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Crystallization and preliminary X-ray analysis of chondroitin sulfate ABC lyases I and II from *Proteus vulgaris*

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Chondroitin sulfate ABC lyases (E.C. 4.2.2.4) are broad-specificity glycosaminoglycan-degrading enzymes. Their preferred substrates are chondroitin sulfate and dermatan sulfate, which are broken down to short oligosaccharides. *Proteus vulgaris* produces two such lyases, ABC lyase I and II, with molecular weights of 112–113 kDa. Diffraction-quality crystals of both enzymes have been obtained by the hanging-drop vapour-diffusion method. ABC lyase I crystallizes in space group $P2_12_12_1$, with unit-cell parameters a = 49.3, b = 95.1, c = 230.0 Å, Z = 4, and diffracts to 1.9 Å resolution. Crystals of ABC lyase II belong to space group $P1$, with unit-cell parameters a = 64.2, b = 64.3, c = 142.1 Å, α = 95.7, β = 98.1, γ = 95.5°, Z = 2; diffraction extends to at least 2.1 Å.

1. Introduction

Glycosaminoglycans (GAGs) are polymers of repeating disaccharide units of hexosamine and glucuronic acid or iduronic acid and are found in most cases as part of extracellular matrix (ECM) proteoglycans (Jackson *et al.*, 1991). Many bacteria produce inducible enzymes capable of degrading the GAG component of the ECM. These enzymes utilize two different enzymatic mechanisms: hydrolysis or lytic β-elimination. Bacterial GAG lyases have been divided into several groups based on their substrate specificity (reviewed by Ernst *et al.*, 1995). Relatively little is known about the details of their catalytic mechanism, although a model for general steps involved in β-elimination catalysis has been proposed (Gacesa, 1987). Structures of only two GAG lyases have been reported: chondroitinase AC, specific for chondroitin 4-sulfate and 6-sulfate (Féthière *et al.*, 1999), and chondroitinase B, specific for dermatan sulfate (Huang *et al.*, 1999), both determined in our laboratory. Their three-dimensional structures are very different. Chondroitinase B belongs to the β-helix fold superfamily together with such enzymes as pectin and pectate lyases (Yoder *et al.*, 1993; Mayans *et al.*, 1997) and polygalacturonase (Pickersgill *et al.*, 1998). Chondroitinase AC has an N-terminal domain with the $(\alpha\alpha)_5$ topology, reminiscent of the $(\alpha\alpha)_6$ topology of glucosyl hydrolases from families 5, 8, 9 and 15, and a C-terminal domain composed of a four-layered β-sheet (Féthière *et al.*, 1999).

Chondroitin sulfate ABC lyase (chondroitinase ABC; E.C. 4.2.2.4) has broad specificity and is capable of degrading both chondroitin sulfate and dermatan sulfate, thus combining the activities of two separate and structurally unrelated enzymes, chondroitinase AC and chondroitinase B. This is of special interest from the enzymatic viewpoint, since the active centres of the two substrates differ in stereochemistry: iduronic and glucuronic acid are epimers distinguished by the configuration of the carboxylate group.

P. vulgaris produces two chondroitinases ABC, both of which have been purified to homogeneity and biochemically characterized (Hamai *et al.*, 1997). Chondroitinase ABC I (MW = 113 kDa) was cloned and sequenced (Sato *et al.*, 1994; NCBI GenBank PIDg1095454) and was shown to be an endolytic enzyme with a pH optimum near 8. Chondroitinase ABC II (MW = 112 kDa; NCBI GenBank PIDg1828879) acts exolytically and also has a pH optimum of 8. Although the amino-acid sequence of this protein has not yet been completed, data available so far suggest that the chondroitinase ABC II is identical (or closely related) to the 'chondroitinase subcomponent' described by Khandke *et al.* (1995). The two *P. vulgaris* chondroitinases share a low level of sequence identity (26%) which extends throughout their entire lengths. It is thus likely that their fold will be similar. Interestingly, they also show a low level of identity (~20%) in their C-terminal parts to the C-terminal domain of chondroitinase AC and several bacterial hyaluronate lyases (Féthière *et al.*, 1999). However, there is no detectable sequence homology within their N-terminal segments. Some homology has also been found to chondroitinase ABC 2 from *Bacteroides thetaiotaomicron*. Although only one sequence of ~600

Table 1
Data-collection statistics.

Values for the last shell are given in parentheses.

