Crystallization of diene lactone hydrolase in two space groups: structural changes caused by crystal packing

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Diene lactone hydrolase (DLH) is a monomeric protein with a simple α/β -hydrolase fold structure. It readily crystallizes in space group *P*₂₁₂₁₂₁ from either a phosphate or ammonium sulfate precipitation buffer. Here, the structure of DLH at 1.85 Å resolution crystallized in space group *C*2 with two molecules in the asymmetric unit is reported. When crystallized in space group *P*₂₁₂₁₂₁ DLH has either phosphates or sulfates bound to the protein in crucial locations, one of which is located in the active site, preventing substrate/inhibitor binding. Another is located on the surface of the enzyme coordinated by side chains from two different molecules. Crystallization in space group *C*2 from a sodium citrate buffer results in new crystallographic protein–protein interfaces. The protein backbone is highly similar, but new crystal contacts cause changes in side-chain orientations and in loop positioning. In regions not involved in crystal contacts, there is little change in backbone or side-chain configuration. The flexibility of surface loops and the adaptability of side chains are important factors enabling DLH to adapt and form different crystal lattices.

1. Introduction

Diene lactone hydrolase (DLH) is a 25.5 kDa monomeric protein belonging to the α/β -hydrolase fold class of enzymes. It was first identified in *Pseudomonas knackmussii* and forms part of the β -ketoadipate pathway that is used by bacteria to degrade otherwise toxic aromatic compounds (Schmidt & Knackmuss, 1980; Ngai *et al.*, 1987). The initial structure of native DLH was determined at 2.8 Å resolution more than 20 years ago (Pathak *et al.*, 1988). It contains 236 amino acids forming seven helices and eight strands of β -sheet; seven of the strands are parallel with strand β 2 antiparallel to the rest. The β -sheet is in the core of the enzyme and is surrounded by the α -helices. Six of the α -helices lie on the outer solvent-accessible domains of the enzyme, while the other lies within the inner core (Pathak & Ollis, 1990). As is characteristic of α/β -hydrolase fold enzymes, DLH contains a catalytic triad consisting of a nucleophilic cysteine (Cys123), a histidine (His202) and an aspartic acid (Asp171) (Pathak *et al.*, 1991). The enzyme is also active with a serine replacing the nucleophile (C123S; Cheah, Ashley *et al.*, 1993; Cheah, Austin *et al.*, 1993).

In the years since the first structure was published, DLH has been studied both mechanistically and from an engineering perspective (Kim *et al.*, 2005; Walker *et al.*, 2000, 2012), with most studies resulting in crystallization and structural analysis. There are currently ten DLH structures in the Protein Data Bank, nine of which are obtained from isomorphous crystals in space group *P*₂₁₂₁₂₁ with a single DLH molecule in the asymmetric unit. The one with different morphology (although still in space group *P*₂₁₂₁₂₁) is attributed to the presence of 2-propanol and the bound PMS moiety of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF). Crystals isomorphous to those previously reported were initially grown but dissolved with the addition of PMSF in 2-propanol only to reform *in situ* with new cell dimensions (Robinson *et al.*, 2000).

DLH has been shown to readily crystallize in space group *P*₂₁₂₁₂₁ using either phosphate or sulfate precipitating agents. Here, we report the crystallization and structure of DLH, using citrate as precipitating agent, in space group *C*2 with two molecules in the asymmetric unit. Differences between the new and old structures

Table 1

Summary of diffraction data and refinement statistics.

Values in parentheses are for the highest resolution shell.

