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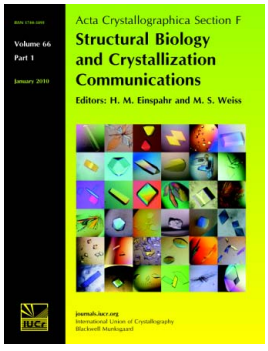

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Crystallization and preliminary X-ray diffraction analysis of *Pseudomonas aeruginosa* phosphorylcholine phosphatase

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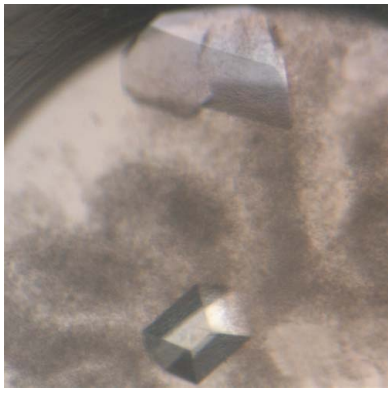

Pseudomonas aeruginosa phosphorylcholine phosphatase (PchP) catalyzes the hydrolysis of phosphorylcholine to produce choline and inorganic phosphate. Phosphorylcholine is released by the action of haemolytic phospholipase C (PlcH) on phosphatidylcholine or sphingomyelin. PchP belongs to the HAD superfamily and its activity is dependent on Mg^{2+}, Zn^{2+} or Cu^{2+}. The possible importance of PchP in the pathogenesis of *P. aeruginosa*, the lack of information about its structure and its low identity to other members of this family led us to attempt its crystallization in order to solve its three-dimensional structure. Crystals of the protein have been grown and diffraction data have been obtained to 2.7 Å resolution. The crystals belonged to the monoclinic space group *C*2, with unit-cell parameters a = 137.16, b = 159.15, c = 73.31 Å, β = 117.89°. Statistical analysis of the unit-cell contents and the self-rotation function suggest a tetrameric state of the molecule with 222 point-group symmetry.

1. Introduction

Pseudomonas aeruginosa colonizes different tissues under varied environmental conditions by the utilization of different mechanisms. One of these mechanisms may involve the breakdown of the host cell's membrane phospholipids through the sequential and coordinated action of haemolytic phospholipase C (PlcH) and phosphorylcholine phosphatase (PchP). The action of PlcH on phosphatidylcholine or sphingomyelin produces phosphorylcholine, which is hydrolyzed to choline and inorganic phosphate by the catalytic action of PchP. An increase in the choline concentration in the environment increases the production of PlcH and PchP and thus amplifies the infectious process (Lisa *et al.*, 1994, 2007). PchP belongs to the haloacid dehalogenase (HAD) superfamily and contains its three characteristic conserved sequence motifs. In mature PchP, motifs I, II and III correspond to ^{31}DMDNT^{35}, ^{166}S and ^{242}K/^{261}GDTPDSD^{267}, respectively (Beassoni *et al.*, 2006). HAD-superfamily members (with the exception of the 2-haloacid dehalogenases; Hisano *et al.*, 1996; Ridder *et al.*, 1999) utilize Mg^{2+} as a cofactor during catalysis (Burroughs *et al.*, 2006). However, we have found that PchP activity measured at pH 5.0 using *p*-nitrophenylphosphate or phosphorylcholine as a substrate is dependent on the divalent cations Mg^{2+}, Zn^{2+} or Cu^{2+} (Beassoni *et al.*, 2008). We have also recently shown that when using phosphorylcholine as a substrate at pH 7.4 Mg^{2+} is an activator and Zn^{2+} is an inhibitor of PchP activity (Otero *et al.*, 2010). The inhibition produced by Zn^{2+} at pH 7.4 was interpreted as a change from octahedral to tetrahedral coordination geometry which is produced by hydrolysis of the Zn-hexacoordinated complex (Otero *et al.*, 2010).

