Crystal structures of the metal-dependent 2-dehydro-3-deoxy-galactarate aldolase suggest a novel reaction mechanism

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Carbon–carbon bond formation is an essential reaction in organic chemistry and the use of aldolase enzymes for the stereochemical control of such reactions is an attractive alternative to conventional chemical methods. Here we describe the crystal structures of a novel class II enzyme, 2-dehydro-3-deoxy-galactarate (DDG) aldolase from *Escherichia coli*, in the presence and absence of substrate. The crystal structure was determined by locating only four Se sites to obtain phases for 506 protein residues. The protomer displays a modified (α/β)₈ barrel fold, in which the eighth α -helix points away from the β -barrel instead of packing against it. Analysis of the DDG aldolase crystal structures suggests a novel aldolase mechanism in which a phosphate anion accepts the proton from the methyl group of pyruvate.

Keywords: aldolase/(α/β)₈ barrel/2-dehydro-3-deoxy-galactarate (DDG) aldolase/domain swapping/X-ray crystallography

Introduction

Aldolases catalyze a wide range of aldol condensations. The potential of aldolases is widely recognized in synthetic chemistry (Wong and Whitesides, 1994), in particular the class II enzymes that are more stable. There are numerous examples where aldolases have proven effective in biotransformations and synthetic organic chemistry, such as the synthesis of novel antibiotics (Wagner *et al.*, 1995; Barbas *et al.*, 1997). The aldolases have been classified into two groups of enzymes, depending on the method of enzyme catalysis. The two classes of aldolases proceed by a different reaction mechanism. Class I aldolases have an essential lysine residue that forms a protonated Schiff base with the substrate carbonyl carbon to stabilize the intermediate (Horecker *et al.*, 1972), whereas the class II aldolases are metal-containing enzymes where the substrate is coordinated to a divalent metal cation such as magnesium (Rutter, 1964; Morse and Horecker, 1968). Since the class I enzymes are generally found in higher organisms and class II enzymes are found in bacteria and other lower organisms, these enzymes are targets of inhibitors that may have anti-bacterial properties.

2-dehydro-3-deoxy-galactarate (DDG) aldolase catalyzes the reversible aldol cleavage of DDG to pyruvate and tartronic semialdehyde (Figure 1). The enzyme is part of the catabolic pathway for D-glucarate/galactarate utilization in *Escherichia coli* (Hubbard *et al.*, 1998). DDG aldolase has a considerable advantage with respect to other aldolases due to its low substrate specificity and its ability to condense a wide range of aldehydes with pyruvic acid (Fish and Blumenthal, 1966). Elucidation of the reaction mechanism of DDG aldolase may pave the way to progress in rational protein engineering towards altered substrate specificity and generation of new catalysts for synthetic chemistry. In order to elucidate the structural features that determine the specificity of DDG aldolase and its mechanism, we have solved the three-dimensional structure of the substrate-free DDG aldolase at 1.8 Å resolution, and its complex with Mg²⁺ and pyruvate at 2.6 Å resolution.

Results and discussion

The structure of the DDG aldolase protomer (subunit molecular weight of 27.4 kDa) adopts a modified (α/β)₈ barrel fold (Figure 2). Only seven α -helices embrace the β -barrel. The eighth α -helix (residues 239–254) protrudes from the β -barrel instead of packing against the hydrophobic β -sheet of the β -barrel. The N-terminal α -helix (α 0) caps the N-terminal end of the β -barrel. Without taking into account α -helix α 8, the dimensions of the protomer are ~40 Å in width and 45 Å in height, with a depth of 37 Å down the barrel axis. The core of the β -barrel is circular in cross-section and reminiscent of *N*-acetylneuraminase lyase (Izard *et al.*, 1994). Subunits of the aldolase dimer within the asymmetric unit have virtually identical conformations and can be superimposed by a 180° rotation about an internal 2-fold axis between the subunits. The overall root mean square deviation (r.m.s.d.) for the C α positions of residues 4–256 is 0.39 and 0.23 Å for the substrate-free aldolase and aldolase–pyruvate, respectively. The 506 C α atoms of the complexed aldolase–pyruvate structure align to the substrate-free structure with an r.m.s.d. of 0.4 Å.