	DLH $P2_12_12_1$	DLH $C2$
Data collection		
Unit-cell parameters ($\text{\AA}$, $^\circ$)	$a = 48.2$, $b = 71.0$, $c = 77.5$, $\alpha = \beta = \gamma = 90.0$	$a = 100.2$, $b = 49.1$, $c = 89.6$, $\alpha = \gamma = 90.0$, $\beta = 94.3$
Wavelength ($\text{\AA}$)	1.5418	1.5418
Resolution range ($\text{\AA}$)	77.46–1.65 (1.70–1.65)	89.36–1.85 (1.90–1.85)
Observed reflections	224333 (14494)	113253 (7865)
Unique reflections	31176 (2138)	35229 (2484)
$\langle I/\sigma(I) \rangle$	50.6 (20.8)	16.9 (5.1)
Completeness (%)	95.6 (91.0)	94.0 (88.9)
R_{merge}	0.030 (0.102)	0.063 (0.303)
Refinement statistics		
Resolution ($\text{\AA}$)	19.67–1.65 (1.70–1.65)	19.66–1.85 (1.90–1.85)
No. of reflections	29557 (1894)	33459 (2299)
No. of free R reflections	1578 (98)	1770 (117)
R factor	0.158 (0.145)	0.173 (0.201)
Free R factor	0.172 (0.185)	0.205 (0.263)
No. of non-H atoms	1766	3542
No. of water molecules	246	184
No. of sulfate ions	3	0
Model quality		
R.m.s. deviations from ideal geometry		
Bond lengths ($\text{\AA}$)	0.007	0.008
Bond angles ($^\circ$)	1.090	1.087
Average B factors ($\text{\AA}^2$)		
Overall	10.38	22.12
Main-chain atoms	8.34	20.11
Side-chain atoms	12.11	24.22
Ramachandran plot, residues in (%)		
Favoured regions	97.0	97.4
Allowed regions	3.0	2.6
Disallowed regions	0	0

highlight the effects of precipitating ions and the crystal lattice on the molecular structure.

2. Materials and methods

2.1. Expression, purification and crystallization

DLH was expressed and purified as described previously (Kim *et al.*, 2005). Both the $P2_12_12_1$ and $C2$ crystal forms were obtained using hanging-drop vapour diffusion. The $P2_12_12_1$ crystals were grown using conditions similar to those previously reported (Kim *et al.*, 2005). The hanging drop consisted of 5 μl of 12 mg ml^{-1} protein solution and 3 μl of 0.1 M sodium citrate, 1.2 M ammonium sulfate pH 5.8 over an 800 μl reservoir. Crystals were harvested after 5–10 d growth at 277 K. The $C2$ crystals were grown in an 8 μl hanging drop with a 1:1 ratio of protein to precipitant solutions, where the precipitant was 0.8 M sodium citrate pH 5.8 and the reservoir volume was again 800 μl . Crystals were harvested after 3–5 d growth at 291 K and were then stored at 277 K. Prior to data collection, crystals were transferred to a cryobuffer consisting of the crystallization buffer with the addition of 30% glucose and 5% glycerol.

2.2. Data collection and processing

Data for both crystal forms were collected using Cu $K\alpha$ radiation at a temperature of 100 K. Data were recorded as a contiguous sequence of 1° rotations using a MAR345 detector mounted on a Xenocs GeniX 3D Cu HF micro-beam X-ray generator, with cooling supplied by an Oxford Cryostream 700 series. 160 frames were collected for the $C2$ crystal and 180 for the $P2_12_12_1$ crystal. The $C2$ crystal required 60 min exposure per frame, whereas the $P2_12_12_1$ crystal required 5 min exposure per frame. Data-collection statistics are listed in Table 1.

2.3. Structure determination and refinement

The $C2$ crystal structure (PDB entry 4p93) was solved by molecular replacement using *Phaser* (McCoy *et al.*, 2007) as implemented in the *CCP4* suite of programs (Winn *et al.*, 2011). PDB entry 1zi6 (Kim *et al.*, 2005) was used as a search model. The search yielded a single correct solution (rotation function Z -score = 23.5, translation function Z -score = 59.9, log-likelihood gain = 4031). Further refinement was undertaken using *REFMAC5* (Murshudov *et al.*, 2011). The final refinement statistics are listed in Table 1. The $P2_12_12_1$ structure (PDB entry 4p92) was simply refined against the same known structure (PDB entry 1zi6). Crystal contacts for both crystal forms were identified using *CONTACT* (Tadeusz Skarzynski, Imperial College London), the structures were superimposed using *SUPERPOSE* (Krissinel & Henrick, 2004) and the difference in accessible surface area due to crystal contacts was calculated using *AREAIMOL* (Saff & Kuijlaars, 1997) in *CCP4*.

