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Crystallization and X-ray diffraction data analysis of leukotriene A_4 hydrolase from *Saccharomyces cerevisiae*

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The *Saccharomyces cerevisiae* leukotriene A_4 (LTA_4) hydrolase (*sc*LTA_4 hydrolase) has been crystallized in order to study the two activities of LTA_4 hydrolase in an evolutionary perspective. Single well diffracting crystals are obtained after switching from the hanging-drop method to liquid–liquid diffusion in capillaries using PEG 8000 as precipitant. These crystals belong to space group $P2_12_12_1$, with unit-cell parameters a = 70.8, b = 98.1, c = 99.2 Å. Intensity data to 2.3 Å resolution were collected from a native *sc*LTA_4 hydrolase crystal using synchrotron radiation. A molecular-replacement solution was obtained using the human LTA_4 hydrolase structure and the program *BEAST*.

1. Introduction

Leukotriene A_4 (LTA_4) hydrolase is a pivotal enzyme in the biosynthesis of leukotrienes, a family of lipid mediators implicated in several inflammatory and hypersensitivity reactions (Samuelsson, 1983). It is a soluble monomeric zinc metalloenzyme with a molecular mass of about 69 kDa. LTA_4 hydrolase catalyses the hydrolysis of the epoxide LTA_4 to the pro-inflammatory LTB_4, which is a potent chemotaxin and leukocyte-activating agent (Samuelsson, 1983; Ford-Hutchinson, 1990). Furthermore, the enzyme possesses an aminopeptidase activity towards certain arginyl di- and tripeptides *in vitro*. However, the physiological substrate or role of this activity is presently unknown (Haeggström *et al.*, 1990; Minami *et al.*, 1990; Orning *et al.*, 1994). The enzyme has a wide tissue distribution and has been purified from several mammalian sources. Very little is known about the evolution of LTA_4 hydrolase and the phylogenetic relationship between its two catalytic activities. In fact, the formation of LTB_4 has been described in birds, frogs and fish, but never in invertebrate species (Habenicht *et al.*, 1989; Green, 1987; Green *et al.*, 1987; Pettitt *et al.*, 1991; Knight *et al.*, 1995).

In order to study these two activities in an evolutionary perspective, the structure of a LTA_4 hydrolase homologue from yeast, *Saccharomyces cerevisiae* (*sc*LTA_4 hydrolase), would be of great interest. The similarity between the human and yeast enzymes is more than 50% (42% identity). In contrast to the human enzyme, the yeast homologue has a normal aminopeptidase activity but only a residual epoxide hydrolase activity. It has a higher turnover number for the aminopeptidase activity and a decreased product specificity for LTA_4 hydrolysis (Kull *et al.*, 1999). Also, the substrate specificity of the aminopeptidase activity is different. The yeast enzyme has a preference for Leu as the N-terminal residue, while the human enzyme prefers an Arg residue. Surprisingly, the aminopeptidase activity in the yeast enzyme is strongly stimulated by LTA_4 in a fashion that suggests the presence of a lipid-binding pocket located at the active centre of the enzyme and presumably overlapping with the catalytic sites (Kull *et al.*, 1999). *sc*LTA_4 hydrolase was recently shown to convert LTA_4 into LTB_4, further corroborating the notion that this enzyme is an early ancestor of its mammalian counterparts (Kull *et al.*, 2001).

It is difficult to predict from sequence alignments which residues are responsible for the catalytic differences. Therefore, the structure of the yeast enzyme is important for further understanding of the enzyme's physical and functional properties. Aside from that, it may also give us further insight into the evolutionary pathway of these enzymes and the way in which the two reactions have been integrated in the same enzyme. The *sc*LTA_4 hydrolase has been cloned, expressed and further characterized (Kull *et al.*, 1999, 2001). Here, we describe the purification, crystallization by a liquid–liquid diffusion technique and preliminary structural studies of *sc*LTA_4 hydrolase.

2. Materials and methods

2.1. Protein expression and purification

In order to avoid problems with translational variants, variant IV of the *sc*LTA_4 hydrolase was used for the expression and purification of *sc*LTA_4 hydrolase (Kull *et al.*,

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1999). Consequently, 40 codons were removed from the 5′-region of the original cDNA, which positions the Met at position −40 as the translation-initiation site. For rapid purification on nickel-affinity chromatography, a six-histidine tag was attached immediately after the start codon, resulting in the plasmid pT3_*sc*LTA4H-40his. The pT3_*sc*LTA4H-40his plasmid was transformed into competent *Escherichia coli* (JM 101) cells. Expression and preparation of crude protein extracts was performed as described in Medina *et al.* (1991). The protein was purified to apparent homogeneity as described in Kull *et al.* (2001).