	ChS ABC I	ChS ABC II
Space group	$P2_12_12_1$	$P1$
Resolution (Å)	20–1.9	20–2.1
Last shell (Å)	1.93–1.90	2.14–2.10
No. of observations	805344	175900
No. of unique reflections	78220	115019
Completeness (%)	91.3 (82.4)	89.8 (54.9)
R_{sym}†	0.068 (0.248)	0.078 (0.237)
Overall mean $I/\sigma(I)$	15.5	7.6

† $R_{sym} = \sum|I_i - \langle I\rangle|/\sum I_i$, where I_i stands for the intensity of the ith observation and $\langle I\rangle$ for the mean intensity of the reflection.

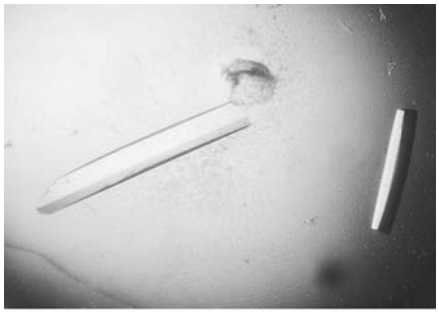
amino acids can be found in the NCBI GenBank, the literature reports isolation and purification (Linn *et al.*, 1983) and cloning (Guthrie *et al.*, 1985) of two such lyases with molecular weights of 104 and 108 kDa, respectively.

We describe here the preparation of diffraction-quality crystals of chondroitinase ABC I and chondroitinase ABC II and their characterization. These crystals are suitable for high-resolution structure determination.

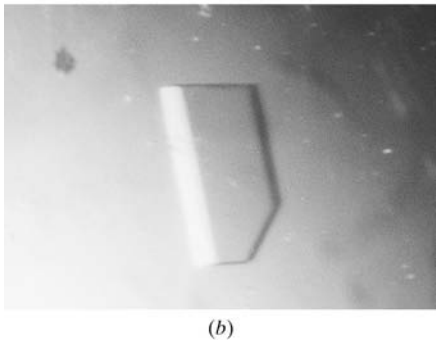
2. Methods and results

2.1. Protein purification

Chondroitin sulfate ABC lyases I and II were purified from *P. vulgaris* NCTC 4636 following a previously described procedure (Hamai *et al.*, 1997). Briefly, bacterial cells were extracted with 5%(*w*/*v*) polyoxyethylene lauryl ether in phosphate buffer pH 7.0. The supernatant was loaded on a CM-Sepharose column and eluted with a linear gradient of 0–0.2 *M* NaCl. Two major protein peaks appeared in the elution profile (designated I and II in order of elution), both with chondroitin sulfate lyase activity. These enzyme fractions were pure according to SDS–polyacrylamide gel electrophoresis.



(*a*)



(*b*)

Figure 1
(*a*) Crystals of chondroitin sulfate ABC lyase I, (*b*) crystals of chondroitin sulfate ABC lyase II.

2.2. Crystallization

Protein samples of chondroitinase ABC I and ABC II were concentrated to approximately 10 mg ml^{-1} by ultrafiltration using a Centricon concentrator (Amicon) with a 30 kDa cutoff. At the same time, the buffer was changed to 20 m*M* Tris–HCl pH 8.0. Protein concentration was estimated by the method of Bradford, using BSA as a standard (Bradford, 1977). The concentrated protein solution was divided into 100 µl aliquots and stored at 253 K until further use. The hanging-drop method was used for crystallization. Each drop was suspended over 1 ml of reservoir solution and contained 5 µl protein solution mixed with 5 µl of reservoir solution. All crystallizations were carried out at room temperature.

Initial crystallization conditions were found using the sparse-matrix method with screens I and II from Hampton Research (Laguna, Niguel, CA, USA) and were further optimized by a systematic search around the initial conditions.

2.2.1. Chondroitin sulfate ABC lyase I. The best crystals were obtained from 15%(*v*/*v*) polyethylene glycol (PEG) 3350, 0.2 *M* ammonium acetate, 0.2 *M* magnesium acetate, 0.1 *M* Tris–HCl pH 8.0 and appeared after approximately three weeks. They grew as elongated plates (Fig. 1*a*) and often occurred in fan-like clusters spreading from a single nucleation site. To obtain single crystals and to decrease the time needed to obtain them, the macroseeding method was employed. A seed crystal was washed in a solution containing 10%(*v*/*v*) PEG for approximately 10 min and then transferred with a nylon loop (Hampton Research, Laguna Niguel, CA, USA) to a new drop. These crystals grew to maximum dimensions of 0.1 × 0.3 × 1.0 mm within one month. They belong to orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 49.3, b = 95.1, c = 230.0 Å, and diffract to 1.9 Å resolution in the synchrotron beam. Assuming one molecule in the asymmetric unit, the V_m value is 2.7 Å^3 Da^{-1}, which is within the expected range (Matthews, 1968).