3. Results and discussion

3.1. Crystal packing and overall structure

The new $C2$ crystal form was found to contain two copies of DLH in the asymmetric unit, designated *A* and *B*. These molecules have different crystal environments and are related by a noncrystallographic twofold axis perpendicular to the unique axis (Fig. 1). The solvent content of the $C2$ crystals was 41%, while the corresponding value for the $P2_12_12_1$ crystals was 47%. The $C2$ crystals did not diffract as well as the $P2_12_12_1$ crystals, but data of sufficient quality could be collected to enable determination of an accurate structure. The average error in the $C2$ coordinates was found to be 0.13 $\text{\AA}$ as determined by the diffraction-component precision index (DPI) value for the model (Cruickshank, 1999), while the corresponding error for the $P2_12_12_1$ structure was 0.08 $\text{\AA}$.

The overall DLH structure was very similar in either the $P2_12_12_1$ (4p92) or $C2$ (4p93) crystal forms. The r.m.s. displacements after superimposing the two chains of the $C2$ structure with the $P2_12_12_1$ structure were 1.02 $\text{\AA}$ for chain *A* and 0.85 $\text{\AA}$ for chain *B*. When the superposition was limited to main-chain atoms, the r.m.s. displacements were 0.43 and 0.41 $\text{\AA}$ for the *A* and *B* chains, respectively. The r.m.s. difference obtained when superimposing molecule *A* on molecule *B* was 0.96 $\text{\AA}$ for all atoms and 0.37 $\text{\AA}$ for main-chain atoms. The differences between the three structures were not uniform and were concentrated principally in the loop regions and at crystallographic interfaces.

The conditions used to obtain either crystal morphology were similar in two important aspects: the pH was the same (pH 5.8) and the crystallization buffers were both high in salt (1.2 M ammonium sulfate and 0.8 M sodium citrate). Ionic strength and pH are two of the most important variables affecting crystal growth (Zeppezauer, 1971; Feher & Kam, 1985); this being the case, it is surprising that isomorphous crystals were not obtained. It should be noted that although the $C2$ crystals were initially grown at a slightly higher temperature, they were subsequently stored at the same temperature as the $P2_12_12_1$ crystallization with no change in morphology.

3.2. Crystal contacts and the role of sulfate ions in the $P2_12_12_1$ crystal

In the $P2_12_12_1$ crystals each molecule interacts with six others through three interfaces (Supplementary Fig. S1¹). The interface

¹ Supporting information has been deposited in the IUCr electronic archive (Reference: SW5074).

Figure 1

Crystal packing of DLH in space groups $P2_12_12_1$ (a), (b) and (c), and $C2$ (d), (e) and (f) shown along the a , b and c axes of the unit cells.

containing the most interactions is between the residues of helices 2 and 7 of neighbouring molecules. Arg81, Glu82 and Gln89 of helix 2 form hydrogen bonds to Ser215, Glu222 and Arg223 of helix 7 as well as parallel π - π stacking between His172, which lies on a loop protruding over the active site, and Trp50 located in helix 1. In addition, there is a sulfate ion that is ligated by side chains from both molecules. It lies between the N-terminal end of helix 6, 2.84 Å from the peptide backbone of Ala176, where it is likely to be stabilized by the α -helix dipole and the exterior side of helix 1 from the neighbouring molecule, where it forms hydrogen bonds with the side chains of Arg45 and Ser49. The remaining interfaces are minor, involving only two to three residues. One involves Gln9 of strand β 1 and Glu78 and Gln76 of loop 6; the other involves Ser22 of loop 3 and Ala188 of loop 13.

