as well as those located on the loops following \( \alpha \)-helices \( \alpha_1, \alpha_{6a}, \) and \( \alpha_6 \). This interface implicates 27 residues that participate in hydrophobic interactions, 12 residues (Asn\(^{25}\), Glu\(^{28}\), Tyr\(^{25}\), Gly\(^{26}\), Arg\(^{28}\), Thr\(^{26}\), Arg\(^{25}\), Pro\(^{27}\), Phe\(^{27}\), Arg\(^{27}\), Tyr\(^{28}\), and Arg\(^{25}\)) that promote 19 intersubunit hydrogen bonds, and three electrostatic interactions between residues Glu\(^{70}\) and Arg\(^{86}\), Glu\(^{28}\), and Lys\(^{27}\), and Asp\(^{27}\) and Arg\(^{25}\). Only one electrostatic interaction is observed across the crystallographic related subunits, between residues Glu\(^{7}\) and Arg\(^{27}\). Finally, some hydrophobic interactions are also formed across the \( P \) axis-related subunits involving residues Met\(^{1}\), Glu\(^{2}\), Glu\(^{2}\), Ala\(^{7}\), Glu\(^{2}\), and Arg\(^{7}\).

Open and Closed Conformations—\( \text{Taq} \) FBP aldolase crystallizes using ammonium sulfate as the precipitating agent at physiological pH in space group P2 or at pH 4 in space group \( \text{I} \). The SeMet-substituted protein crystals are tetragonal, with two protomers in the asymmetric unit and diffraction to 2.8 Å Bragg spacing. Diffraction to 2.3 Å Bragg spacing was observed from native \( \text{Taq} \) FBP aldolase monodisperse crystals with four protomers in the asymmetric unit. The two protomers in the tetragonal cell are in the closed conformation, which contains one small loop (residues 175–190) and one large loop (residues 134–152) that close over the active site. These two loop regions show that weak electron density and residues 138–149 and 176–188 are disordered in the SeMet model. In contrast, native monoclinic protein crystals in the presence of the cofactor Co\(^{2+}\) comprise one protomer in the closed conformation shown in Fig. 3B, with continuous electron density for residues 1–139 and 148–305 and three protomers in the open conformation having electron density for all 305 residues shown in Fig. 3A. Application of the crystallographic 2-fold symmetry thus generates one tetramer having all four protomers in the open conformation and one tetramer with two protomers in the open conformation and two protomers in the closed conformation.

Cobalt in the Active Site—The catalytic cobalt in \( \text{Taq} \) FBP aldolase was identified based on its coordination and peak size in \( F_o - F_c \) electron density maps. During refinement, difference Fourier electron density maps were calculated from models comprising all atoms except those subsequently identified as cobalt cations. The three strongest peaks in the difference electron density maps were 24–28 times over the noise level. These three peaks are the best candidates for the binding sites of the catalytic Co\(^{2+}\) in the three subunits in their open conformation within the asymmetric unit. Interestingly, the protomer in the closed conformation has two mutually exclusive binding sites for cobalt metal cations (Fig. 4A). This dual binding is mediated by a conformational transition involving side chain rotations that occur following chelation by histidine residues 81, 178, and 208 (Fig. 4A). Because these two mutually exclusive Co\(^{2+}\) cations are only partially occupied, their corresponding peaks in the difference electron density maps are only half the height (≈14 times the noise level) of that of the single cobalt ion having full occupancy found in the other three subunits within the asymmetric unit that display the open conformation. Ligands for the two mutually exclusive cobalt cations in the closed protomer include nitrogen atoms from His\(^{151}\) (2.3 and 2.5 Å), His\(^{178}\) (2.1 and 2.4 Å), and His\(^{208}\) (2.2 and 2.4 Å) and one oxygen atom from a water molecule (2.3 and 2.8 Å), respectively (Fig. 4A). In contrast, candidate ligands for the single cobalt cation found in the three open protomers include not only imidazole rings of His\(^{81}\) (2.2, 2.3, and 2.3 Å), His\(^{178}\) (2.0, 2.1, and 2.2 Å), His\(^{208}\) (2.3, 2.3, and 2.4 Å), and one oxygen atom from each three water molecules (2.3, 2.4, and 2.4 Å) but also the carboxylate of Glu\(^{125}\) (2.2 Å in all three protomers; Fig. 4B).