Helix swapping between protomers

Each protomer is tightly associated with a non-crystallographically 2-fold-related subunit. In each subunit, the eighth α -helix packs along the exposed hydrophobic region of the β -sheet of a 2-fold-related protomer. This helix swapping results in an intertwined dimer with one α -helix of each protomer replaced by the identical α -helix from another protomer to form complete (α/β)₈ barrels. The swapped helices engage in an extensive dimer interface. Domain swapping has been observed in several proteins and provides a mechanism for forming oligomeric proteins from their monomers (Bennett *et al.*, 1995). The

Fig. 1. The reactions catalyzed by DDG aldolase (EC 4.1.2.20). Components are depicted in the Fisher projection. The equilibrium constant lies far in the direction of cleavage (Fish and Blumenthal, 1966).

DDG aldolase structure is the second example where dimer association of $(\alpha/\beta)_8$ barrels is achieved through swapping of the eighth α -helix. Such an arrangement has previously been reported for phosphoenolpyruvate mutase (Huang *et al.*, 1999).

Hexamer assembly

In solution, the enzyme functions as a hexamer (Blackwell *et al.*, 1999) with point group symmetry 32 (Figure 3A). The crystallographic triad axis coincides with the oligomer's 3-fold axis. The dimensions of the globular-shaped hexamer are ~ 85 Å along the triad and 75 Å across the 3-fold axis. The intersubunit interactions are more extensive across the oligomer's dyad than triad. Almost all dyad intersubunit contacts arise from the swapped α -helices. Dimer contacts involve one salt link between Asp42 and Lys255 and nine intersubunit hydrogen bonds. Several hydrophobic side chains also participate in the interactions along the 2-fold axis and include residues Ile21, Leu27, Ile31, Val35 and Leu36 located on α -helix α_1 ; Ala39, Phe41; Val236, Gly237, Leu240, Val241, Phe243, Leu250, Ala251 and Phe254 located on the C-terminal α -helix (α_8) and its preceding loop. Dimerization results in a total buried surface area of 2974 Å².

In contrast, trimer contacts involve only four hydrogen bonds and one salt link between Glu49 and Lys85. Compared with dyad interactions, triad interactions implicate fewer hydrophobic residues: Pro52, Ile55, Ile59, Val82, Pro116, Gly122, Val123, Phe139, Ala182, Leu187, Gly188 and Leu240. The two distinct and complementary protomer surfaces implicated in trimer formation bury a total of 1492 Å² of the solvent-accessible surface: 783 Å² of one protomer and 709 Å² of the 3-fold-related protomer. DDG aldolase oligomerization could thus entail a mechanism involving association of stable dimers for hexamer formation.

Active site

As with other $(\alpha/\beta)_8$ barrel structures, the active site is located in the depression at the C-terminal end of the β -barrel. One side of the catalytic cavity is further lined by residues following α -helix α_5 of a 3-fold-related protomer (Figure 3B), in particular Ser124 and Val125.

DDG aldolase requires a divalent metal ion (such as Mg²⁺, Co²⁺ or Mn²⁺) for catalysis (Fish and Blumenthal, 1966). The catalytic metal was identified on the basis of its octahedral coordination and was modeled as a magnesium, because of the high concentration of magnesium sulfate in the protein storage buffer. The enzyme activity is maximal in potassium phosphate buffer (Fish and Blumenthal, 1966). Indeed, crystals were only obtained in the presence of phosphate buffer (Blackwell *et al.*, 1999). The substrate-free DDG aldolase crystal structure revealed two phosphate anions bound within the active site pocket (Figure 4A). One of them, the 'first' phosphate, coordinates the catalytic magnesium cation (2.3 Å). The 'first' phosphate refined to an average temperature factor of 23.7 Å², while the 'second' phosphate refined to a higher value of 36.6 Å². A water-mediated interaction is observed between the two phosphates. Three water molecules also coordinate the Mg²⁺ at a distance of 2.4, 2.2 and 2.4 Å, respectively. Only two of the metal's ligands are contributed by the protein, namely the side chains of Asp179 (2.2 Å) and Glu153 (2.2 Å). This arrangement suggests that phosphate plays a role in the enzyme's activity in binding the divalent cation to the active site.

