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Expression, purification, crystallization and preliminary X-ray characterization of the class B acid phosphatase (AphA) from *Escherichia coli*

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The class B non-specific acid phosphatase AphA from *Escherichia coli* has been expressed in *E. coli* and purified following a new protocol. ESI mass spectroscopy shows that the purified enzyme solution contains two polypeptides with molecular weights differing by 185 Da corresponding to two different cleavage sites of the signal peptide from the AphA *E. coli* precursor. Despite the solution heterogeneity, X-ray quality crystals have been obtained. However, the crystals have a tendency to give polymorphs and to lose long-range order with time while maintaining an intact crystal habit. Crystals have been grown in space groups *I*222 and *C*2 with three different unit cells and different asymmetric unit contents. Diffraction data to 1.6 Å resolution have been collected with synchrotron radiation at ESRF and DESY.

1. Introduction

The non-specific acid phosphatase/phosphohydrolase AphA from *E. coli* belongs to the so-called molecular class B of acid phosphatases, which are homotetrameric metalloenzymes formed of monomers of about 25 kDa. These enzymes are completely unrelated to the phosphatases of molecular class A in terms of both sequence and metal dependency, but have significant similarity to the evolutionarily more distant class C acid phosphatases (Rossolini *et al.*, 1998). Analysis of the sequences of class B and C acid phosphatases has provided further evidence of the presence of domains that are highly conserved in other bacterial, eukaryotic and archaeal enzymes, leading to the definition of a large superfamily of phosphohydrolases named 'DDDD' after the presence of four invariant aspartate residues (Thaller *et al.*, 1998).

The AphA enzyme is secreted in the periplasmic space of *E. coli* and consists of four identical subunits with a molecular mass of about 100 kDa, as estimated by gel-permeation chromatography. The AphA enzyme catalyzes the dephosphorylation of a broad variety of organic phosphomonoesters, including 5′- and 3′-nucleotides, 2′-deoxy-5′-nucleotides, aryl phosphates, glycerol 2-phosphate and phytic acid, and is also able to catalyze phosphate transfer to hydroxyl groups of organic compounds. Its activity is inhibited by EDTA, nucleosides and Ca^{2+}, while being stimulated by Mg^{2+} (Thaller *et al.*, 1997). Here, we report the expression, purification and preliminary characterization by X-ray crystallography of the *E. coli* AphA enzyme.

Class B acid phosphatases are widespread in the bacterial world, being found in members of the families *Enterobacteriaceae* and *Pasteurellaceae* (phylum BXII) and also in *Streptococcus pyogenes* (phylum BXIII) (Thaller, Berlutti *et al.*, 1995; Thaller, Lombardi *et al.*, 1995). One of their physiological functions could be to scavenge phosphate esters that otherwise would not cross the cytoplasmic membrane (Uerkvitz & Beck, 1981). In *E. coli*, a role for the AphA enzyme in parental strand recognition of the DNA-replication origin has also been suggested (Reshetnyak *et al.*, 1999). No crystal structure of a class B acid phosphatase is yet available; of the phosphatases of the DDDD superfamily, only the structure of phosphoserine phosphatase from the archaeon *M. jannaschii* (class PSPase) has been published (Wang *et al.*, 2001); the human phosphoserine phosphatase (class PSPase) has been subjected to X-ray diffraction analysis (Peeraer *et al.*, 2002).

2. Experimental and results

2.1. Enzyme expression and purification

The plasmid pATac (Thaller *et al.*, 1997) was used for overproduction of AphA in the *E. coli* DH5α host (Sambrook *et al.*, 1989). The enzyme was extracted from cells grown aerobically at 310 K in Super Broth (Ausubel *et al.*, 1999) supplemented with 44.5 m*M* potassium phosphate buffer pH 7.2 and ampicillin (250 µg ml^{-1}) for plasmid selection. The culture was induced with isopropyl-β-D-thiogalactopyranoside (IPTG; 0.5 m*M* final concentration) when A_{600} reached a value of

0.5. Cells were collected 15 h after induction and the enzyme was extracted from the periplasmic space as described previously (Thaller *et al.*, 1997). The enzyme was precipitated from the extract by adding 30%(*w*/*v*) PEG 6000, resolubilized in buffer *A* (10 m*M* Tris–HCl pH 7.45, 1 m*M* $MgCl_2$; approximately 1/50 of the original culture volume) and loaded onto a DEAE-Sepharose FF column (Amersham–Pharmacia Biotech) equilibrated with buffer *A*. The flowthrough fractions containing the enzymatic activity were concentrated approximately sevenfold by ultrafiltration using a Centriprep YM-30 device (Millipore). The concentrated enzyme preparation was diluted 1:10 in buffer *B* [10 m*M* MES–NaOH pH 6.0, 1 m*M* $MgCl_2$, 1%(*w*/*v*) PEG 6000] and loaded onto a Source 15S column (Amersham–Pharmacia Biotech) equilibrated with the same buffer. The bound enzyme was eluted with a linear NaCl gradient (0–0.8 *M*) in the same buffer. Fractions containing phosphatase activity, eluted at 300 m*M* NaCl, were pooled and concentrated by ultrafiltration using a Centricon device (Millipore; 10 kDa cutoff) to a final concentration of $\sim$6 mg ml^{-1}. Phosphatase activity was monitored using *p*-nitrophenyl phosphate as a substrate as described previously (Thaller *et al.*, 1997). All purification steps were monitored by SDS–PAGE analysis (Laemmli, 1970) (Fig. 1).