2.2. Crystallization by the hanging-drop vapour-diffusion method

The *sc*LTA_4 hydrolase was concentrated to 10 mg ml^{-1} in 10 m*M* Tris–HCl buffer at pH 7.5 by ultrafiltration using an YM3 membrane (Amicon, Beverly, USA) with a nominal molecular-weight cutoff of 3000 Da. Crystals were initially grown by hanging-drop vapour-diffusion techniques using Hampton Research Crystal Screens 1 and 2 at both 277 K and room temperature. Crystallization solutions were prepared by mixing 2 µl of protein solution with 3 µl of reservoir solution in a 24-well culture plate. The hanging drop on the cover glass was vapour-equilibrated against 0.5 ml of the reservoir solution in each well of the tissue-culture plate. Screens were stored at both 277 K and room temperature, respectively, and observations were made 1 day after setup and at weekly periods thereafter.

2.3. Crystallization by capillary liquid–liquid diffusion method

In this method, 5 µl of protein solution is layered on top of 5 µl PEG solution in an S80 melting-point capillary (Modulohm, Denmark). Each solution is spun down at 800 rev min^{-1} in a swing-out centrifuge for a period of 60 s. When the second solution is spun down, a sharp boundary between the two layers forms owing to the higher density of the precipitant solution. The open end of each tube is subsequently sealed by melting the glass. The crystallization conditions for the crystals grown in capillaries were initially adapted from the best conditions that produced the original crystals in the hanging-drop experiments.

2.4. Cryocooling and data collection

The crystals are very sensitive to radiation damage. In order to collect complete data sets, cryocooling is necessary.

Table 1
Data-collection statistics.

Values in parentheses represent the values for the highest resolution shell.

Space group	$P2_12_12_1$
Unit-cell parameters (Å, °)	a = 70.8, b = 98.1, c = 99.2, $\alpha = \beta = \gamma$ = 90
Wavelength (Å)	1.0854
Resolution (Å)	20–2.3 (2.38–2.3)
Unique reflections	29531 (2629)
$I/\sigma(I)$	5.2 (14)
Redundancy	2.2 (1.5)
Data completeness (%)	94.8 (86.0)
$R_{\rm merge}$	0.087 (0.243)

Crystals were removed from the capillaries by gravity into a 5 µl drop of mother liquor [100 m*M* Tris–HCl pH 7.3, 13%(*w*/*v*) PEG 8000] containing 2.5 mg ml^{-1} *sc*LTA_4 hydrolase. The crystals were then soaked for 5 s with the stepwise addition of increasing amounts of cryoprotectant solution [crystallization liquor complemented with 20%(*w*/*v*) L-(+)-2,3-butanediol and 2.5 mg ml^{-1} *sc*LTA_4 hydrolase] to the drops of mother liquor containing protein and the crystals. The crystal cryostabilization proceeded over four steps, with the addition of 2, 5, 10 and finally 15 µl cryoprotectant. The crystal was then scooped up in a rayon loop and flash-frozen in cold nitrogen gas (100 K) and diffraction data were collected from a single crystal (1.6 × 0.2 × 0.2 mm) on a 345 mm 150 µm phosphoimaging-plate detector (MAR Research) at beamline I711, Max-lab, Lund, Sweden. The data were indexed and integrated with *DENZO* (Otwinowski & Minor, 1997) and merged with *SCALEPACK* (Otwinowski, 1993). Data-collection and processing statistics can be found in Table 1.

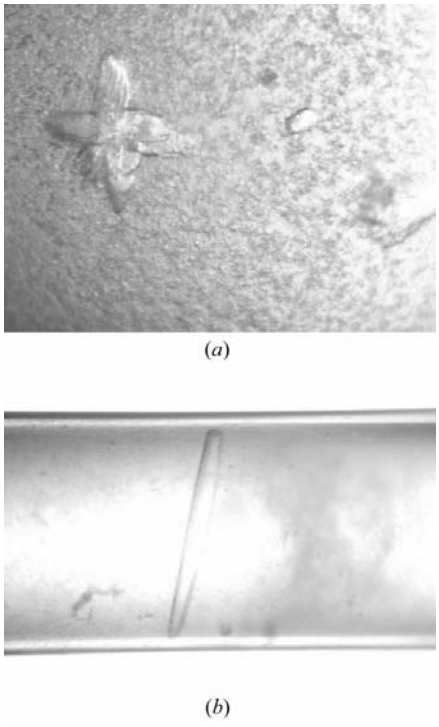

Figure 1
(*a*) Multi-crystal of *sc*LTA_4 hydrolase grown by hanging-drop vapour-diffusion methods. (*b*) A crystal of *sc*LTA_4 hydrolase grown by capillary liquid–liquid diffusion methods in space group $P2_12_12_1$, with approximate dimensions 1.6 × 0.2 × 0.2 mm. In contrast to the crystals grown by hanging-drop vapour-diffusion methods, crystals grown by capillary liquid–liquid diffusion methods can be rapidly reproduced and diffract to a higher resolution.

