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Crystallization and preliminary X-ray crystallographic studies on the class II cholesterol oxidase from *Burkholderia cepacia* containing bound flavin

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Burkholderia cepacia cholesterol oxidase (ChoS) is a 58.7 kDa molecular-weight flavoenzyme which has been categorized as a 3β-hydroxysteroid oxidase converting the 3β-hydroxyl group of a range of hydroxysteroids to the corresponding ketone. Analysis of enzymes with this activity has shown that two classes of cholesterol oxidase can be defined. Enzymes belonging to class I contain non-covalently bound FAD, whereas the class II enzymes contain FAD covalently bound to an active-site histidine. Despite catalysing the same chemical reaction, the class I and class II enzymes show no sequence similarity and have a different molecular architecture. Crystals of a recombinant class II enzyme from *B. cepacia* have been grown by the hanging-drop vapour-diffusion method using polyethylene glycol as a precipitating agent. The crystals belong to space group $P3_121$, with unit-cell parameters $a = b = 119.6$, $c = 101.1$ Å, and have one subunit in the asymmetric unit. These crystals diffract to at least 2.0 Å resolution at the Daresbury SRS and are suitable for a full structure determination. Ultimately, analysis of the structure of *B. cepacia* ChoS may allow the characteristics and structural features which contribute to its suitability as a diagnostic reagent for the detection of cholesterol and unresolved mechanistic features of the class II enzymes to be understood.

1. Introduction

Cholesterol oxidase (3β-hydroxysteroid oxidase; EC 1.1.3.6), a key enzyme in cholesterol metabolism, is an FAD-dependent enzyme which catalyses the oxidation of 3β-hydroxyl groups of steroids to the corresponding ketone. This enzyme has an important application in clinical assay kits for the determination of serum levels of cholesterol in a coupled system with cholesterol esterase and peroxidase (Allain *et al.*, 1974). Biochemical studies of cholesterol oxidase from *Brevibacterium sterolicum* have suggested that two classes of enzyme can be distinguished (Croteau & Vrielink, 1996). The class I enzymes have been shown to contain non-covalently bound FAD. In contrast, the class II enzymes contain FAD covalently linked to an active-site histidine *via* the C8α atom of the flavin isoalloxazine ring. Despite catalysing the same chemical reaction, these two classes of enzyme show no significant sequence similarity.

Cholesterol oxidase (ChoS) from *Burkholderia cepacia* oxidizes various 3β-hydroxysteroids with the activity for cholesterol being greatest (Doukyu & Aono, 1998). This enzyme is a particularly attractive target for the development of a cholesterol assay because the Michaelis constant of the *Burkholderia* sp. ChoS enzyme is lower than that of other commercially available oxidases. Biochemical studies have shown that a further favourable feature of the *Burkholderia* sp. ChoS is the finding that in the organic solvents used in the clinical assay the enzyme is both stable and shows an oxidation rate enhanced by more than threefold (Doukyu & Aono, 1998). Analysis of the sequence of the cholesterol oxidase from *B. cepacia* showed no similarity to the class I enzymes. Rather, this enzyme shows 46% sequence identity to the class II cholesterol oxidase from *Br. sterolicum*, indicating that the *B. cepacia* enzyme is a class II cholesterol oxidase containing covalently bound FAD.

The structures of representative class I enzymes containing non-covalently bound FAD have been determined for the enzymes from both *Br. sterolicum* (Vrielink *et al.*, 1991) and *Streptomyces* sp. (Yue *et al.*, 1999). More recently, the structure of the *Br. sterolicum* class II cholesterol oxidase containing covalently bound FAD from has been solved to 1.7 Å resolution and has shown that the fold of this enzyme is unrelated to that of the class I enzyme (Coulombe *et al.*, 2001). Analysis of the class II *Br. sterolicum* ChoS has shown that the active site consists of a cavity sealed off from the exterior of the protein. Moreover, a

novel feature suggested for this enzyme from the structure is the observation that a water-filled hydrophobic channel extending to the flavin moiety may act as the entry point for oxygen to the catalytic site during the oxidative half-reaction. Control of access by oxygen to this cavity is suggested to involve concerted movements of key arginine, glutamate and isoleucine residues which were observed to occupy multiple conformations in the structure (Coulombe *et al.*, 2001). In order to contribute to a full understanding of the mechanism of enzymes belonging to this class and, in particular, to the nature of the conformational changes which control gating to molecular oxygen, we have initiated crystallographic studies on *Burkholderia* sp. ChoS. Furthermore, the structure might also provide important insights into the molecular features of *B. cepacia* cholesterol oxidase that make it stable in the presence of organic solvents and therefore suitable as a diagnostic reagent for cholesterol. In this paper, we report the crystallization and preliminary X-ray analysis of ChoS.