This protocol reproducibly yielded 3.5–5.5 mg of purified protein per litre of culture. The purity of the AphA preparation was estimated to be higher than 99.9% by SDS–PAGE (Fig. 1, lane 3). ESI mass spectroscopy of the purified protein revealed the presence of two different polypeptides of molecular masses of 23 528 and 23 713, corresponding to two different cleavage sites of the signal peptide from the AphA *E. coli* precursor (Swiss-Prot ID P32697). The lower molecular-mass species was the predominant form and resulted from the removal of a 25-amino-acid signal peptide, while the higher molecular-mass species, corresponding to the removal of a 23-amino-acid signal peptide, was present in a smaller amount (about 20%).

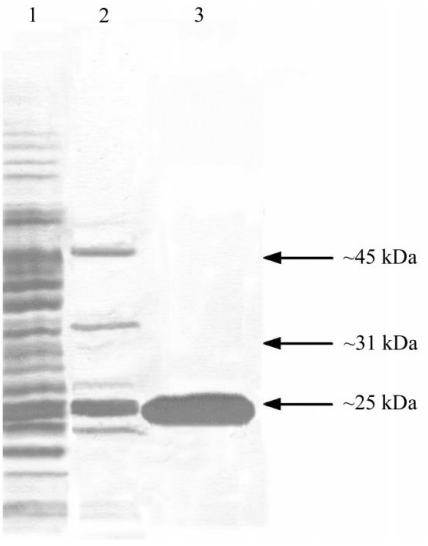

Figure 1
SDS–PAGE analysis of the purification steps of the *E. coli* AphA protein. Lane 1: crude extract obtained using the modified spheroplasting technique. Lane 2: flowthrough enzyme-containing fraction from the DEAE-Sepharose FF column. Lane 3: enzyme-containing fraction eluted from the Source 15S column.

2.2. Crystallization

Early reports of the crystallization of the same enzyme from *S. typhimurium* LT2 (82% sequence homology), the crystal structure of which has never been published, suggested that AphA crystals could be obtained from polyethylene glycol (Uerkvitz & Beck, 1988). Indeed, preliminary screening (Screen 1, Hampton Research, CA, USA; Jancarik & Kim, 1991) indicated PEG 4000–8000 solutions in different buffers at pH values close to 7.0 as the most promising precipitants. However, crystallization of AphA has posed several difficulties, which are likely to arise from the presence of two molecular species in the purified enzyme preparations.

The crystallization conditions were optimized by varying the PEG type and concentration, the pH and the buffer. Eventually, X-ray diffraction-quality crystals were obtained at room temperature with the sitting-drop method from 10 mg ml^{-1} enzyme solutions in HOAc/NaOAc buffer (5 m*M*) pH 7.2 using as precipitant 17–22% PEG 6000 solutions in the same buffer. The presence of divalent metal cations from $MgCl_2$, $ZnCl_2$ or $CoCl_2$ (1–50 m*M*) and of other additives such as 0.1 m*M* β-octyl glucoside or 0.6–1.0%(*w*/*v*) spermine·HCl was essential to obtain large ordered crystals. The drops were prepared by mixing 2 µl of the protein solution with 2 µl of the precipitant solution and were equilibrated with a well containing 100 µl of the precipitant solution. Crystals of average dimensions of between 0.1 and 0.4 mm were commonly grown in the above conditions. Fig. 2 shows a typical drop containing AphA crystals of different sizes.

The AphA crystals are characterized by a marked tendency to give polymorphs even within the same crystallization drop. Indeed, we have obtained data sets indexed in two space groups and three different unit cells, as reported in Table 1. The occurrence of *I*222 or *C*2 crystals, as well as the different unit cells, was apparently a random phenomenon that was independent of the crystallization conditions. This indicates that the AphA tetramer might undergo small conformational rearrangements with a change in the local point symmetry of the tetrameric molecule and that the conformers can pack equally well, reaching close free-energy minima. In the *I*222 cell the asymmetric unit consists of only one subunit, suggesting that the point symmetry of the tetramer is most probably 222, whereas in the *C*2 cell 3 (Table 1) this symmetry is lowered to point group 2, leading to two or four independent monomers in the asymmetric unit. It is also interesting to note that the densest packings are those occurring in the lower symmetry space group.

Table 1
Crystal data for *E. coli* AphA.