DLH crystallized in space group $P2_12_12_1$ has sulfate ions that form crystal contacts between protein molecules within the lattice framework. In crystals obtained using a phosphate precipitation buffer, phosphates replace sulfates in these positions. There are three coordinated sulfate molecules in the lattice. One forms part of an interface, as described above. The second and third sulfate molecules are coordinated by a single DLH molecule rather than at interfaces. One is positioned near the start methionine with an O atom (O4) 2.72 Å from the N-terminus. The final sulfate ion is positioned at the opening of the enzyme active site and is coordinated by Arg81, Ser203, Arg206 and Ser209 (Fig. 2). The latter residues reside on a loop containing the His202 of the catalytic triad. This loop functions

as a cover for the active site, creating a crevice inaccessible to bulk solvent. The location of this sulfate ion causes problems when trying to soak substrates into the enzyme active site.

3.3. New crystallographic interfaces in the $C2$ crystal

In the $C2$ crystal lattice each molecule interacts with eight others, giving rise to eight unique interfaces that involve 21 chain A residues and 21 chain B residues (Supplementary Fig. S2). The main interface involves residues Gln67, Glu70, Thr71 and Ala72 from loop 6 and Arg79, Gln80, Gln83 and Lys86 for helix 2 hydrogen bonding to the same residues from a neighbouring molecule. This interface is between a chain A and a chain B molecule; therefore, although the hydrogen-bonding residues involved are the same, the bond distances are different. For example, the distance of the hydrogen bond between Thr71 from chain A and Gln83 from chain B is 3.22 Å, whereas the bonding distance when the chains are reversed is 3.78 Å. Similarly, the distance between Ala72 from chain A and Arg79 from chain B is 3.16 Å compared with 2.65 Å between Arg79 from chain A and Ala72 from chain B . These differences are significantly larger than the error as determined by the DPI value.

There are two interfaces that lie along the crystallographic twofold symmetry axis, one between chain A molecules and one between chain B molecules. The crystallographic interface between chain A molecules involves hydrogen bonding between Gln89 and Ala176; additionally, parallel-displaced π - π stacking is observed between

Figure 2
Overlay of structures and active site with and without a sulfate ion bound.

Tyr85 and His172. The crystallographic interface between chain *B* molecules consists of a weak hydrogen bond of 3.25 Å between Gln89 and Glu184. The interactions at the interface of molecules *A* and *B* in the asymmetric unit involve Arg105 and His109 from helix 3 of molecule *A* with Ser49 from helix 1 as well as Thr207 of molecule *B*.

The first of the four remaining interfaces has hydrogen bonding between Glu184 and Arg223 from helices 6 and 7 as well as His195 from strand β 8 of chain *A*, with residues Glu4, Ser7 and Thr15 near the N-terminus of chain *B*. The second consists of hydrogen bonds between Thr15 and Ser7 of chain *A* and Glu184 and Gly187 of chain *B*. The third interface involves hydrogen bonding between Arg45 and Ser49 of helix 1 and Ser22 from chain *A* and Arg105, His109 and Pro111 of helix 3 and loop 8 of chain *B*. The final interface involves a single interaction between Lys160 and Asp226 of neighbouring chain *B* molecules.

3.4. Structural changes

The r.m.s. displacement plot for residues of the $P2_12_12_1$ structure superimposed with molecule *A* of the *C2* crystal highlights regions with the most variation (Fig. 3*a*). Similar differences were noted between molecules *A* and *B* of the *C2* crystal. The backbone segment that has the most variation for both chains of the *C2* structure is the section between residues 22 and 27 that comprise loop 3. Ser22 is involved in hydrogen bonding to a different residue in the *C2* crystal

form than in the $P2_12_12_1$ crystal form. In the $P2_12_12_1$ crystal it hydrogen bonds to the backbone of Ala188 at a distance of 2.97 Å, while in molecule *A* of the *C2* crystal it interacts with Arg105 of molecule *B* at a distance of 3.18 Å. Another significant region of difference is between residues 169 and 185 that comprise loop 12 and helix 6. As mentioned previously, the DLH interface involving helix 6 is along the crystallographic twofold symmetry axis for the *C2* crystal. Residues His172 and Ala176 are involved in interactions at this interface and the change in side-chain orientation to establish hydrogen bonds and accommodate the new interface is reflected in both the main-chain and side-chain difference values. Overall, where the difference is in side-chain orientation, it is observed that most differences are for charged residues that are involved in interactions at the protein–protein interfaces within the crystals.

