Acta Cryst. (1998). D**54**, 279–280

Crystallization and preliminary analysis of chondroitinase AC from *Flavobacterium heparinum*†

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(*Received 31 March 1997; accepted 17 June 1997*)

Abstract

Chondroitinase AC (E.C. 4.2.2.5) overexpressed in its host, *Flavobacterium heparinum*, was crystallized by vapor diffusion using polyethylene glycol methyl ether as precipitant. It crystallizes in the space group $P4_32_12$ or its enantiomorph with $a = b = 87.1$ and $c = 193.1$ Å and one molecule in the asymmetric unit. Crystals diffract to a maximum of 2.5 Å resolution on a rotating-anode source. Screening for heavy-atom derivatives identified a lead compound that binds to a single site on the protein. Further screening is in progress.

1. Introduction

Glycosaminoglycans (GAG's) are acidic polymeric saccharide structures composed of uronic acid and hexosamine. They are considered to be the most negatively charged naturally occurring biopolymers because of their uronic acid backbone and extensive sulfation. GAG's are linked to core proteins through Ser residues (Jackson *et al.*, 1991; Ernst *et al.*, 1995) forming proteoglycans that are present in the extracellular matrix and on the surface of many cell types. These act as a protective barrier to diffusion and are involved in the modulation of cell signalling. Proteoglycans also play a critical role in the pathophysiology of certain basement-membrane-related diseases, metastasis and angiogenesis (Jackson *et al.*, 1991; Ernst *et al.*, 1995). The ability to control GAG depolymerization might, therefore, have important repercussions on the evolution of certain diseases.

Two main classes of enzymes are responsible for the initial catabolism of GAG's: the lyases and the hydrolases (Linhardt *et al.*, 1986; Ernst *et al.*, 1995). Lyases are predominantly of microbial origin, where their primary role is to provide the organism with nutrients for growth rather than for tissue invasion (Linhardt *et al.*, 1986). Isolated from soil and intestinal bacteria where substrate is abundant, they act through an eliminative mechanism (Linhardt *et al.*, 1986) while mammalian hydrolases use a hydrolytic mechanism (Ernst *et al.*, 1995). Polysaccharides likely to be substrates for these lyases bear a carboxylate group on the C atom adjacent to the glycosidic linkage (Jackson *et al.*, 1991). *Flavobacterium heparinum*, a non-pathogenic Gram-negative bacteria, constitutively produces three classes of cell associated lyases: heparinases, chondroitinases, and hyaluronidases. One of them, chondroitinase AC, is responsible for the eliminative cleavage of chondroitin-4-sulfate, chondroitin-6-sulfate and hyaluronic acid to yield disaccharides and unsaturated uronic acid (Ernst *et al.*, 1995; Gu *et al.*, 1995). This enzyme is a monomer with a molecular weight of 79 kDa (as assessed by electrospray mass spectrometry), a p*I* of 8.8, and two *O*-glycosylation sites (Laliberté *et al.*, 1996). Its catalytic activity is influenced by divalent cations and the presence of other GAG's (Ernst *et al.*, 1995; Yamagata *et al.*, 1968), such as heparin and dermatan sulfate. Lyases from different sources vary in substrate specificity, and catalytic parameters (Ernst *et al.*, 1995; Hiyama & Okada, 1976, 1977; Gu *et al.*, 1993), indicating possible variations in their catalytic mechanisms.

To date there are no structural data for enzymes of this class. In order to understand the molecular aspects of the substrate specificity and catalytic mechanism of these lyases we have undertaken the structure determination of chondroitinase AC from *F. heparinum*. We report here the crystallization and preliminary results toward determination of the structure of this lyase.

2. Methods and results

2.1. *Protein purification*

Chondroitinase AC from *F. heparinum* ATCC 13125 was purified following the method of Gu *et al.* (Gu *et al.*, 1995). A broth from 3 l fermentation was centrifuged, the cell pellet was lysed by sonication, and the supernatant was subjected to two successive chromatographic steps, SP-Sepharose, and hydroxyapatite. This procedure yielded 375 mg of chondroitinase AC. The enzyme was 98.6% pure as determined by sodium dodecyl sulfate–electrophoresis and reverse-phase high-pressure liquid chromatography. This preparation was dialyzed against 20 m*M* Tris–HCl, pH 8.0, concentrated in a centriprep concentrator (Amicon Co.) with a 10 kDa cut-off, and stored in aliquots at 203 K.

