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Crystallization and preliminary crystallographic studies of *Streptococcus pyogenes* cysteine protease precursor

Robert Janowski,[a] Grzegorz Bujacz,[b,c] Dieter Gerlach[d] and Mariusz Jaskolski[a,c]*

[a]Department of Crystallography, Faculty of Chemistry, A. Mickiewicz University, Grunwaldzka 6, 60-780 Poznan, Poland, [b]Faculty of Food Chemistry and Biotechnology, Technical University of Łodz, Stefanowskiego 4/10, 93-923 Łodz, Poland, [c]Center for Biocrystallographic Research, Institute of Bioorganic Chemistry, Polish Academy of Sciences, Noskowskiego 12/14, 61-704 Poznan, Poland, and [d]Institute of Medical Microbiology, Friedrich-Schiller University, Semmelweisstrasse 4, D-07743 Jena, Germany

Correspondence e-mail: mariuszj@amu.edu.pl

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Streptococcal protease precursor, secreted by the human pathogen *Streptococcus pyogenes*, becomes activated to a cysteine protease. The precursor and the mature enzyme appear to contribute to *S. pyogenes* virulence. The precursor protein was crystallized in the form of very thin flexible flakes. X-ray diffraction data were collected to 3.15 Å resolution at 100 K using synchrotron radiation. The crystals are monoclinic, space group $P2_1$, with unit-cell parameters $a = 41.6$, $b = 136.0$, $c = 156.7$ Å, $\beta = 95.7^\circ$, and contain four copies of the protein in the asymmetric unit.

1. Introduction

The ability of numerous microorganisms to cause disease relies upon the highly regulated expression of secreted proteins. The bacterium *Streptococcus pyogenes*, a Gram-positive group A *Streptococcus*, secretes several pyrogenic exotoxins (SPE) also known as superantigens of types A, B or C. They are believed to be responsible for a wide spectrum of biological reactions, such as skin reaction (Uchiyama *et al.*, 1994), scarlet fever (Hooker & Follensby, 1934), change in the permeability of the blood–brain barrier (Schlievert & Watson, 1978), lymphocyte stimulation, enhancement of lethal endotoxic shock or reduction in the number of leukocytes (Hribalova *et al.*, 1980). They are unique in their ability to induce damage to heart tissue (Schwab *et al.*, 1955). Superantigen activation of T-cells leads to increased secretion of specific cytokines by lymphocytes and monocytes, accumulation of which can result in acute shock and illness characteristic of streptococcal toxic shock syndrome (Stevens, 1995; Herman *et al.*, 1991).

SPE B is a 40 kDa streptococcal protease precursor (SPP) that can be activated to the 27.6 kDa streptococcal cysteine protease in an autocatalytic process following reduction with mercaptoethanol as well as by trypsin or subtilisin digestion of the N-terminal 118-residue prosegment (Elliott & Liu, 1970). The SPP protein was recorded for the first time by Elliott (1945). The total sequence of the zymogen (371 amino acids) was reported by Hauser & Schlievert (1990). Earlier reports (Tai *et al.*, 1976; Yonaha *et al.*, 1982) were characterized by an omission of 34 residues (Ser84–Ile117). On the basis of its biological activities, the mature enzyme is also referred to as interleukin-1 β-convertase, *Streptococcus* peptidase A or streptopain.

SPE B appears to contribute to *S. pyogenes* pathogenesis by cleavage or activation of host proteins and release of bacterial proteins from the cell surface. It degrades human fibronectin and vitronectin, two proteins involved in maintaining the integrity of the extracellular matrix and interactions between cells (Kapur *et al.*, 1993). The activation of interleukin-1β precursor to biologically active interleukin-1β, a major cytokine mediating inflammation and shock, was reported by Herwald *et al.* (1996). It was found that purified streptococcal cysteine protease releases biologically active kinin from its purified precursor protein, H-kininogen, *in vitro* and from kininogens present in the human plasma *ex vivo* (Herwald *et al.*, 1996). It also activates a human matrix metalloprotease, resulting in increased type IV collagenase activity (Burns *et al.*, 1996). SPE B may be responsible for processing monocytic cell urokinase receptor (Wolf *et al.*, 1994), releasing fibrinogen-binding fragments of M1 protein and Ig-binding of the N-terminal fragment of protein H (Berge & Björck, 1995). Streptococcal exotoxin B is irreversibly inactivated with a peptidic diazo inhibitor, Z-Leu-Val-Gly-CHN_2. The inhibitor, which is based on the protease-binding segment of human cystatin C, has been shown in animal tests to inhibit the growth of many *Streptococcus* species without toxic effects (Björck *et al.*, 1989).