Monovalent Cation Binding—Cations, such as NH\(_4^+\) or K\(^+\), are known to activate class II FBP aldolases and in the case of \( \text{Taq} \) aldolase, only NH\(_4^+\) cations activate the enzyme (34). Strong electron density in the difference maps was observed for...
Fig. 3. Superposition of class II aldolase structures. Stereo C$_\alpha$-trace superposition of the *Taq* FBP aldolase protomer bound to Co$^{2+}$ in its open conformation (yellow in A), the closed subunit of the enzyme when bound to yttrium (yellow in B), and the *E. coli* FBP aldolase structure (Protein Data Bank ID code 1DOS; red in A), onto the *E. coli* FBP aldolase structure in complex with the transition analogue phosphoglycolohydroxamate (Protein Data Bank ID code 1B57; red in B). The alignment was performed pairwise using SwissProt (39).

The largest root mean square deviation was 1.33 Å for 221 C$_\alpha$ atoms in common between *E. coli* aldolase and *Taq* aldolase protomer in closed conformation. The large and small loop regions of *Taq* aldolase protomers are illustrated in gray, and those of *E. coli* aldolase are shown in purple. In A are grouped *Taq* and *E. coli* aldolase subunits whose loop conformations correspond to the open position. *B* shows *E. coli* aldolase and *Taq* aldolase subunits with loop conformation in the closed or bound position. Also shown in *B* is substrate FBP that was modeled (using acyclic FBP coordinates of Protein Data Bank entry 1FDJ) into the active site and subjected to energy minimization. The bonds of FBP are drawn in green and shown in ball-and-stick representation. *C*, shows in detail modeling of FBP guided by two sulfate anions (bonds in gray) whose positions were consistent with the phosphate-binding site of the substrate in the acyclic form. The bonds of the transition state analogue, phosphoglycolohydroxamate, are drawn in black.
catalytic cation sites in all four subunits in the asymmetric unit, with peak heights ranging from 7 to 11 standard deviations above the noise level. The putative NH₄⁺/H₁₁₀₀₁ cation sites were modeled with sodium scattering factors (Fig. 4B). The cation is coordinated by the nitrogen atoms of the imidazole ring of His⁷₈ (with distances ranging from 2.8 to 3 Å in the four subunits) and the oxygen atoms of the side chains of Asp⁸₀ (2.8 to 3 Å), Glu¹³₀ (2.8 to 2.9 Å), Asn²₅₁ (3.2 Å), and a water molecule (3 to 3.3 Å).

Novel Cation-binding Site—Taq FBP aldolase crystals were soaked in the presence of an yttrium chloride salt, which identified an additional metal-binding site (Fig. 4C). This metal-binding site has not been previously reported and is uniquely found in the protomer in its closed conformation. The metal is coordinated by two oxygen atoms of the side chain of Asp¹₀² (2.7 Å), one oxygen atom of the carboxylate group of Glu¹³₂ (2.9 Å), and the hydroxyl of Ser¹⁰⁴ (2.8 Å), Wat²⁵⁵ (2.9 Å) and contacts a sulfate anion. The metal refined to a temperature factor below 40 Å². The site was modeled using a strontium scattering factor, which has a scattering factor almost identical to that of yttrium.

Sulfate Binding—Taq FBP aldolase crystallizes from high concentrations of ammonium sulfate as the precipitating agent (27). Under these concentrations, sulfate efficiently competes with phosphate or phosphate-containing compounds for binding to proteins. Two strong peaks of 17–19 times the noise level...
are found in the difference Fourier maps that correspond to two sulfate anions bound to the active site of the protomer in the closed conformation. One sulfate anion engages in hydrogen bonds to the hydroxyl of Ser211, the amides of Gly179, Asp253, and Thr254, as well as to two water molecules (Fig. 4A). Gly179 resides on the small loop that closes over the active site in this protomer. The second sulfate anion is located 10 Å away and makes an electrostatic interaction with the side chain of Arg278 as well as hydrogen bonding to the hydroxyl of Ser69 and three water molecules.

The same two sulfate anions are also found in the three subunits in the asymmetric unit in their open conformation (Fig. 4B). However, because residue Gly179 of the closed protomers is not available for binding to the sulfate anion, this sulfate anion does not bind as tightly as in the protomer in the closed conformation. Difference electron density for this sulfate anion was weaker (with only a half of the signal to noise ratio (∼10) versus to the closed subunit). The occupancy was also reduced to one-half to obtain a refined average temperature factor of 43.9 Å² (compared with 36.2 Å² in the closed subunit, where this anion has full occupancy). By contrast, the second sulfate anion in the active site binds to the open subunit in a fashion similar to that in the closed subunit (Fig. 4B).