Pyruvate binding

The two phosphate anions are replaced by pyruvate, as seen in the aldolase–pyruvate crystal structure (Figure 4B), without any conformational changes required by the protein (Figure 4C). The three carbon atoms of pyruvate are in van der Waals contact with residues Gly176, Ser178 and Leu216. The orientations of the methyl and carboxylate groups of pyruvate were decided upon modeling the condensed substrate into the active site (Figure 4D) and excluding the other possible orientation on the basis of steric hindrance (not shown). This orientation conveniently places the methyl group of pyruvate in a hydrophobic environment and its carboxylate in a polar environment.

The aldolase–pyruvate structure shows that the ligand's carbonyl coincides with the 'first' phosphate anion and analogously coordinates the catalytic magnesium cation. Instead of a water molecule coordinating the Mg²⁺ as seen in the substrate-free structure, the carboxyl of pyruvate fulfills its function. It is therefore not surprising that the pyruvate offering two ligands for coordinating the metal displaces the phosphate anion. Interestingly, the 'second' phosphate anion is not found in the aldolase–pyruvate structure where its pocket is filled with ordered water molecules.

Comparison with other class II aldolase active sites

Numerous crystal structures of class I enzymes have been determined (see Cooper *et al.*, 1996 for references) and there is a good understanding of their substrate recognition and catalytic mechanism. In contrast, the structures of only two class II aldolases have previously been determined,

Fig. 2. (A) Stereo cartoon drawing (Bacon and Anderson, 1988; Kraulis, 1991; Merritt and Murphy, 1994) of the DDG aldolase protomer; α -helices are depicted as yellow helical ribbons and β -strands as blue arrows. The phosphate anions in the active site and the catalytic magnesium are shown. Oxygen atoms are colored in red, nitrogen in blue, sulfur in green, carbon in yellow, phosphate in black and magnesium in light blue. This color coding is used throughout all figures. β -strands β 4 and β 6 are immediately followed by more than one helix of which helix 4' is a short 3_{10} -helix. Prolines 7 and 107, located on the loop preceding the N-terminal α -helix and on α -helix α 4, respectively, are in their *cis* conformations. In addition to the assigned elements of secondary structure, there is a section of sequence that presents an extended conformation. This section involves residues 122–125 residing between helices α 4 and 4'. (B) Stereo C_α trace of the DDG aldolase protomer shown in the same orientation as in (A). Every tenth C_α atom is labeled.

namely *E.coli* fructose-1,6-bisphosphate (FBP) aldolase (Blom *et al.*, 1996; Cooper *et al.*, 1996; Qamar *et al.*, 1996) and *E.coli* fucose-1 phosphate (Fuc) aldolase (Dreyer and Schulz, 1993), the latter enzyme not displaying an $(\alpha/\beta)_8$ barrel fold. In each of these two structures, the catalytic divalent cation has a different environment compared with the magnesium coordination site found in DDG aldolase. In the FBP aldolase structure, three histidine residues and a glutamate coordinate the catalytic zinc. This cation binds at either of two mutually exclusive sites. Rotation of two histidine residues has been suggested to move the zinc from the more buried and fully coordinated site into a more exposed site where it can bind water molecules (Blom *et al.*, 1996). Furthermore, a monovalent metal cation may play a role during the carbon–carbon cleavage step or towards release of the condensed product from the enzyme (Plater *et al.*, 1999). In Fuc aldolase, also in the condensation direction,

substrate binding displaces a glutamate liganded to the catalytic zinc cation. This allows the substrate to coordinate the metal through both hydroxyl and carbonyl oxygen atoms. A tyrosine residue protonates the carbonyl oxygen of the aldehyde. Furthermore, Fuc aldolase utilizes the zinc-chelating motif HXH...H (Vallee and Auld, 1990) for metal coordination.