	Space group *I*222		Space group *C*2	
	Cell 1†	Cell 2†	Cell 3	Cell 4
Unit-cell parameters				
a (Å)	51.26	49.25	91.73	150.58
b (Å)	93.74	93.25	66.50	85.15
c (Å)	138.90	137.49	91.86	75.68
β (°)			121.4	98.4
V_M (Å^3 Da^{-1})	3.4	3.2	2.5	2.6
Monomers per AU	1	1	2	2

† Cells 1 and 2 refer to the same crystal form and are reported with the aim of comparing the quality of the data collected on a laboratory source (cell 1) with respect to a synchrotron source (cell 2).

2.3. Data collection and processing

AphA crystals (Fig. 2) were successfully frozen in liquid nitrogen by adding 12–15%(*v*/*v*) ethylene glycol to the mother liquor as cryoprotectant. Crystals of different forms were tested using a labora-

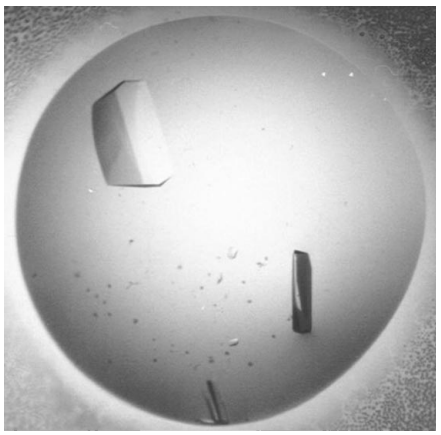

Figure 2
E. coli AphA crystals.

Table 2
X-ray data-collection statistics.

Values in parentheses refer to the high-resolution bin.

	Cell 1	Cell 2	Cell 3	Cell 4
X-ray source	Rotating anode (Cu $K\alpha$)	EMBL X11	ESRF BM30A	EMBL X11
Wavelength (Å)	1.542	0.81	1.138	0.81
Rotation range (°)	0.3	0.8	1.0	1.0
Total No. of reflections	44688	230204	121460	216008
No. of unique reflections	15176	26655	34356	77655
Resolution (Å)	2.2 (2.3–2.2)	1.86 (1.89–1.86)	1.94 (2.02–1.94)	1.85 (1.88–1.85)
Completeness [$I > 2\sigma(I)$] (%)	90.5 (45.2)	98.4 (93.8)	97.7 (89.8)	96.8 (54.5)
$R_{\rm merge}$	0.11 (0.20)	0.068 (0.288)	0.086 (0.153)	0.056 (0.232)
$I/\sigma(I)$	8.2 (2.3)	9.9 (5.0)	4.4 (2.5)	15.0 (3.1)
Temperature (K)	100	100	100	100

tory rotating-anode source coupled with Göbel optics and equipped with a Bruker 1K CCD detector mounted on a three-axis goniometer. The best crystals showed diffraction to 2.0 Å resolution. A *C*2 crystal (cell 4) gave diffraction to 1.6 Å on a synchrotron source. Complete data sets at 100 K were collected at the EMBL beamline X11, at the DORIS storage ring (DESY Hamburg, Germany) and at ESRF beamline BM30A (Grenoble, France) using either MAR image plates or CCD detectors. The latter data collection was performed on a putative osmium derivative. All data were collected with the rotation method with different strategies depending on the experimental setup. In the home laboratory, 0.3° ω scans were performed with the detector fixed at 20−25° in θ. With the larger image plates or CCD detectors, conventional φ scans with the detector fixed at 0° in θ were performed.

We have observed a decay in crystal order with time on several occasions and this observation was mainly associated with the use of detergents, although it also occurred in some of the crystal batches grown with spermine. In such cases, the crystals only showed diffraction within 2–3 weeks of growth, independently of their space group. They subsequently lost any diffraction capability, while maintaining the same habit with no apparent macroscopic sign of degradation. No signs of diffraction decay arising from radiation damage were observed. Table 2 reports the data-collection statistics of the native enzyme in the three crystal forms, comparing the rotating-anode and synchrotron data.

Attempts to solve the structure by molecular replacement using phosphatase domains of known enzymes such as alkaline phosphatase (Kim & Wickoff, 1991), calcium ATPase (Toyoshima *et al.*, 2000) and phosphoserine phosphatase (Wang *et al.*, 2001) have been unsuccessful although, owing to the similarity of the catalyzed reaction, some structural similarity to AphA is expected to occur in the active site of these enzymes. However, the sequence homology of AphA to the other phosphatases of different classes, as well as to any other protein, is extremely low, making the selection of common structural motifs very difficult. Several AphA heavy-atom derivatives are currently being screened to find useful candidates for structure solution by MIR or MAD techniques. MIR phasing has been unsuccessful to date owing to both poor binding and lack of isomorphism of the derivatives. MAD trials on putative gold and osmium derivatives have likewise been unsuccessful.

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