Fig. 3(*b*) shows a temperature-factor analysis for the main-chain atoms of the $P2_12_12_1$ structure and each chain of the *C2* structure. Individual residues have been identified where the *B* factor is large in all structures or there is a significant difference in the value between structures. For example, Gln76 has a large *B* factor in both chains of the *C2* structure (33.0 and 39.0 Å²) but not in the $P2_12_12_1$ (13.2 Å²). In the latter Gln76 is involved in hydrogen bonding to Gln9 of a neighbouring molecule, while in the *C2* structure it does not form crystal contacts. Similarly, Ala176 forms crystal contacts in chain *A* of the *C2* crystal but not in chain *B* or in the $P2_12_12_1$ structure. The high flexibility of solvent-exposed surface loops (3, 6, 8, 12 and 14) is

Figure 3

(a) R.m.s. distance difference plot showing the distance between the $P2_12_12_1$ and chain A of the $C2$ structure for both the main-chain and side-chain atoms. Note that some side-chain r.m.s. difference values are zero; occupancy was set to zero during structure refinement because of high flexibility shown by poor electron density. (b) Average B factors of main-chain atoms for each residue of the $P2_12_12_1$ and both chains of the $C2$ structure. (c) The difference in accessible surface area caused by crystal contacts.

confirmed with peaks in the observed B factors. Fig. 3(c) shows the loss in accessible surface area (ASA) per residue as a result of crystal packing. The different interfaces are reflected here; for example, loop 6 is at a crystallographic interface in both chains of the $C2$ crystal but not in the $P2_12_12_1$ crystal. The total loss in ASA for the enzyme is greater for the $C2$ crystal, with a difference of 3106 Å² for chain A and 2747 Å² for chain B compared with 2303 Å² for the enzyme when in the $P2_12_12_1$ crystal form.

Studies into the influence of the crystal environment on protein structure have identified aspects that are most likely to vary with a changing environment (Zhang *et al.*, 1995). The orientations of side chains on the surface of proteins have been observed to differ among proteins crystallized in multiple space groups, and this is especially true for polar residues (Jacobson *et al.*, 2002). Flexible loop regions

have also been observed to exhibit different conformations when in different crystallographic environments (Kishan *et al.*, 1994). The changes observed for DLH are consistent with these previous observations.

4. Conclusion

This is the first report of DLH crystallizing in a space group other than $P2_12_12_1$. The new $C2$ crystal form can be attributed to the absence of a significant concentration of sulfate or phosphate. In the $P2_12_12_1$ crystals one sulfate ion binds to cause a minor shift in the backbone conformation of a loop that forms part of an interface in both crystal forms. More importantly, a second sulfate ion forms an

important part of an interface. The crucial location of this sulfate in the lattice explains the necessity for a high ammonium sulfate concentration in the crystallization buffer for formation of the orthorhombic $P2_12_12_1$ crystals. Subsequently, it also explains why, in the absence of sulfate or phosphate ions despite a similar ionic strength and pH, the $P2_12_12_1$ lattice previously reported cannot form and DLH crystallizes in a new space group.

The overall structures of a single DLH molecule crystallized in either the $P2_12_12_1$ or $C2$ space groups are very similar. The secondary and tertiary structures can be easily aligned with minimal discrepancy, and this is also the case for both molecules in the $C2$ asymmetric unit. The main differences are in the side-chain orientations, more specifically for surface residues at protein–protein interfaces within the crystal lattice. In regions of the protein where there are no crystal contacts in either crystal form (*i.e.* between residues 110 and 140), there are only very slight differences in residue side-chain orientation and main-chain position. This highlights the fact that the differences in structure caused by crystal contacts as a result of crystal packing are minor. One of the frequent criticisms of crystallography is that crystal contacts distort molecular structure. In the case of DLH, the effects of crystal packing are small.

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