Proposed reaction mechanism

Tritium incorporation studies have shown that DDG aldolase first catalyzes the enolization of pyruvate, and this enzyme-bound enolate then attacks the polarized carbon of the aldehyde (Fish, 1964). Inspection of the active site in the presence of pyruvate does not reveal specific enzyme moieties proximal to the methyl group of pyruvate that are capable of acting as a base to remove its proton. Instead, this group is in van der Waals contact with Leu216 (Figure 4B). Superposition of the substrate-

Fig. 3. (A) Space filling representation of the hexameric DDG aldolase looking down the non-crystallographic dyad. Each of the six subunits is colored differently. (B) Cartoon drawing of DDG aldolase oligomer shown in the same orientation as in (A) illustrating the active site pocket location between two 3-fold-related protomers. For clarity, only four of the six protomers within the hexamer are shown. The two phosphates located in the active site pocket and the catalytic magnesium are shown in space filling representation. The active site pocket is mainly lined by residues belonging to one protomer. The 3-fold-related subunit makes contacts with the 'second' phosphate.

free DDG aldolase structure onto the aldolase–pyruvate structure places the 'second' phosphate in a chemically optimal position to accept the proton of the pyruvate's methyl group (Figure 4C). Arg75 is hydrogen bonded to the 'first' phosphate and a small rotation of its side chain

would allow interaction with the 'second' phosphate. Consequently, the negative charge of the 'second' phosphate would be neutralized and its pK_a possibly altered. In that way, this phosphate could readily accept a proton from the methyl group of pyruvate. The hydrophobic environment prevents water from reaching and reprotonating the methyl group. This postulated first step in the reaction mechanism is consistent with the maximal activity of the enzyme in the presence of phosphate. The emerging negative charge at the C3 atom of pyruvate could be distributed over the mesomeric enediolate structure stabilized by Mg^{2+} . A phosphate anion, intramolecular in this case, has previously been shown to act as a catalyst for α -proton abstraction in FBP aldolase. The α -ketophosphate in the dianionic form of the enzyme's natural substrate, dihydroxyacetone phosphate, catalyzes its own enolization. Changing from phosphate to sulfate prevented intramolecular proton transfer from occurring because sulfate is too weakly basic to accomplish the same function (Periana *et al.*, 1980).

The second step involves attack by the carbanion on the electrophilic carbon on the partially polarized carbonyl group of the aldehyde. We modeled the condensed substrate, DDG, into the active site (Figure 4D). The large number of possible DDG orientations is limited by steric hindrance, in particular at the crucial location of C4. In such a model, the corresponding aldehyde oxygen (O4 at C4 in DDG) is hydrogen bonded to Arg75 and to one of the water molecules coordinating the metal cation. This water molecule is within hydrogen bonding distance of His50. A water molecule responsible for the second step of the reaction mechanism as a proton donor and acceptor would be consistent with the enzyme's ability to accept a wide range of aldehydes as substrates. Both postulated steps of the reaction mechanism have not been encountered amongst this family of enzymes. Our postulated reaction scheme requires verification through further structural analysis of DDG aldolase crystal complexes, in particular capturing the transition state of the enzyme. Such an analysis should provide a molecular template to exploit this family of enzymes in the design and utilization of novel bio-catalysts.

Materials and methods

Preparation of selenomethionyl DDG aldolase

The seleno-L-methionine (Se-Met) isoform of DDG aldolase was produced in the methionine auxotrophic *E.coli* strain B834 (DE3). A single colony of the latter carrying the DDG aldolase gene was added to 10 ml of NMM medium (Budisa *et al.*, 1995) containing 0.3 mM methionine and 100 μ g/ml ampicillin and incubated overnight at 37°C. A 5 ml aliquot was used to inoculate 500 ml of NMM medium, containing 0.3 mM Se-Met and 100 μ g/ml ampicillin. After 15 h, protein expression was induced by adding 0.84 mM isopropyl- β -D-thiogalactopyranoside. Cells were harvested in the stationary growth phase after 41 h. Se-Met DDG aldolase was purified to homogeneity by column chromatography as described (Blackwell, 2000). Electrospray mass spectrometry analysis (K.Lilley, unpublished results) of the Se-Met DDG aldolase indicated substitution of all three methionine residues.

