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Crystallization of the oligopeptide-binding protein AppA from *Bacillus subtilis*

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AppA is the membrane-anchored extracellular receptor component of an ABC transporter responsible for the uptake of oligopeptides into *Bacillus subtilis*. AppA has been overexpressed as a cleavable maltose-binding protein fusion in *Escherichia coli*. Following removal of the fusion portion, AppA has been crystallized from morpholinoethanesulfonic acid-buffered solutions at pH 6.5 containing polyethylene glycol and zinc acetate. A complete X-ray diffraction data set extending to 2.3 Å spacing has been collected.

1. Introduction

Peptides can serve as the sole source of carbon and nitrogen for bacteria. In enteric bacteria such as *Escherichia coli* and *Salmonella typhimurium*, three peptide transporters with overlapping specificities have been characterized: a dipeptide permease (*dpp*), a tripeptide permease (*tpp*) and an oligopeptide permease (*opp*). The peptide transporters belong to the ATP-binding cassette (ABC) family. In these peptide-uptake systems, a soluble periplasmic receptor protein delivers substrates into a set of four membrane components which mediate translocation to the cytoplasm (Higgins, 1992). The receptor proteins define the substrate specificity of the transporter, which in the case of peptide permeases is broad indeed (Wilkinson & Verschueren, 2002).

In Gram-positive bacteria such as *Bacillus subtilis*, as well as being nutrient sources, peptides also play notable roles in the signalling pathways controlling responses to a deteriorating cellular environment (Lazazzera & Grossman, 1998; Pottathil & Lazazzera, 2003). In *B. subtilis*, the dipeptide permease and the oligopeptide permease are retained; however, there is a second ABC-type oligopeptide-uptake system, App (short for 'another peptide permease'; Koide & Hoch, 1994). The reason for this redundancy in oligopeptide transport is not understood, although it is known that *opp* and *app* are differentially regulated (Koide *et al.*, 1999). In the absence of a periplasm, the binding-protein components of the three transporters DppE, OppA and AppA are lipoproteins anchored to the membrane (Sutcliffe & Russell, 1995).

The periplasmic substrate-binding proteins of peptide transporters are of particular interest in view of their broad substrate specificity. Genetic studies suggest that Opp from *S. typhimurium* binds peptides 2–5 residues in length regardless of their sequence. Studies using isothermal titration calorimetry indicate that OppA binds tripeptide and tetrapeptide ligands with the highest affinity and that dipeptides and pentapeptides are bound much more weakly (Sleigh *et al.*, 1997, 1999). In *B. subtilis*, much less is known about the substrate specificity of the peptide permeases; however, several lines of evidence suggest that App and/or Opp can transport longer peptides. Firstly, unlike in *E. coli* and *S. typhimurium*, there is no outer membrane in Gram-positive bacteria to limit the size of extracellular solutes that can gain access to the receptors for transport. Secondly, imported pentapeptides are a key part of regulatory circuits controlling sporulation and competence development (Lazazzera *et al.*, 1997; Perego, 1997; Perego & Brannigan, 2001). Thirdly, binding of nonapeptides has been shown to signal competence pathways (Magnuson *et al.*, 1994). Fourthly, binding of the peptide RPPGFSPFR to *B. subtilis* AppA heterologously expressed in *Lactococcus lactis* has been demonstrated in fluorescence studies (Picon & van Wely, 2001). To explore how longer peptides are accommodated in peptide-transporter proteins, we have crystallized AppA from *B. subtilis* as a first step in structure determination.

2. Expression and purification

AppA was expressed as a C-terminal fusion to maltose-binding protein (MBP) in the plasmid pMALp2 (New England BioLabs). The *appA* gene was amplified from *B. subtilis* JH14116 chromosomal DNA (a gift from J. A. Hoch) by PCR with a high-fidelity DNA polymerase (Vent, Stratagene) using primers designed to introduce convenient restriction sites for cloning (*Eco*RI and *Bam*HI) and alter the codon encoding Cys+1 of mature AppA to Ser (AGC). Equivalent clones have also been

produced for the *oppA* and *dppA* genes from *B. subtilis* strain IG20 (168 Trp$^-$).