2.2.2. Chondroitin sulfate ABC lyase II. The best crystallization conditions for this protein were 10%(*v*/*v*) PEG monoethyl ether 5000, 0.2 *M* ammonium acetate, 0.2 *M* magnesium acetate, 50 m*M* cacodylate buffer pH 6.5. These crystals grew in bunches. Employing macroseeding as above was essential for reducing the nucleation and for obtaining single crystals of diffraction quality. These crystals had the shape of thin rhombic plates (Fig. 1*b*) and grew to maximum dimensions of 0.2 × 0.3 × 0.3 mm in about a month. They belong to the triclinic space group $P1$, with unit-cell parameters a = 64.2, b = 64.3, c = 142.1 Å, α = 95.7, β = 98.1, γ = 95.5°. Although these lattice points can be described by a monoclinic C-centred cell (with unit-cell parameters a = 86.2, b = 95.1, c = 142.1 Å, α = 90, β = 100.1, γ = 90°), the intensities show only approximate twofold symmetry, inconsistent with the $C2$ space group (see below). They diffract to 2.1 Å resolution using a rotating-anode generator and have a V_m value of 2.7 Å^3 Da^{-1}, assuming two molecules in the unit cell.

2.3. Data collection and analysis

Diffraction data for orthorhombic chondroitinase ABC I crystals were measured at the X8C beamline, Brookhaven National Laboratory, Upton, NY. The crystal was immersed in a cryoprotecting solution (the same as the mother liquor but containing 30% PEG 3350) for several hours, scooped into a nylon loop and flash-frozen to 100 K in a nitrogen stream generated by a Cryostream (Oxford Cryosystems, Oxford, England). Data were collected on a Quantum-4 CCD area detector at a wavelength of 1.009 Å in 1° oscillation frames. The frames were processed with *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). Data-collection statistics are shown in Table 1.

Diffraction data for crystals of chondroitin ABC lyase II were collected on an R-AXIS IIC image-plate detector mounted on a Rigaku RU-300 X-ray generator operated at 50 kV and 180 mA. Mother liquor with the concentration of PEG monoethyl ether 5000 increased to 30% was used as a cryoprotectant. Crystals were flash-frozen to 100 K in a nitrogen stream (Oxford Cryosystems) and data were collected in 1° oscillations to a maximum resolution of 2.1 Å (Table 1). Processing in the $P1$ space group led to an overall R_{sym} of 0.078 and an R_{sym} in the last shell (2.14–2.10 Å) of 0.237.

$\langle I/\sigma(I)\rangle$ in the last shell was 2.2. Attempts to process frames in the *C*2 space group led to an overall $R_{\rm sym}$ of 0.17, with $\langle I/\sigma(I)\rangle$ less than 1.0 beyond 2.4 Å resolution owing to high standard deviations. Therefore, the correct choice of space group is *P*1.

In accordance with the expectation of two molecules in the asymmetric unit for chondroitinase ABC II based on the V_m value and pseudo-*C*2 symmetry, the self-rotation function (program *POLARRFN*; Collaborative Computational Project, Number 4, 1994) showed a strong peak at $\kappa = 180.0$ with 36% intensity of the peak at the origin, indicating twofold non-crystallographic symmetry. The pseudo-symmetry axis is approximately parallel to the [1, −1, 0] diagonal direction.

In summary, both crystals diffract to high resolution and are suitable for structure-determination studies.

2.4. Heavy-atom derivative screening

Initial screening identified two potential heavy-atom derivatives for chondroitinase ABC I. Although this protein contains no cysteine residues, CH_3HgCl binds to specific sites within the crystal. Soaking the crystals in a cryoprotectant containing 1 m*M* and 5 m*M* CH_3HgCl resulted in two derivatives with different numbers of binding sites. Difference Patterson maps calculated at 5 Å resolution revealed several strong peaks in the Harker sections that could be easily interpreted. Screening for additional derivatives is in progress.

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