Crystallization and data collection

Crystals of Se-Met DDG aldolase were obtained in the same way as the native enzyme (Blackwell *et al.*, 1999). These crystals also belong to space group *R*32 with two polypeptide chains in the asymmetric unit, a solvent content of 0.48 and a volume to protein mass ratio (V_m) of 1.34 $\text{\AA}^3/\text{Da}$. Co-crystals of DDG aldolase in complex with pyruvate were

Fig. 4. Stereo views of ligands binding to DDG aldolase. The bonds of the ligands are shown in pink while the bonds of the enzyme are shown in white. For clarity, water molecules (drawn as red spheres) are not labeled. The magnesium site is shown and possible ligands coordinating the Mg^{2+} are indicated. (A)–(D) are in the same orientation. (A) Residues in contact with the two phosphate anions bound to the active site pocket as seen in the substrate-free DDG aldolase structure. Final σ_A -weighted $F_o - F_c$ omit electron density map for ligands bound to the enzyme. The contour level of the electron density map is 3σ and the resolution is 1.8 Å. Four solvent molecules and the catalytic magnesium interact with the ‘first’ phosphate and five water molecules are hydrogen bonded to the ‘second’ phosphate. Ser124’ and Val125’ belong to a 3-fold-related protomer. (B) Residues in contact with pyruvate. Final σ_A -weighted $F_o - F_c$ omit electron density map for pyruvate bound to the enzyme. The contour level of the electron density map is 3σ and the resolution is 2.6 Å. The ligand’s carbonyl and carboxyl residue 2.5 and 2.4 Å, respectively, away from the magnesium. All contacts are made by one subunit within the hexamer. (C) Superposition of the substrate-free structure (white) onto the aldolase–pyruvate complexed structure (gray) to illustrate the possible role of the ‘second’ phosphate in the reaction mechanism (gray dotted line). The anion’s oxygen is 3.4 Å away from the methyl carbon atom. The current distance of 3.9 Å between Arg75 and the ‘second’ phosphate is easily decreased to hydrogen bonding distance by a slight side chain movement without steric hindrance and/or by moving the ‘second’ phosphate deeper into the active site. (D) Modeling of the condensed substrate, DDG, into the active site based upon the aldolase–pyruvate structure. The carboxylate at C6 of DDG fills the cavity occupied by the ‘second’ phosphate. A possible role during catalysis for the solvent molecule bridging the ligand’s O4 and His50 is indicated (gray dotted line).

grown under similar conditions to native DDG aldolase (Blackwell *et al.*, 1999) with the inclusion of 10 mM ligand to the crystallization drop. Crystals were cryoprotected by including 35% glycerol in the mother liquor. A CCD detector was used to collect data from a single, flash-frozen crystal of selenomethionyl protein at three wavelengths at beam line BM14 at ESRF, Grenoble. An X-ray fluorescence spectrum was recorded and used to select the wavelength optima for subsequent multiple anomalous dispersion (MAD) data collection. Data were collected at 0.9785 Å (the inflection point of the fluorescence spectrum, f' minimum), 0.9783 Å (f'' maximum) and 0.8855 Å (remote high-energy wavelength). All three data sets were collected from the same crystal at a crystal-to-detector distance of 240 mm and using 0.5° oscillations per image. The unusually low percentage of methionine residues in DDG aldolase did not allow structure determination from MAD data sets collected as 1° oscillations. The data were collected using inverse beam geometry with each set of data measured in a single pass. 1.82 Å resolution data at the three wavelengths were processed independently using the programs DENZO and SCALEPACK (Otwinowski and Minor, 1997). Data statistics are given in Table I. Aldolase–pyruvate X-ray data were collected at 100 K on a CCD detector on beam line 9.6 at SRS, Daresbury, with the wavelength set to 0.87 Å and processed with DENZO and SCALEPACK (Otwinowski and Minor, 1997). X-ray data were collected from a DDG aldolase native crystal at 100 K using a CuK α rotating-anode source with an R-Axis IV imaging plate and processed as described previously (Blackwell *et al.*, 1999).

Structure determination

The MAD data (Table I) were scaled together with the CCP4 (1994) program SCALEIT. Four Se sites of six expected in the asymmetric unit were determined using SOLVE (Terwilliger and Berendzen, 1996). The N-terminal Se-Met was not found and the first three residues are disordered in both refined structures. Initial phases were calculated with the program MLPHARE (CCP4, 1994) and were improved by 100 cycles

of solvent flattening and gradual phase extension from 3.7 to 2.5 Å resolution using the program DM (CCP4, 1994). The final R -factor for the phase extension at 2.5 Å resolution was 0.33. The atomic model was constructed using the program O (Jones *et al.*, 1991). The crystal structures of the native enzyme and the enzyme in complex with pyruvate were solved by molecular replacement using the Se-Met DDG aldolase structure as a search model. The highest solution for the native (aldolase–pyruvate) structure showed a peak at 16.6 (17.6) times the r.m.s.d. from the mean with an R -factor of 0.40 (0.39).