E. coli B834 cells harbouring pMALp2-AppA were grown with shaking at 310 K in 2 l LB medium supplemented with 100 µg ml^{-1} ampicillin to an optical density of 0.6 at 600 nm. Expression of recombinant fusion protein was induced by the addition of IPTG to 0.3 m*M* and further incubation at 298 K for 14 h. Periplasmic extracts were prepared by an osmotic shock procedure (Neu & Heppel, 1965). A tenth of the culture volume of 1 *M* Tris–HCl pH 8.5 was added and the cultures were shaken for a further 20 min to release lipopolysaccharides and permeabilize the outer membrane. Cells were harvested by centrifugation and resuspended in 200 ml 30 m*M* Tris–HCl pH 7.5, 2 m*M* EDTA, 40% sucrose. 30 min later, the cells were pelleted and resuspended in 100 ml of ice-cold deionized water. Following centrifugation, the supernatant containing the osmotic shock fluids was retained and stored frozen at 253 K.

MBP-AppA fusion protein was purified by anion-exchange chromatography using Q-Sepharose media and resolved with a linear gradient of 0–0.5 *M* NaCl in 50 m*M* Tris–HCl pH 8.5. Fractions containing fusion protein were identified by SDS–PAGE, pooled and dialysed against 50 m*M* Tris–HCl pH 8.5. The protein was cleaved with factor Xa (Denzyme) at a ratio of 1 mg protease to 100 mg fusion protein for at least 8 h at 291 K. AppA was purified from the cleaved mixture by anion-exchange chromatography as above but using a Mono-Q column (Pharmacia). Typical yields were 18 mg of purified AppA per litre of cell culture.

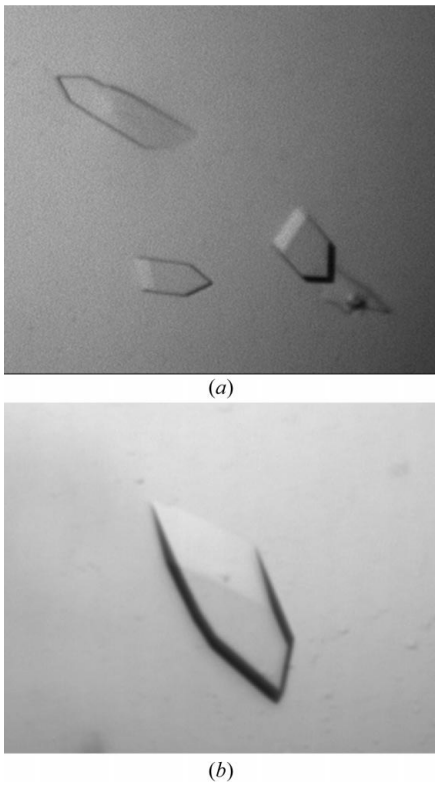

Figure 1
Photomicrographs of typical AppA crystals grown from 16–18% PEG 8000, 0.1 *M* MES pH 6.5 and 0.2 *M* zinc acetate. Crystals reached final dimensions of 0.08 × 0.08 × 0.15 mm in about a week at 291 K.

3. Crystallization

Crystallization of AppA was carried out using the hanging-drop vapour-diffusion method and a variety of commercially available screens (Brzozowski & Walton, 2001; Jancarik & Kim, 1991). Prior to crystallization, the purified protein was concentrated using a 30 kDa molecular-weight cutoff ultrafiltration membrane (Filtron) to 8–10 mg ml^{-1} in 20 m*M* Tris–HCl pH 7.5. Crystals grew when 1 µl drops of protein solution were mixed with an equal amount of reservoir solution containing 16–18% polyethylene glycol (PEG) 8000, 0.1 *M* morpholinoethanesulfonic acid (MES) buffer pH 6.5 and 0.2 *M* zinc acetate (Fig. 1). The crystals did not grow at pH values higher than 6.5 or with lower concentrations of zinc acetate.

