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Cloning, purification, crystallization and preliminary crystallographic analysis of SecA from *Enterococcus faecalis*

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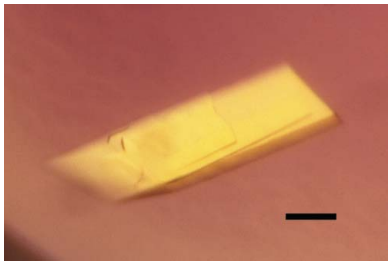

The gene coding for SecA from *Enterococcus faecalis* was cloned and overexpressed in *Escherichia coli*. In this protein, the lysine at position 6 was replaced by an asparagine in order to reduce sensitivity towards proteases. The modified protein was purified and crystallized. Crystals diffracting to 2.4 Å resolution were obtained using the vapour-diffusion technique. The crystals belong to the monoclinic space group $C2$, with unit-cell parameters a = 203.4, b = 49.8, c = 100.8 Å, $\alpha = \gamma = 90.0$, $\beta = 119.1^\circ$. A selenomethionine derivative was prepared and is currently being tested in crystallization trials.

1. Introduction

In bacteria, preprotein transport across the inner membrane is assisted by the multisubunit protein complex translocase, consisting of the heterotrimeric membrane protein SecYEG and the peripheral membrane-associated SecA (de Keyzer *et al.*, 2003). SecA is an ATP-dependent molecular motor that drives the movement of preproteins through the SecYEG translocon in a stepwise fashion concomitant with large conformational changes and with its own membrane-insertion/deinsertion reaction coupled to ATPase activity.

Although a wealth of data is available on the genetics and biochemistry of SecA (Vrontou & Economou, 2004) and the crystal structures of the SecA proteins from *Bacillus subtilis* (Hunt *et al.*, 2002; Osborne *et al.*, 2004) and *Mycobacterium tuberculosis* (Sharma *et al.*, 2003) have been described, the oligomeric state of the active protein is still a subject of controversy. In solution, SecA forms a homodimer under physiological conditions. It has been described as being translocation-active in a dimeric state (Driessen, 1993; de Keyzer *et al.*, 2005), while other studies have proposed the dissociation of the dimer during translocation (Duong, 2003; Or *et al.*, 2005). Several lines of evidence indicate that depending on salt, temperature (Woodbury *et al.*, 2002), specific lipids and signal peptide analogues (Benach *et al.*, 2003), SecA exists in a dynamic equilibrium of monomeric, dimeric and higher oligomeric states.

SecA is essential in bacteria and there is no SecA analogue in human or animals. Thus, it represents a potential target for antimicrobial agents. Increasing resistance of pathogenic bacteria towards common antibiotics makes it necessary to develop new agents for the therapy of infectious diseases. For this purpose, the knowledge of the three-dimensional structure of the target protein is a prerequisite. The pathogenic microorganism *Enterococcus faecalis* presently causes the majority of human enterococcal infections and is therefore a suitable target for antimicrobial therapy (Huycke *et al.*, 1998). In this paper, we describe the cloning, overexpression and crystallization of the SecA protein from *E. faecalis*.

2. Materials and methods

2.1. Cloning, expression and purification

The *secA* gene was amplified by PCR using *E. faecalis* DNA as template and the oligonucleotides EFSecA-NcoI and EFSecA-HindIII as primers. The amplificate (2563 bp) was digested with *Nco*I and *Hin*dIII. The resulting fragment was purified and ligated to the plasmid expression vector pNCO113 (Stueber *et al.*, 1990) previously

treated with the same restriction enzymes, affording the expression plasmid pNCO-EFSecA.

The plasmid pNCO-EFSecA served as a template for a PCR with the oligonucleotides EFSecA-K6N-NcoI and EFSecA-XcmI-271 as primers. Both the purified PCR product (294 bp) and the vector pNCO-EFSecA were digested with the restriction endonucleases *Nco*I and *Xcm*I, yielding fragments with 260 and 5670 bp, and were then ligated together. The resulting plasmid (pNCO-EFSecA-K6N) was used as a template for PCR with the oligonucleotides EFSecA-SplI-2240 and EFSecA-HIS6-HindIII as primers. The amplificate (356 bp) and the vector pNCO-EFSecA-K6N were digested with the restriction endonucleases *Spl*I and *Hin*dIII, yielding fragments of 332 and 5621 bp, and were ligated together, yielding the expression plasmid pNCO-EFSecA-K6N-C-HIS6.

All ligation mixtures were transformed into *Escherichia coli* strain XL-1 Blue. Plasmids reisolated from XL-1 strains were sequenced using the method of Sanger (GATC Biotech, Konstanz, Germany; for oligonucleotides, strains and plasmids, see supplementary material[1]).