For a long time it was believed that streptococcal erythrogenic toxin B (SPE B) and streptococcal protease precursor (SPP) are two different proteins with very high sequence homology which share several properties (toxicity). The identity of *Streptococcus* pyrogenic exotoxin B (SPE B, also called erythro-

genic toxin type B) and the streptococcal proteinase precursor was first reported by Gerlach *et al.* (1983). Southern hybridization analysis of the wild-type strain has shown that only a single copy of the *speB* gene is present in the chromosome of *S. pyogenes* (Chaussee *et al.*, 1993). It was also noted by the same authors that inactivation of the *speB* gene results in the absence of both SPE B and SPP. Searching the complete genome of this pathogen (Ferretti *et al.*, 2001) confirmed that only one gene is present and that there are no other nucleotide sequences suggesting a similar protein in group A *Streptococcus*.

X-ray crystallographic techniques have led to the elucidation of several superantigen structures: SPE C (Roussell *et al.*, 1997), SPE A1 (Papageorgiou *et al.*, 1999), SPE A (Earhart *et al.*, 2000), SPE H (Arcus *et al.*, 2000) and SPE B (Kagawa *et al.*, 2000). The structures of these proteins are very important from the medical point of view as targets in the rational design of drugs against the pathologies caused by *S. pyogenes*. In contrast to the active-site mutant (Cys→Ser) studied by Kagawa *et al.* (2000), the SPE B protein studied in this report represents the wild-type form.

2. Materials and methods

2.1. Protein preparation

Streptococcal protease precursor was isolated from bacterial strain B220 according to the procedure described by Gerlach *et al.* (1983). The streptococci were grown in 5 l yeast extract broth at 303 K and pH 6.0. After killing the bacteria with 0.15% H_2O_2 and centrifugation, the zymogen was precipitated at 75% ammonium sulfate saturation. The precipitate was solubilized in H_2O containing 5 µ*M* sodium iodoacetate and recrystallized at pH 8.0. Another solubilization in 0.02 *M* acetate buffer pH 5.2 containing 5 µ*M* sodium iodoacetate (buffer *A*) was followed by dialysis and final purification on a HiLoad S Sepharose column (Pharmacia) using a linear gradient of buffer *A* to buffer *B* (buffer *A* plus 1 *M* NaCl). The protein was eluted at 0.15 *M* NaCl. Crystalline precipitate was obtained by dialysis against 75% ammonium sulfate pH 8.0, as described by Elliott (1950). The protein used for crystallization tests was stored in the form of a suspension in 75% saturated ammonium sulfate solution containing 5 µ*M* sodium iodoacetate.

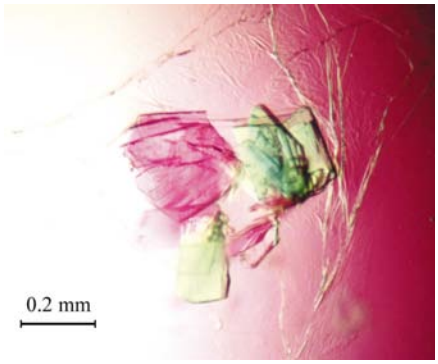

Figure 1
Crystals of *S. pyogenes* cysteine protease precursor.

2.2. Crystallization

The protein was transferred in a three-step centrifugation procedure from the ammonium sulfate solution to 10 m*M* Tris–HCl buffer pH 8.5 supplemented with 75 m*M* NDSB [3-(1-pyridino)-1-propane sulfonate]. The final protein concentration was 20 mg ml^{-1}. Preliminary crystallization conditions were established at 292 K using the hanging-drop vapour-diffusion technique (McPherson, 1982) and sparse-matrix screens from Hampton Research (Jancarik & Kim, 1991). Bunches of extremely thin crystalline flakes appeared within a few days from solution containing sodium cacodylate buffer pH 6.5, 0.2 *M* zinc acetate and 18% PEG 8K. Only slight improvement of the crystal quality was achieved with the addition of a detergent (nonyl-β-D-glucopyranoside) from the Hampton Research Detergent Screen (McPherson *et al.*, 1986) in the proportions 1.6 µl protein, 0.4 µl detergent and 2 µl reservoir solution (Fig. 1).