2.2. *Crystallization*

Chondroitinase AC is *O*-glycosylated on Ser306 and Ser433 (Laliberté *et al.*, 1996); no attempts were made to deglycosylate the enzyme prior to crystallization. Initial conditions for crystallization were obtained using the hanging-drop method with the sparse-matrix screen from Hampton Research (California) (Jancarik & Kim, 1991). Previously stored protein was thawed, and used directly for crystallization. The reservoir contained 1 ml of the precipitating solution and the drop was composed of 2 µl reservoir mixed with 2 µl of protein stock solution. Small crystals were formed under most of the screening conditions containing polyethylene glycol (PEG) as precipitant. Subsequently, PEG-monomethylether (PEG-MME) was chosen as a precipitant and larger crystals were obtained by the microseeding technique (Thaller *et al.*, 1985) with careful adjustment of the protein and precipitant concentrations. Small

† NRCC publication No. 39963. JF is a postdoctoral fellow from the Medical Research Council of Canada.

crystals obtained from 18% PEG-MME (average M_r = 2000), 80 mM ammonium acetate, pH 6.5 were used as the initial seeds. Nucleation was controlled by the addition of sodium acetate to the mother liquor. The best conditions for crystal growth were obtained with an initial protein concentration of 10 mg ml^{-1}, a reservoir containing 1 ml of 15–17% PEG-MME (average M_r = 2000), 80 mM ammonium acetate buffer, pH 6.5, and 400 mM sodium acetate. Drops for seeding were prepared by mixing 6 µl of reservoir and 3 µl of protein solution. Single diffraction quality crystals grew at 291 K to a size of 0.3 × 0.2 × 1.0 mm after 3 weeks (Fig. 1).

2.3. *Data collection*

Native data were collected from a single crystal on a R-AXIS IIC area detector mounted on a RU-300 Rigaku rotating-anode generator. Oscillation images with 1.5° oscillation range were processed with programs *DENZO* and *SCALEPACK* (Otwinowski, 1993; Minor, 1993). Data collection at room temperature indicated a rapid decay of the crystals upon exposure to the X-rays. Data were, therefore, collected from frozen crystals (Rodgers, 1994) as follows: crystals were cryoprotected in mother liquor to which 25% glycerol was added, mounted in a nylon loop (Hampton Research, California), flash frozen in liquid nitrogen, and transferred to a nitrogen stream maintained at 120 K (Oxford Cryosystems Cryostream, Oxford, UK). Under these conditions, the crystals showed very little radiation decay.

A 2.5 Å native data set has been collected. Processing of 122 455 observations led to 25 463 independent reflections (fivefold redundancy) with an $R_{\rm merge}$ of 0.059. The data is 96% complete to 2.5 Å with an $\langle I\sigma(I)\rangle$ of 19.9. In the high-resolution shell (2.59–2.50 Å) 60% of reflections have $I>5\sigma(I)$.

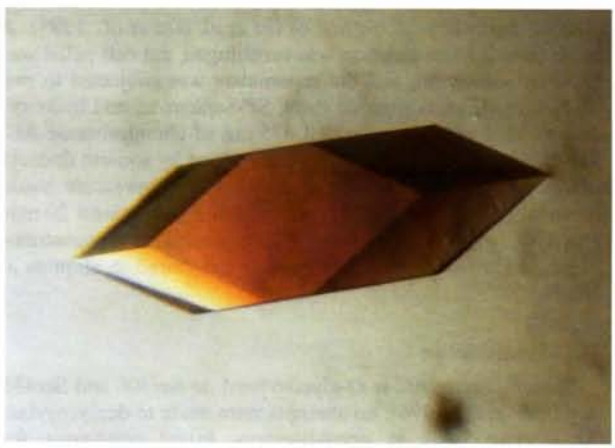

Fig. 1. Tetragonal crystal of chondroitinase AC from *F. heparinum*.

The crystals belong to the tetragonal system and have unit-cell parameters of $a = b = 87.1$, $c = 193.1$ Å. The systematic absences indicate space group $P4_32_12$ or its enantiomorph. Assuming one molecule in the asymmetric unit, a V_m value of 2.3 Å^3 Da^{-1} is obtained, within the normal range for protein crystals (Matthews, 1968).

2.4. *Heavy-atom derivatives screening*

A promising derivative data set was obtained from a crystal soaked for 40 h in a solution containing 40 mM lead acetate. This data set is 97% complete to 2.8 Å resolution, with a $\langle I/\sigma(I)\rangle$ of 13 and an $R_{\rm merge}$ of 0.067. This crystal was isomorphous to the native crystal and the data set scaled to the native set with an $R_{\rm iso}$ of 0.094. Despite this low $R_{\rm iso}$ value an isomorphous difference Patterson map, calculated with the program *PHASES* (Furey & Swaminathan, 1997), showed several strong peaks on Harker sections, all of which could be interpreted in terms of a single heavy-atom site. Parameters of the heavy atom were refined with the program *PHASES*95. Search for additional derivatives is under way.

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