Residues Arg116 and His123 at the C-terminal end of α-helix 4 bind a third sulfate anion in all four protomers within the asymmetric unit. The site is located at the solvent surface of each protomer and is distant from the active site. Again, the electron density was also weak for this ligand, and the occupancy was set to one-half in all subunits to obtain a refined average temperature factor of 43.9 Å². In the current model, the sulfate ion accepts hydrogen bonds from the side chains of Arg116 (2.7 Å) and His123 (2.8 Å), as well as from two water molecules (2.7 and 2.9 Å) in each protomer. In the closed conformation, an additional sulfate ion complexes with Arg135 side chain, one water molecule, and the yttrium ion (shown in Fig. 4C).

**DISCUSSION**

*Molecular Details of Hyperthermostability—Electrostatic interactions are the major determinant in hyperthermostability of a protein* (35, 36). *Taq* aldolase, which is stable at 90 °C for several hours (37), has 27 intramolecular salt bridges in its tertiary structure. Of these, only 10 similar interactions are observed in the *E. coli* FBP aldolase crystal structure. In particular, an intricate network of ion pairs between α-helices αi and αq in the *Taq* FBP aldolase crystal structure is replaced by two single salt bridges in the *E. coli* structure. Glutamate residues, Glu39 (corresponding to Glu47 in *E. coli* aldolase), Glu39 (Lys51 in *E. coli* aldolase), Glu286 (Thr339 in *E. coli* aldolase), and Glu289 (Ala343 in *E. coli* aldolase) surround lysine residues Lys289 (Ile342 in *E. coli* aldolase) and Lys293 (Glu346 in *E. coli* aldolase) that results in multiple electrostatic interactions. In *E. coli* aldolase, residue substitution, although yielding two compensating electrostatic interactions, Lys51–Glu346 and Glu47–Arg335, excludes formation of an ion-pairing network. In a sequence alignment of 18 class II FBP aldolase sequences (38), only 10 of 45 residues involved in electrostatic intramolecular interactions observed in the *Taq* aldolase structure are invariant.

Three intermolecular ion pairs in *Taq* FBP aldolase are observed between subunits related by the Q dyad. Only one of these (Arg58–Asp79) are found in the analogous *E. coli* structure (Lys71–His90). In the thermophile structure, Arg58 is involved in a network of ion pairs involving Glu66 and Arg69 and the 2-fold related residues (Arg38, Glu66, Arg69, and Asp79). Additional strong interactions were also found across subunits related by the P dyad. In particular, the side chains of Glu7 and Arg72 are within hydrogen bonding distance (2.8 Å) and the sulfur atoms of the 2-fold related Met1 participate in van der Waals’ interactions. No electrostatic interactions were found in the *Taq* FBP aldolase structure across the remaining interface. Protomers related by the R dyad involve, however, a considerable number of hydrophobic contacts involving residues Tyr65, Leu97, Arg107, Leu107, Arg108, Phe111, Ala121, Ala124, Val125, and Val127.
Additional structural features that contribute to thermostability are short loops and close packing as exemplified by fewer cavities (36). Pairwise superposition of the crystal structures of class II FBP aldolase from *E. coli* and *T. aquaticus* with SwissProt (39) is shown in Fig. 3; the largest root mean square deviation was 1.33 Å for 221 Cα atoms in common between *E. coli* aldolase and Taq aldolase protomer in closed conformation. The superpositions demonstrate that the Taq aldolase structure has shorter loops and a higher degree of secondary structure, creating a more compact structure that is consistent with structural attributes found in thermostable proteins. Overall, loop residues make up 158 amino acids in the crystal structure has shorter loops and a higher degree of secondary to 125 residues in *Taq* enzyme, whereas this amount is reduced to 125 residues in Taq aldolase. For instance, the loop connecting α-helices α5 and α6 in the *E. coli* structure consists of 10 residues (residues 70–79), whereas only three residues (residues 56–58) are engaged in a tight turn on the thermostable structure. In addition, the loop following α-helix α9 in the *E. coli* structure involves 11 additional residues (residues 127–137) not present in Taq aldolase. The *E. coli* structure has also 10 additional residues without secondary structure at its N terminus compared with the Taq structure. By contrast, α-helix α7, and nine additional residues comprising the sequence 219PELVERFRASGGEIGEAA232 in Taq aldolase are not present in the *E. coli* structure. Hydrophobic amino acids from this sequence pack against α-helices α9α10 and αα2 that are antiparallel to each other and create an arm that protrudes from the barrel (Fig. 1). The arm appears to mediate oligomerization because of the large number of hydrophobic amino acids making intersubunit van der Waals’ interactions with the Q-related protomer (Fig. 2A). The arrangement results in residues on α-helix α9α10 of the Q-related protomer interacting with residues residing on α-helix α2α3. The inserted sequence of 17 residues thus not only enhances subunit stability but also promotes dimer formation and is consistent with the enhanced thermostability of Taq aldolase.