2.3. Data collection and processing

Diffraction data were measured using synchrotron radiation (EMBL, c/o DESY, Hamburg, beamline X11, $\lambda = 0.89190$ Å) and a MAR Research CCD detector. A very thin crystal (0.2 × 0.1 × 0.01 mm) was mounted in a nylon-fibre loop and flash-frozen at 100 K in a nitrogen-gas stream (Teng, 1990). Unusual mechanical properties of the crystals were observed during the manipulations. They were highly elastic and behaved like rubber. To separate it from a cluster, a single flake had to be pulled out rather than broken off. For cryoprotection, the reservoir solution was prepared with 25% PEG 400 instead of 18% PEG 8K. The crystal was soaked in the cryoprotectant solution for a few minutes before mounting and freezing. The data were collected to 2.5 Å resolution (300 images of 0.4° oscillation). The anisotropy of the crystal was reflected in the high anisotropy of the diffraction pattern and the limit of diffraction depended on the crystal orientation (Figs. 2*a* and 2*b*). The images were indexed, integrated and scaled using the *HKL* program package (Otwinowski &

Table 1
X-ray data-collection and processing statistics.

Values in parentheses correspond to the last resolution shell.

Space group	$P2_1$
Unit-cell parameters (Å, °)	$a = 41.6$, $b = 136.0$, $c = 156.7$, $\beta = 95.7$
Temperature (K)	100
Resolution (Å)	15.0–3.15
No. of observations	374048
No. of unique reflections	24889
Completeness (%)	83.5 (74.8)
$\langle I/\sigma(I)\rangle$	5.9 (1.8)
$R_{\rm int}$†	0.136 (0.290)

† $R_{\rm int} = \sum_h \sum_j I_{hj} - \langle I_h\rangle| / \sum_h \sum_j I_{hj}$, where I_{hj} is the intensity of observation j of reflection h.

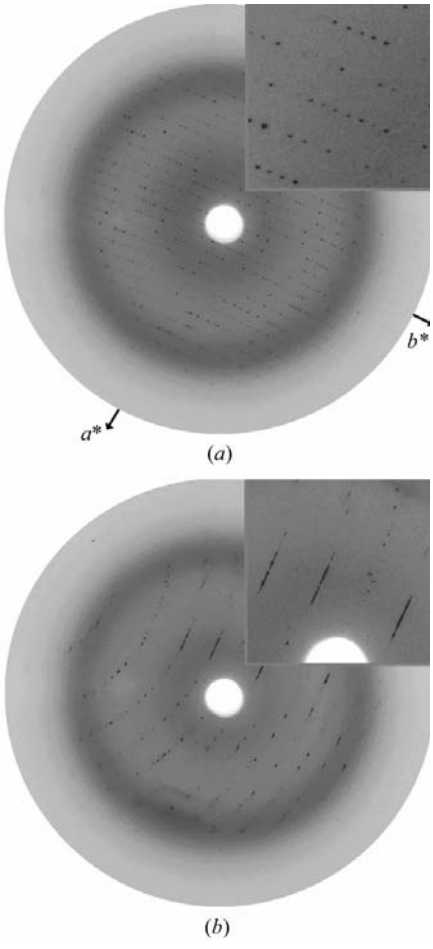

Figure 2
Two diffraction images (0.4° oscillation) corresponding to perpendicular directions in reciprocal space. (*a*) corresponds to the *hk*0 layer, with approximate directions of the *a** and *b** axes indicated. (*b*) was recorded after 90° rotation around the horizontal spindle axis. The insets illustrate the excellent definition of the reflections in (*a*) and their smearing into lines in (*b*). The edge of the detector corresponds to 2.5 Å *d* spacing.

Minor, 1997). Owing to the poor quality of the high-angle data, the final scaling was limited to 3.15 Å. 374 048 observations were merged into a unique data set consisting of 24 889 reflections. This data set is 83.5% complete and is characterized by $R_{\rm int} = 0.136$ and $\langle I/\sigma(I)\rangle = 5.9$. Table 1 summarizes the data-collection and processing statistics.