Crystallographic refinement

The substrate-free DDG aldolase and aldolase–pyruvate structures were refined with the program CNS (Brünger *et al.*, 1988, 1998) using standard protocols. The free R -value (Brünger, 1992) was monitored throughout the refinement. Table I lists the final model parameters. The electron density was continuous for the main chain of all residues. Water molecules were initially identified in $F_o - F_c$ maps and screened for reasonable geometry and refined thermal factor <50 Å². Table I shows the crystallographic and free R -factors for all models and all observed reflections within the resolution range indicated. A Ramachandran plot analysis using the program PROCHECK (CCP4, 1994) of the native (aldolase–pyruvate) structure indicates that 92% (90.2%) of all the residues lie in most favorable regions and 8% (9.8%) in additional allowed regions. This structure analysis also showed that all stereochemical parameters are better than expected at the given resolution.

Coordinates

The atomic coordinates have been deposited in the Protein Data Bank (PDB ID codes 1dxs and 1dxl for the native and aldolase–pyruvate structures, respectively).

Table I. Crystallographic data and refinement statistics

Data collection	Edge	Peak	Remote	Native	Aldolase–pyruvate
Wavelength (Å)	0.9785	0.9783	0.8855	1.54	0.87
Resolution (Å)	1.82	1.82	1.82	1.8	2.6
Cell dimensions: <i>a</i> (Å), α	93.1, 85.3°	93.1, 85.3°	93.1, 85.3°	93.2, 87.6°	92.8, 85.8°
Total data	1 391 298	1 386 204	1 386 456	869 856	232 660
Unique data	47 621	47 695	47 830	46 241	16 162
Redundancy	29.2	29.1	28.9	18.8	14.4
Overall completeness	0.996	0.997	0.996	0.927	0.934
Completeness (last shell)	0.992	0.994	0.993	0.599	0.727
$F^2 > 3\sigma(F^2)$ (%)	81.5	83.3	78.7	87.2	72.1
R_{merge}^a (overall)	0.046	0.049	0.043	0.098	0.059
R_{merge}^a (last shell)	0.375	0.336	0.415	0.231	0.167
Average $F^2/\sigma(F^2)$	11.5	11.8	10.5	23.4	14.2
Crystallographic refinement	Native	Aldolase–pyruvate			
No. of reflections	46 201	15 344			
Final model parameters					
no. of amino acid residues	506	506			
no. of protein atoms	3822	3822			
no. of solvent molecules	656	245			
resolution range (Å)	20–1.8	20–2.6			
<i>R</i> -factor ^b (overall)	0.171	0.173			
<i>R</i> -factor ^b (last shell)	0.214	0.240			
<i>R</i> _{free} ^c (overall)	0.194	0.233			
<i>R</i> _{free} ^c (last shell)	0.260	0.322			
average main-chain <i>B</i> -factor (Å ²)	15.8	35.3			
average side-chain <i>B</i> -factor (Å ²)	16.1	35.8			
average water molecule <i>B</i> -factor (Å ²)	28.5	32.2			
average ligand <i>B</i> -factor (Å ²)	28.1	44.5			
R.m.s.d. from ideal geometry					
covalent bond lengths (Å)	0.005	0.006			
bond angles (°)	1.5	1.3			

$$^a R_{\text{merge}} = \frac{\sum_{\text{unique reflections}} \left(\sum_{i=1}^N |I_i - \bar{I}| \right)}{\sum_{\text{unique reflections}} \left(\sum_{i=1}^N I_i \right)}$$

$$^b R\text{-factor} = \frac{\sum_{hkl} ||F_{\text{obs}}| - |F_{\text{calc}}||}{\sum_{hkl} |F_{\text{obs}}|}$$

^cThe free *R*-factor is a cross-validation residual calculated using 5% of the native data that were randomly chosen and excluded from the refinement.

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