Considering the molecular model based on the crystal structure of *Methanococcus jannaschii* phosphoserine phosphatase (PSP; Wang *et al.*, 2001), the hexacoordination of Mg^{2+} occurs using a pair of electrons contributed by O atoms from the carboxylate groups of Asp31 and Asp262, the backbone C═O of Asp33, one O atom of the phosphate moiety (O_1-P) of the substrate and the O atoms of two water molecules (Beassoni *et al.*, 2008; Otero *et al.*, 2010). The interactions between Mg^{2+}, Zn^{2+} or Cu^{2+} and the most important

residues of motifs I, II and III in the catalytic pocket of recombinant *P. aeruginosa* PchP have been determined (Beassoni *et al.*, 2006, 2008). Several enzymes belonging to the HAD superfamily have been structurally and enzymatically characterized, including PSP from *Methanococcus janaschii* (Wang *et al.*, 2001), human mitochondrial deoxyribonucleotidase (Rinaldo-Matthis *et al.*, 2002), β-phosphoglucomutase from *Lactobacillus lactis* (Lahiri *et al.*, 2002), DNA 3′-phosphatase from *Saccharomyces cerevisiae* (Deshpande & Wilson, 2004) and class B acid phosphatase and histidinol phosphate phosphatase from *Escherichia coli* (Calderone *et al.*, 2004; Rangarajan *et al.*, 2006). With the exception of the model proposed for PchP (Beassoni *et al.*, 2008; Otero *et al.*, 2010), the absolute lack of structural information on this enzyme and its low homology to other members of the HAD superfamily beyond the active site led us to focus our attention on the study of its physicochemical properties and on its crystallization (Beassoni *et al.*, 2010). However, some difficulties were encountered in this previous work using protein that was purified from inclusion bodies and refolded; the crystals that were yielded diffracted to low resolution (4 Å), preventing further analysis. Therefore, in this paper we used a recombinant His-tagged soluble protein folded in its native state that can be produced in high yield and that allowed us to crystallize PchP. Here, the diffraction data and preliminary crystallographic analysis to 2.7 Å resolution are reported.

2. Materials and methods

2.1. Protein expression and purification

The $pchP_{327}$ gene was subcloned in pET15b (Novagen) and the pET15-*pchP* vector was obtained for the expression of PchP as an N-terminal fusion to an MGSSHHHHHHSSGLVPRGSH tag (Otero *et al.*, 2010). The PchP His-tagged protein was expressed in *E. coli* BL21 CodonPlus (Stratagene) and purified as described in Otero *et al.* (2010). Briefly, 1 l of culture was grown at 310 K in LB medium supplemented with 150 µg ml^{-1} ampicillin and 0.5% glucose until the OD_{550} reached 0.6–0.8 and was then induced with 0.4 m*M* IPTG for 18 h at 291 K for protein expression. After expression, cultures were harvested and the cells were broken by osmotic shock and mechanical disruption with sonication. The 6×His-PchP present in the clarified extracts was purified by affinity chromatography using HisLink™ Ni-resin (Promega) according to the manufacturer's instructions. The purified enzyme was dialyzed against 10 m*M* Tris–HCl pH 8.0 containing 30%(*v*/*v*) glycerol and was stored at 253 K. The protein yield was 18–20 mg per litre of culture. The specific activity of this preparation, measured using phosphorylcholine and Mg^{2+} as described in Otero *et al.* (2010), was 61 µmol inorganic phosphate per minute per milligram of protein.

For crystallization experiments, pure fractions were pooled and extensively dialyzed against 200 m*M* NaCl, 10 m*M* Tris–HCl pH 8.0 to remove the glycerol or 200 m*M* NaCl, 20 m*M* bis-tris pH 5.5 in order to also modify the pH. The high concentration of NaCl in the dialysis buffer was essential to prevent protein precipitation. The enzyme was then concentrated to approximately 10 mg ml^{-1} at 277 K using a 10 kDa cutoff Amicon Ultra-4 protein concentrator (Millipore Corporation). The homogeneity of the protein was confirmed by analytical SEC and 15%(*v*/*v*) SDS–PAGE. The final protein concentration was determined using the theoretical molar extinction coefficient (ε = 69 915 M^{-1} cm^{-1}) calculated with the *ProtParam* tool for physicochemical parameter prediction, which is available on the *ExPASy* server (Gasteiger *et al.*, 2005).