**Induced Fit Movements in Class II FBP Aldolases—Class II FBP aldolases undergo induced fit movements during catalysis.** Cα backbone traces for Taq aldolase protomers in open and closed conformation (shown in Fig. 3) suggest that the small loop (residues 175–190) and a larger loop (residues 134–152) undergo conformational changes upon active site ligand binding. Comparison of the crystal structure of *E. coli* class II FBP aldolase determined to 1.6 Å (21) and 2.5 Å (24) as well as in complex with phosphoglycolohydroxamate to 2.7 Å (26), which resembles the one-diolate transition state of the dihydroxyacetone phosphate substrate, corroborates a lid closure mechanism mediating ligand binding. In the unbound *E. coli* structures, these flexible loops exhibited positional disorder to various extents, and both small and large loops display an open conformation, shown in Fig. 3A. In the case of the large loop, its conformation, although open, has shifted somewhat toward a closed position compared with the more open conformation observed in the thermostable enzyme. In the bound *E. coli* structure, the small loop closes over the active site (shown in Fig. 3B) and adopts a conformation observed in the Taq enzyme. Furthermore, as in the Taq aldolase closed protomer conformation, a number of residues of the equivalent large loop region of the *E. coli* enzyme could not be traced in the ligand complex, supporting enhanced flexibility by residues in the large loop and originating most likely from fewer positional constraints as a result of the conformational change by the adjacent small loop.

What is the mechanism that is responsible for lid closure? The small loop conformations point to occupancy of the sulfate-binding site as a means by which to influence the stability of the small loop in its closed conformation and hence lid closure. The three protomers in their open conformation bind only weakly to the sulfate anion that interacted with the small loop in the closed position as indicated by partial occupancy and consistent with a high millimolar *Kd* (1.7 m ammonium sulfate concentration used in crystallization conditions). In contrast, the protomer in its closed conformation exhibits full occupancy sulfate binding because of lid closure, indicating tighter sulfate ion binding. The protomer in the closed conformation cannot open its lid because of steric hindrance of Ile139 with the symmetry-related Val143. Similarly, the lid position in the three subunits having an open conformation is also stabilized by crystal contacts. In particular, Val143 interacts with the symmetry-related Phe138, and the carbonyl of Ala144 is hydrogen-bonded to the side chain of the symmetry-related Arg164. The crystal contacts by stabilizing distinct loop conformations clearly show that lid closure enhances sulfate anion affinity. Conversely, the two distinct conformational states are not inconsistent with an induced fit mechanism whereby lid closure is promoted by ligand attachment.

The sulfate anion-binding sites have enabled the identification of the FBP binding mode in the Taq aldolase active site. The position of the C1-phosphate moiety in the structure of the *E. coli* enzyme in complex with the transition state analogue, phosphoglycolohydroxamate, coincides with that of a sulfate ion present in all protomers of our structure (Fig. 3C) and that mediates lid closure. Furthermore, mutagenesis of Arg278 in *E. coli* aldolase perturbs FBP C6-phosphate binding (40), suggesting that the sulfate anion interacting with the equivalent Arg278 in all Taq aldolase protomers delineates the C6-phosphate binding locus. Superposition of the FBP C1- and C6-phosphate moieties with the appropriate sulfate anion-binding sites is consistent with binding by the acyclic keto form of FBP in both open and closed conformations. The FBP orientation, shown in Fig. 4C, is free of steric conflicts, and loop closure indeed traps the C1-phosphate, whereas the C6-phosphate is able to interact with Arg278. Additionally, FBP C2 and C4 hydroxyls hydrogen bond with Asp80 and Asp253, respectively. The equivalent aspartate residues in the *E. coli* structure, Asp109 and Asp268, respectively, when mutated compromise FBP as well as dihydroxyacetone phosphate binding (41) validating the docking of the acyclic keto form of FBP in this orientation. The docked FBP conformation corresponding to the nascent dihydroxyacetone phosphate portion of FBP also mimics the binding observed for the transition state analogue in the active site of *E. coli* aldolase, shown in Fig. 3C (26). Unique to the closed conformation, FBP docking allows interaction by the C3 keto with the C5 cation bound furthest from Glu132, i.e., the solvent-accessible hence exterior binding site, consistent with a reaction mechanism where the cation is able to polarize the keto moiety. Cyclic forms of FBP did not allow superposition of phosphates moieties with the binding sites for the sulfate oxyanions.