3. Results and discussion

The crystals belong to the monoclinic space group $P2_1$, with unit-cell parameters $a = 41.6$, $b = 136.0$, $c = 156.7$ Å, $\beta = 95.7^\circ$. These parameters differ from those reported by Kagawa *et al.* (2000) for the crystals of pyrogenic exotoxin B Cys47Ser active-site mutant ($a = 46.7$, $b = 116.6$, $c = 144.7$ Å, $\beta = 94.2^\circ$; space group $P2_1$). The mutant structure, deposited in the Protein Data Bank with code 1dki, contains four protein chains in the asymmetric unit. Analysis of the Matthews volume (Matthews, 1968) for the present crystal form of the native protein indicates the presence of three to six molecules in the asymmetric unit. Molecular-replacement calculations using the program *MOLREP* (Vagin & Teplyakov, 1997) and one monomer of the 1dki structure identified four molecules in the asymmetric unit with acceptable crystal packing (solvent content 54.7%, 2.7 Å^3 Da^{-1}). Electron-density maps calculated after several cycles of *REFMAC* refinement (Murshudov *et al.*, 1997) are clear for all four molecules and in addition are interpretable in some of the regions that were not visible in the mutant structure. In particular, the Ile56–Gly59 surface loop in the prosegment domain has very clear definition in all four copies of the protease precursor in the present structure. As an example, Fig. 3 illustrates the electron density in this area for one of the molecules.

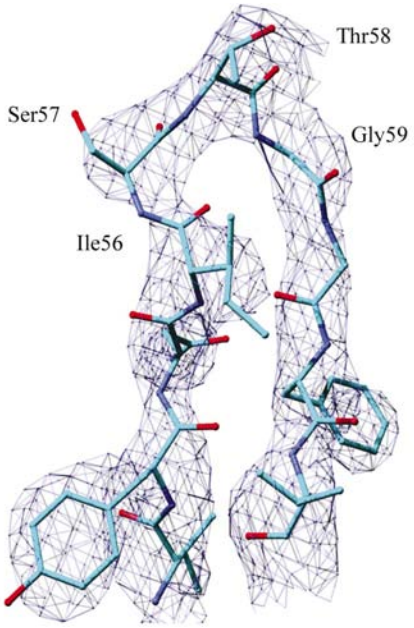

Figure 3
$2F_o - F_c$ electron-density map contoured at the 1.2σ level for a solvent-exposed loop in the prosegment domain of monomer *D*. Residues not visible in the mutant structure (1dki; Kagawa *et al.*, 2000) are labelled.

The active-site residue Cys47 (in mature protease numbering; 165 including the prosegment sequence) is well defined in the electron-density map in all copies of the molecule. Because of the use of sodium iodoacetate in the storage buffer, the catalytic thiol group is covalently modified. The modification is visible in three of the molecules as at least one extra C atom attached to the S^γ.

Superpositions of the C^α traces of the four mutant molecules reported by Kagawa *et al.* (2000) upon the present wild-type molecules yielded r.m.s.d. values between 0.60 and 0.76 Å (average 0.68 Å). This is rather high when compared with the analogous r.m.s. deviations calculated between the independent molecules in each structure (1dki, 0.24 Å; present structure, 0.30 Å). The elevated cross r.m.s.d. value can be partly explained by the presence of different-length gaps in the models. However, it is also related to the arrangement of the molecules in the crystal lattice, which is different in the two crystal structures. For instance, regions that are involved in intermolecular contacts in one structure are solvent exposed in the other and the mutual orientations of the individual molecules are different. Crystal packing of the mutant structure is rather compact and the solvent regions between the molecules are small. The Matthews volume of the wild-type structure is 13% larger and in consequence the contacts between the molecules are weaker. In the present structure, the protein molecules are packed in separated alternating layers consisting of molecules $A + B$ and $C + D$. This laminar arrangement of the molecules perpendicular to the *c* direction might be the reason why the crystals grow in the form of very thin plates.

At the present stage, the refinement is characterized by R and $R_{\rm free}$ of 0.237 and 0.291, respectively.

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