The reisolated plasmid pNCO-EFSecA-K6N-C-HIS6 was transformed into M15(pREP4) *E. coli* competent cells, affording the recombinant *E. coli* strain M15(pREP4)-pNCO-EFSecA-K6N-C-HIS6. The cells were grown at 310 K to an $OD_{600\,nm}$ of 0.7 in LB medium, induced with 2 m*M* IPTG and incubated for 3 h. Cells were harvested, washed in 50 m*M* Tris–HCl pH 7.5, 20% glycerol and stored at 253 K. Cells were resuspended in 50 m*M* Tris–HCl, 1 m*M* PMSF, 20% glycerol and sonicated (5 × 30 s) followed by ultracentrifugation at 40 000 rev min^{-1} in a Ti-70 rotor (Beckman). The protein EFSecA-K6N-HIS6 was purified by affinity chromatography on Ni–NTA agarose (HisTrap, Amersham Biosciences) with a linear gradient of 10–500 m*M* imidazole in 50 m*M* Tris–HCl pH 7.5 and 10% glycerol. All data described in this paper were obtained from EFSecA-K6N-HIS6, which is hereafter referred to as EFSecA.

Selenomethionine labelling of the EFSecA was performed as described by Bader *et al.* (2004) and the protein was purified as described above. The incorporation of selenomethionine (∼78%) was demonstrated by MALDI–TOF/TOF [Bruker Daltonics; determined MW 98 100–98 200 Da (SeMet-labelled EFSecA); calculated MW 97 163 Da (EFSecA); calculated MW 98 430 Da (SeMet-EFSecA, fully labelled)].

2.2. Crystallization

For crystallization experiments, a solution of 10 mg ml^{-1} EFSecA in 50 m*M* Tris–HCl pH 7.5, 50 m*M* NaCl and 10%(*v*/*v*) glycerol was used. In initial crystallization attempts, commercial screens from Hampton Research (Crystal Screen, Crystal Screen 2, Crystal Screen Lite, Membfac and Natrix) were tested. Crystallization experiments were performed at 293 K using the vapour-diffusion technique with sitting drops equilibrated against 1 ml reservoir solution. The conditions in the initial screenings which promoted crystallization were retested and optimized. All experiments were designed and monitored using the program *XtalBase* (W. Meining; http://www.xtalbase.net). In cocrystallization experiments with AMP-PNP and ADP, 0.5 µl of a 10 m*M* solution of the respective ligand was added to 1 µl protein solution and 1 µl reservoir solution.

2.3. Data collection and preliminary crystallographic analysis

Diffraction data were collected on the I711 beamline at MAX-Lab (Lund, Sweden) equipped with an Oxford Cryosystem and a MAR Research imaging plate. Prior to data collection, crystals of interest went through three cycles of a gradual exchange of the mother liquor by a solution containing 25%(*v*/*v*) PEG 8000, 300 m*M* sodium cacodylate pH 6.5, 100 m*M* magnesium acetate and 10%(*v*/*v*) PEG 400. The crystals were then placed in a nylon loop and flash-cooled to 105 K. Two data sets, one to a resolution limit of 2.4 Å and another one to 3.5 Å, were collected from a single crystal at a wavelength of 1.092 Å and a temperature of 105 K under atmospheric pressure. The oscillation ranges were 1 and 5°, respectively, and exposure times were approximately 30 s. The data were indexed, scaled and merged using the *HKL* package programs *XDISP*, *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The program *Phaser* was used for attempts to determine phases *via* molecular replacement (Read, 2001).

3. Results and discussion

3.1. Protein expression and purification

Plasmids carrying the engineered gene EFSecA-K6N-C-HIS6 under the control of a T5 promotor and *lac* operator directed the synthesis of a 97 kDa protein in a recombinant *E. coli* strain. Expression and purification of the wild-type EFSecA (845 amino acids) yielded peptides lacking the N-terminal five and six amino acids as determined by mass spectroscopy. Because of the presence of a lysine–lysine motif in the amino-acid sequence at position 6 and 7, the wild-type protein appeared to be highly sensitive to proteolysis at this position. Mimicking the sequence of *B. subtilis* SecA, lysine 6 was replaced by an asparagine (K6N mutant). Edman degradation showed an intact N-terminus of the K6N mutant. A hexahistidine tag was introduced at the C-terminus to allow rapid purification and the resulting peptide was used in crystallization trials.