4. Data collection

The crystals were characterized using Cu $K\alpha$ X-ray radiation from a Rigaku RU-200 rotating-anode generator with a MAR Research image-plate scanner as a detector. The crystals were frozen at 120 K using a cryocooling system (Oxford Cryosystems). The cryoprotectant solution contained 15% PEG 8000, 0.2 *M* zinc acetate, 0.1 *M* MES pH 6.5 and 25% glycerol. Crystals belong to space group $P2_12_12$, with unit-cell parameters a = 121.5, b = 90.9, c = 54.7 Å. Assuming one 57.9 kDa protein molecule in the asymmetric unit, the specific volume V_M is 2.6 Å^3 Da^{-1} and the solvent content is 52.5% (Matthews, 1968). The crystals diffract to at least 2.7 Å using an in-house diffractometer and to 2.3 Å using a synchrotron-radiation source. Complete data have been collected to 2.3 Å resolution on beamline ID14 station 1 at the European Synchrotron Radiation Facility (ESRF; λ = 0.934 Å) with a CCD ADSC Quantum 4 detector. The data were processed using *DENZO* (Otwinowski & Minor, 1997) and *SCALEPACK* (Collaborative Computational Project, Number 4, 1994). X-ray data statistics can be found in Table 1.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	0.934
Temperature (K)	100
Range collected (°)	90
Oscillation angle (°)	0.5
No. crystals used	1
Space group	$P2_12_12$
Unit-cell parameters (Å, °)	a = 121.46, b = 90.92, c = 54.74, $\alpha = \beta = \gamma$ = 90
Unit-cell volume (Å^3)	604535.38
Solvent content (%)	52.5
Matthews coefficient (Å^3 Da^{-1})	2.6
Total No. unique reflections	28321
Total No. observed reflections	173803
Resolution (Å)	30.0–2.30
Completeness (%)	99.6 (100.0)
Multiplicity	5
$I/\sigma(I)$	16.46 (5.23)
R_{merge}	0.080 (0.397)

5. Discussion

The gene encoding AppA was cloned from *B. subtilis* strain JH14116, as standard laboratory-type strains of this organism have a single base deletion in their *appA* genes, leading to a frameshift mutation and hence inactive protein (Koide & Hoch, 1994). The gene was modified to alter the first amino acid (Cys) to serine, as owing to the absence of an outer membrane in Gram-positive organisms AppA is anchored as a lipoprotein to the outer face of the membrane. The encoded fusion protein was MBP-IEKR-IESF-S-AppA. Cleavage with factor Xa generated authentic *B. subtilis* AppA in which the amino-terminal cysteine was replaced by the pentapeptide IESFS.

The structures of the receptor proteins for two peptide-transport systems, the oligopeptide-binding protein OppA of *S. typhimurium* and the dipeptide-binding protein DppA from *E. coli*, are known, each having been solved with and without bound peptides (Dunten & Mowbray, 1995; Nickitenko *et al.*, 1995; Sleigh *et al.*, 1997; Tame *et al.*, 1994). Both proteins are made up of three domains that contribute to two lobes that close over the ligand, burying it in a binding process often likened to the closing of a venus flytrap (Mao *et al.*, 1982). It will be interesting to establish whether AppA does the same. In OppA from *L. lactis*, which has been shown to bind peptides longer than 20 residues, it seems unlikely that complete enclosure of the ligand within the protein takes place (Lanfermeijer *et al.*, 2000). We are also curious to see if, as is common with other ABC receptors, the peptide-binding protein has co-purified with an endogenous ligand. This might lead to insights into peptide signalling in bacteria, which may

have implications wider than control of sporulation and competence in *Bacillus*. Other systems are regulated by imported peptides, including induction of conjugation in *Enterococcus* (Leonard *et al.*, 1996) and control of virulence factors in the *B. cereus* group (Slamti & Lereclus, 2002).

The crystals of AppA from *B. subtilis* grown here are clearly suitable for structure determination. However, we have not succeeded in solving the structure of AppA by molecular replacement using the coordinates (in open or closed conformations) of OppA from *S. typhimurium* or DppA from *E. coli* as search models. These proteins share 22 and 26% sequence identity with AppA, respectively. We are therefore searching for heavy-atom derivatives to enable structure solution by either isomorphous replacement or anomalous dispersion methods.

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