The loop formed by residues 134–152 has undergone, in the closed subunit conformation shown in Fig. 3B, a significant conformational change compared with the open position shown in Fig. 3A that flips it toward the active site. Lue138 in the open subunit conformation is repositioned 9.4 Å, based on Cα coordinates, closer to the active site and whose side chain, pointing toward the active site interior, would provoke a steric clash with the His177 side chain, located on the small loop, were it positioned as in the open conformation in Fig. 3A. Lid closure thus requires coordinate movement of both small and large loops. The displacement by the His177 side chain (5.2 Å using Cα coordinates) into the closed conformation remodels the
Co\(^{2+}\) binding site with respect to the binding site observed in protomers in the open conformation; the Co\(^{2+}\) cation at the interior site (closest to Glu\(^{132}\)) now interacts only indirectly with Glu\(^{132}\) through an intervening water molecule, Wat\(^{446}\). As a result, Glu\(^{132}\) and Wat\(^{446}\) are able to promote synergistic binding of the yttrium cation with the sulfate anion, implying that cations in presence of oxyanions could activate class II aldolases by enhancing the stability of the closed conformation.

Exchange by the transition metal cation between the two mutually exclusive binding sites is not stericly hindered, involving merely small side chain rotations by the chelating histidine residues. The emerging picture of the catalytic cycle in class II aldolases is therefore that of a two-stage process involving merely small side chain rotations by the chelating histidine residues. The emerging picture of the catalytic cycle in class II aldolases is therefore that of a two-stage process involving merely small side chain rotations by the chelating histidine residues. The emerging picture of the catalytic cycle in class II aldolases is therefore that of a two-stage process.

**Metal Cation Preference**—One outstanding question is the basis for metal cation preference by class II FBP aldolases. In the thermophilic enzyme, the largest activation occurs with metal cofactors Co\(^{2+}\) and Fe\(^{2+}\), whereas in the mesophilic enzyme, Zn\(^{2+}\) displays highest activation. Inspection of the active site shows that the Co\(^{2+}\) cation is hexa-coordinated in the open conformation in Taq aldolase, whereas at the equivalent interior site in the *E. coli* aldolase structure, the Zn\(^{2+}\) cation is tetra-coordinated. Both cations coordinate the same number of nitrogen atoms from histidine residues but differ as to the number of coordinating oxygen atoms. The Co\(^{2+}\) cation coordinates an additional water molecule and interacts in a bidentate manner with the Glu\(^{132}\) carboxylate side chain rather than binding in a monodentate manner as observed in the mesophilic enzyme.

The composition of the coordination sphere of two cations thus entails a greater preference for oxygen lone pairs by Co\(^{2+}\) cation that is consistent with greater hardness of the Co\(^{2+}\) cation as a Lewis acid compared with Zn\(^{2+}\) (42). Residues comprising the active site and coordinating the Zn\(^{2+}\) and Co\(^{2+}\) cations are identical in the mesophilic and thermophilic class II aldolases, and superposition of the active site reveals no significant structural differences. The cation binding preference thus appears to be determined by hardness of the metal cation as Lewis acid and possibly by subtle structural differences between the two enzymes.

Greater active site coordination by the Co\(^{2+}\) cation in the thermophilic enzyme compared with Zn\(^{2+}\) suggests enhanced active site integrity at higher temperatures. A similar consideration would also apply to the reaction trajectory. The Zn\(^{2+}\) cation in *E. coli* aldolase complexes the transition state analogue, phosphoglycerolhydroxamate, by interaction through the C\(_2\) and C\(_3\) oxygens as well as the active site histidine residues (26). Stability of a similar transition state architecture in the thermophilic enzyme would be enhanced at higher temperatures by interaction with the Co\(^{2+}\) cation. Enhanced active site integrity is supported by the ~15-fold reduction in activity using Zn\(^{2+}\) as a metal cofactor in the thermophilic enzyme (35). Furthermore, the higher activation by Fe\(^{2+}\) compared with Co\(^{2+}\) in Taq aldolase (35) concurs with the even greater hardness of the Fe\(^{2+}\) cation, supporting the hypothesis that hardness of the metal cofactor enhances active site integrity at high temperatures.
Induced Fit Movements and Metal Cofactor Selectivity of Class II Aldolases:
STRUCTURE OF THERMUS AQUATICUS FRUCTOSE-1,6-BISPHOSPHATE ALDOLASE
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