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Crystallization and preliminary X-ray crystallographic analysis of the protease inhibitor ecotin in complex with chymotrypsin

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Ecotin, a homodimeric protein composed of 142-residue subunits, is a novel protease inhibitor present in the periplasm of *Escherichia coli*. It shows a broad inhibitory specificity towards a group of serine proteases and binds two molecules of protease to form a tetrameric complex in a distinct chelation mechanism. The ecotin–chymotrypsin complex has been crystallized in the triclinic space group $P1$ with unit-cell parameters a = 57.29, b = 57.39, c = 79.75 Å, α = 91.49, β = 88.63 and γ = 112.45°. The asymmetric unit contains the whole tetrameric complex, consisting of two molecules of chymotrypsin bound to the ecotin dimer, with a corresponding crystal volume per protein mass (V_M) of 2.58 Å^3 Da^{-1} and a solvent fraction of 48.9%. The crystals diffract beyond 2.0 Å with Cu $K\alpha$ X-rays and are very stable in the X-ray beam. Native X-ray data have been collected from a crystal to approximately 2.0 Å Bragg spacing.

1. Introduction

Ecotin, an *Escherichia coli* periplasmic protein, is a potent inhibitor of pancreatic serine proteases such as trypsin, chymotrypsin and elastase (Chung *et al.*, 1983). It is also a powerful inhibitor of human neutrophil elastase and various blood-coagulation proteases, including factor Xa, factor XIIa and plasma kallikrein (Seymour *et al.*, 1994; Ulmer *et al.*, 1995). However, it does not inhibit any of the known proteases from *E. coli* and, therefore, has been suggested to play a role in protecting the bacteria from exogenous proteases found in the mammalian gut (Chung *et al.*, 1983). Ecotin exists in solution as a homodimer consisting of two identical subunits of 142 amino-acid residues and it binds to two protease molecules to form a tetrameric complex (Chung *et al.*, 1983; Seymour *et al.*, 1994; Lee *et al.*, 1991; McGrath *et al.*, 1991). The P1 reactive site of ecotin was determined to be Met84 (Seymour *et al.*, 1994; McGrath *et al.*, 1991). However, this P1 reactive-site residue was found not to be the key determinant of the specificity toward target proteases, since replacement of Met84 with Arg, Glu, Ile or Tyr showed little or no effect on the ability of ecotin to inhibit the pancreatic serine proteases (Seong *et al.*, 1994).

The three-dimensional structure of ecotin, first determined in a tetrameric complex with a mutant rat trypsin (McGrath *et al.*, 1994), revealed that the monomer has a modified jelly-roll fold and that Met84 (the P1 residue) is located on an extended surface loop. It also revealed that dimerization is achieved through its extended C-terminal arm. The dimeric structure of ecotin provides two binding sites for trypsin: a primary binding site from one subunit and a secondary binding site from the other (McGrath *et al.*, 1995). Subsequently, the crystal structures of free ecotin as well as an ecotin–collagenase complex have been reported (Shin *et al.*, 1996; Perona *et al.*, 1997). The intrinsic flexibility of the four surface loops in ecotin which comprise the binding sites and the relative adjustment between the primary and the secondary sites have been suggested to play an important role in the broad inhibitory specificity (Yang *et al.*, 1998). However, in order to gain a more complete understanding of the structural basis for the ecotin's broad specificity, further structural information on the complexes of ecotin with a variety of proteases is essential. Here, we report the crystallization of the ecotin–chymotrypsin complex and the collection of X-ray data to approximately 2.0 Å resolution as a first step toward its structure determination.

2. Experimental

2.1. Preparation of the complex and its crystallization

Ecotin was overexpressed and purified as described previously (Chung *et al.*, 1983; Lee *et al.*, 1991). α-Chymotrypsin from bovine pancreas was purchased from Sigma (C3142). Ecotin (20 mg) was incubated with a slight molar excess of α-chymotrypsin (35 mg) at 277 K in an acetate buffer (50 mM sodium acetate pH 5.2). The incubation mixture was subjected to FPLC Superdex G-75 chromato-

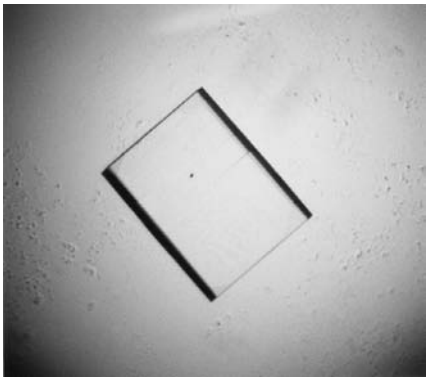

Figure 1
A triclinic crystal of ecotin–chymotrypsin complex grown from an ammonium sulfate solution. Its approximate dimensions are 0.5 × 0.4 × 0.1 mm.

graphy (1.6 × 60 cm) to eliminate chymotrypsin fragments generated by autolysis. The fractions containing the ecotin–chymotrypsin complex were concentrated by ammonium sulfate precipitation and were dialyzed against distilled water. Crystallization was performed by the hanging-drop vapour-diffusion method at 296 K. Hanging drops were prepared by mixing equal volumes of the protein solution (15 mg ml^{-1}) and the reservoir solution.

2.2. X-ray data collection

X-ray data were collected at 290 K on a FAST area-detector system (Enraf–Nonius) using the *MADNES* software (Messerschmidt & Pflugrath, 1987). Graphite-monochromated Cu $K\alpha$ X-rays from a rotating-anode generator (Rigaku RU-200) running at 40 kV and 70 mA, were used with a 0.3 mm focus cup and a 0.6 mm collimator. The reflection intensities were obtained by the profile-fitting procedure (Kabsch, 1988) and the data were scaled by the Fourier scaling program (Weissman, 1982).

3. Results

The thin plate-like crystals grew to dimensions of 0.5 × 0.4 × 0.1 mm (Fig. 1) within 8 d at 296 K from a reservoir solution containing 2.0 *M* ammonium sulfate, 100 m*M* zinc acetate and 50 m*M* sodium acetate (pH 5.2). The diffraction pattern from the crystals extended to 1.85 Å and the crystals were very stable in the Cu $K\alpha$ X-rays from the rotating-anode generator. Therefore, they are suitable for high-resolution structure determination. A set of native data extending to approximately 2.0 Å were collected from a single crystal. The data set consisted of 93261 measurements of 54331 unique reflections, with an $R_{\rm merge}$ of 7.1%. The crystal belongs to triclinic space group *P*1 with unit-cell parameters a = 57.29, b = 57.39, c = 79.75 Å, α = 91.49, β = 88.63 and γ = 112.45°. The merged data set is 83.3% complete in the resolution range 10.0–2.0 Å. The completeness for the 2.1–2.0 Å shell is 52.9% and very incomplete (13.5%) data to 1.91 Å have also been included in the merged data. Assuming one tetrameric complex in the asymmetric unit, a crystal volume per protein mass (V_M) of 2.58 Å^3 Da^{-1} and a solvent content of 48.9% by volume are obtained. These are within the commonly observed ranges (Matthews, 1968). Structure determination will be achieved by the molecular-replacement method.

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