2. Materials and methods

The overexpression and initial purification of the *B. cepacia* ChoS were initially carried out as previously described (Doukyu & Aono, 1998). However, to further purify the protein before crystallization trials, an additional step of preparative gel-filtration chromatography was carried out using a Superdex 200 HiLoad 16/60 column (Pharmacia) equilibrated with 10 m*M* PBS buffer pH 8.0. After this step, the enzyme was concentrated to 10 mg ml^{-1} (estimated by the Bradford assay; Bradford, 1976) in 10 m*M* Tris–HCl pH 8.5. The protein sample was then centrifuged at 25 000*g* for 10 min to clarify the solution before initiating any crystal trials.

Preliminary crystallization experiments were carried out at 290 K with the Hampton Crystal Screens I and II and the PEG ion screen using the hanging-drop vapour-diffusion technique, mixing 2 µl of protein solution and an equivalent volume of reservoir solution. Promising trials were refined to give optimum conditions, the best of which were based on a reservoir solution consisting 28% PEG 400, 0.1 *M* Na HEPES pH 7.5 and 0.2 *M* calcium chloride. Under these conditions, crystals with the morphology of square plates of dimensions up to 0.2 × 0.25 × 0.2 mm were obtained after two weeks. Preliminary X-ray analysis of the crystals, flash-frozen using the reservoir buffer as a cryoprotectant, showed that they diffracted well to high resolution. Subsequently, a complete data set was collected to 2.0 Å resolution at 100 K, using 1° rotation frames and X-rays with a wavelength of 0.978 Å on an ADSC Quantum 4 detector at station PX 14.2 at the Daresbury SRS. The data were processed using the *DENZO*/*SCALEPACK* package (Otwinowski & Minor, 1997).

Table 1
Data-collection statistics for the 2.0 Å ChoS data set.

Values in parentheses indicate values for the highest resolution shell.

Resolution (Å)	15–2.0 (2.15–2.0)
No. of unique reflections	45559
Completeness (%)	88.9 (91)
$R_{\rm merge}$ (%)	0.059 (0.348)
$I/\sigma(I) > 3$ (%)	82.6 (58.7)
Multiplicity (%)	4.48 (4.30)

3. Results and discussion

Autoindexing and scaling of the data using *DENZO*/*SCALEPACK* and analysis of the pattern of systematic absences is consistent with a primitive trigonal crystal system, identifying the space group as $P3_121$ (or its enantiomorph $P3_221$), with unit-cell parameters $a = b = 119.6$, $c = 101.1$ Å. Significant reflections were observed to the edge of the image-plate detector and a high-quality data set was collected to this resolution (Table 1). Considerations of possible values of $V_{\rm M}$ suggest that the asymmetric unit could either contain a monomer with a $V_{\rm M}$ of 3.5 Å^3 Da^{-1} or a dimer with a $V_{\rm M}$ of 1.75 Å^3 Da^{-1}, both of which lie within the range observed by Matthews (1977), with the former being more probable. Gel-filtration studies on ChoS strongly suggest that this enzyme is a monomer in PBS buffer solution, consistent with this conclusion.

In order to provide a completely independent solution to the structure, we initially carried out a heavy-atom screen which yielded a number of very promising derivatives based on either mercury, gold or gadolinium. Using these derivatives a preliminary map at 2.4 Å resolution was calculated and showed a clear molecular boundary. Whilst this work was in progress, the coordinates for the class II ChoS from *Br. sterilocum* were deposited in the Protein Data Bank (Berman *et al.*, 2000) (PDB code 1i19). A preliminary attempt to solve the structure by molecular replacement gave a solution in space group $P3_121$ corresponding to a monomer in the asymmetric unit, which made acceptable packing interactions in the cell. Given the quality of the derivatives and in order to examine the conformation of key residues at the active site in a manner in which any bias is minimized, we have chosen to proceed with the structure determination using an isomorphous replacement approach. Ultimately, it is hoped that a complete solution of this structure will lead to a better understanding of the enzymes belonging to the cholesterol oxidase family and so help in the design of more efficient clinical tools for the assay of cholesterol.

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