To determine the quaternary structure, EFSecA was analyzed by analytical ultracentrifugation and size-exclusion chromatography. Molecular weights in the region of 110 kDa were found, indicating the monomeric state of the protein. Electron microscopy of negatively stained EFSecA showed particles with a diameter of 5–6 nm, in agreement with the monomeric form. In the absence of reducing agents, protein preparations also contained also a small fraction of dimeric species which could be separated by size-exclusion chromatography or native gel electrophoresis. In the presence of 10 m*M* DTT the dimeric species disappeared, indicating the reduction of an intermolecular disulfide bridge. The existence of a stable monomeric form of the SecA protein from *E. faecalis* is in contrast to the dimeric

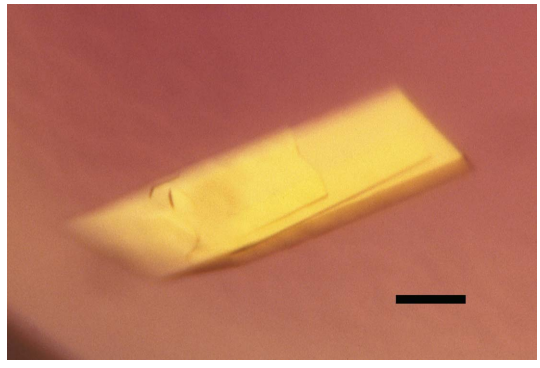

Figure 1
Crystal of native SecA from *E. faecalis*. The scale bar corresponds to 0.1 mm. See text for a description of crystallization conditions.

[1] Supplementary material has been deposited in the IUCr electronic archive (Reference: PU5137).

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Space group	*C*2
Unit-cell parameters (Å, °)	a = 203.4, b = 49.8, c = 100.8, α = 90.0, β = 119.1, γ = 90.0
Resolution (Å)	50–2.7 (2.75–2.7)
No. of observations	346722
Unique reflections	24812
Completeness	100.0 (100.0)
$\langle I\rangle/\langle\sigma(I)\rangle$	28.498 (1.746)
$R_{\rm merge}$†	0.061 (0.624)
Subunits per AU	1
$V_{\rm M}$ (Å^3 Da^{-1})	2.3

† $R_{\rm merge} = \sum|I - \langle I\rangle|/\sum I$.

proteins from *E. coli* and *B. subtilis* observed under similar conditions (Driessen, 1993; Takamatsu *et al.*, 1992; Ding *et al.*, 2003).

3.2. Crystallization

EFSecA was crystallized in sitting drops *via* the vapour-diffusion method. The largest crystals were obtained when 1 µl reservoir solution (15–30% PEG 8000, 0.1–0.3 *M* sodium cacodylate pH 6.5, 0.1–0.4 *M* magnesium acetate) was mixed with 1 µl protein solution [10 mg ml^{-1} EFSecA in 50 m*M* Tris–HCl pH 7.5, 50 m*M* NaCl and 10%(*v*/*v*) glycerol] and equilibrated against 1 ml reservoir solution. In successful experiments, the crystallization drops developed phase separation within 6 d and plate-shaped crystals with an edge length of up to 800 µm within two weeks (Fig. 1). Crystals were also obtained in the presence of ADP and grew under similar conditions to the native protein. However, these crystals diffracted X-rays to a resolution of only about 8 Å. Crystals were also obtained by cocrystallization with AMP-PNP. These crystals were generally twinned and were too small for data collection.

3.3. X-ray diffraction experiments

Crystals of EFSecA diffracted X-rays to 2.4 Å, but were merged to 2.7 Å, as the merging statistics indicated an increased $R_{\rm merge}$ and a fall-off of $I/\sigma(I)$ to below 1 at higher resolution (Table 1). The crystal lattice was determined to be monoclinic and the space group was *C*2, with unit-cell parameters a = 203.4, b = 49.8, c = 100.8 Å, $\alpha = \gamma$ = 90.0, β = 119.1°. The corresponding unit-cell volume is 892 243 Å and the Matthews coefficient calculated for one molecule per asymmetric unit is $V_{\rm M}$ = 2.3 Å^3 Da^{-1}, corresponding to 46.5% solvent content. The assumption of one molecule seems reasonable, as the calculated $V_{\rm M}$ is close to the most probable $V_{\rm M}$ found in proteins (Kantardjieff & Rupp, 2003; Matthews, 1968) and because the sample was found to be monomeric in electrophoresis and electron-microscopic studies. In attempts to solve the structure *via* molecular replacement, various models originating from the published structures of SecA from *B. subtilis* (PDB code 1m6n; Hunt *et al.*, 2002) and *M. tuberculosis* (PDB code 1nkt; Sharma *et al.*, 2003) were used as search models. These attempts resulted in a unique properly packed clear solution, but with resulting electron density of only moderate quality. The density did not improve upon density modification. The nucleotide-binding domains I and II were generally best defined, whereas other domains or parts of them were ill-defined or not even visible (*e.g.* the extended helix in the helical scaffold domain). In total, only about 30% of the density allowed the determination of the approximate location and orientation of individual residues. The lack of acceptable density prompted us to attempt solving the structure using anomalous diffraction data collected from crystals grown from SeMet-enriched SecA. Such a modified SecA sample has been prepared and is currently being tested for crystallization.

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