2.5. Molecular replacement

Since *sc*LTA_4 hydrolase has 42% identity at the amino-acid level with the human enzyme, the structure was solved by molecular-replacement methods using the program *BEAST* (Read, 2001). The human LTA_4 hydrolase (PDB code 1hs6; Thunnissen *et al.*, 2001) without water molecules was used as a search model. The rotation search (resolution range 15–2.3 Å) gave a peak of 6.7σ above the mean. A subsequent translation search using the same resolution limits gave a position 11.2σ above the mean. Further steps of model building and refinement are currently in progress.

3. Results and discussion

We have succeeded in the crystallization and subsequent X-ray diffraction data analysis of *sc*LTA_4 hydrolase. Initially, crystals were grown by hanging-drop vapour-diffusion techniques using Hampton Research Crystal Screens 1 and 2. One solution (No. 36) from Hampton Research Crystal Screen 1 [8%(*w*/*v*) PEG 8000 in 100 m*M* Tris–HCl pH 8.5] produced promising results. After routine optimization, the best condition was determined to be 100 m*M* Tris–HCl pH 7.25, 7–7.5%(*w*/*v*) PEG 8000 and ∼4 mg ml^{-1} protein. In this early work, most crystals were small, tended to grow as multi-crystals or show surface defects (Fig. 1*a*) and diffracted poorly (∼3.5 Å) using an in-house rotating-anode X-ray facility. In addition, reproducibility of the crystallization was a major problem, since only 2% of the drops contained crystals that were usable for diffraction.

In order to improve the reproducibility of crystal formation and to obtain better diffracting crystals, the purification procedure was taken into consideration; however, the original procedures proved to give the best crystals. Moreover, extensive trials with hanging-drop, sitting-drop and macro-seeding techniques as well as addition of

additives resulted only in small or poor crystals. Finally, long rod-shaped well diffracting crystals (Fig. 1*b*) were obtained by the capillary liquid–liquid diffusion method, incubating at 295 K.

Further optimization gave optimal crystallization conditions for liquid–liquid diffusion experiments consisting of a precipitant solution containing 100 m*M* Tris–HCl pH 7.2–7.4, 12–14%(*w*/*v*) PEG 8000 and a protein solution containing ∼6 mg ml^{-1} in 10 m*M* Tris–HCl buffer. Crystals of average dimensions 1.2 × 0.1 × 0.1 mm^{-1} grew in 8–10 weeks at room temperature. Since the conditions are not that different from the conditions that gave the best crystals using the hanging-drop method, it shows that screening the method of crystallization can be worthwhile in order to obtain better quality crystals.

The long rod-shaped crystals of *sc*LTA$_4$ hydrolase diffract well to 2 Å at room temperature using synchrotron radiation, but they are very radiation-sensitive. Only a few frames from each crystal can be collected before the crystals lose all diffraction intensity. Therefore, a cryoprotectant search for *sc*LTA$_4$ hydrolase was performed using the Hampton Research Cryoprotectant Kit.

L-(+)-2,3-Butanediol was found to be the most promising cryoprotectant. Success was only achieved when the mother liquor as well as the cryosolution were complemented with 2.5 mg ml^{-1} *sc*LTA$_4$ hydrolase.

Moreover, the addition of the cryoprotectant to the mother liquor has to be in a stepwise manner. The first increment of cryoprotectant to the drop was of 2 µl, the second 5 µl, the third 10 µl and the last 15 µl. These increments were allowed to diffuse through the mother liquor and crystal for at least 5 s. A faster increase in L-(+)-2,3-butanediol concentration invariably led to rupture of the crystals.

Data were processed to 2.3 Å resolution with a completeness of 94.8%. The crystals were found to be primitive orthorhombic and the space group was assigned as $P2_12_12_1$, with unit-cell parameters a = 70.8, b = 98.1, c = 99.2 Å, $\alpha = \beta = \gamma = 90^\circ$. Table 1 summarizes the data statistics.

We now have crystals of *sc*LTA$_4$ hydrolase that diffract to a resolution of 2.3 Å, which in turn has allowed us to solve the structure by molecular-replacement methods. Further steps of model building and refinement are in progress. The structure will be important for further understanding of the physical and functional properties of the enzyme. Furthermore, it will hopefully unravel which parts of the active site are critical for allosteric binding and turnover of LTA$_4$.

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