2.2. Analytical size-exclusion chromatography (SEC)

Analytical SEC experiments were performed at 295 K using a Superdex 200 10/300 GL column (GE Healthcare) equilibrated with 100 m*M* NaCl, 10 m*M* $MgCl_2$, 30 m*M* sodium acetate/acetic acid pH 5.0. Calibration of the column was performed with a set of standard globular proteins: thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa) and vitamin B_{12} (1.35 kDa). Eluted peaks were monitored by UV absorption at 280 nm.

2.3. Sedimentation equilibrium

Sedimentation-equilibrium experiments were performed in a Beckman Optima XL-A ultracentrifuge using an An-50 Ti rotor and six-channel Epon charcoal centrepieces (optical pathlength 12 mm).

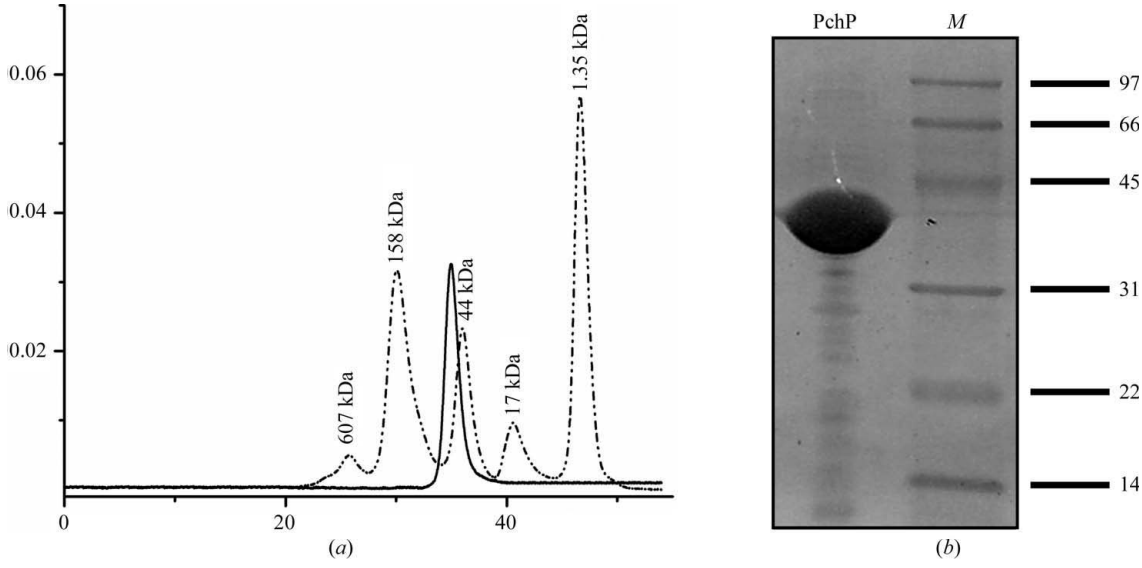

Figure 1
(*a*) Analytical SEC chromatogram of PchP. The solid line shows elution corresponding to PchP in the presence of 100 m*M* NaCl, 10 m*M* $MgCl_2$, 30 m*M* sodium acetate/acetic acid pH 5.0. The dashed line shows elution corresponding to molecular-weight markers (kDa; Gel Filtration Standard, Bio-Rad). (*b*) 15% SDS–PAGE of PchP. The first lane corresponds to PchP incubated with 100 m*M* NaCl, 10 m*M* $MgCl_2$, 30 m*M* sodium acetate/acetic acid pH 5.0. Samples were loaded under reducing conditions. Lane *M* contains molecular-weight markers (kDa; Low-Range, Bio-Rad). The gel was stained with Coomassie Brilliant Blue (Bio-Rad).

Table 1
X-ray diffraction data-collection statistics.

Values in parentheses are for the highest resolution shell. A single crystal was used for measurement for each data set.

Wavelength (Å)	1.54
Resolution (Å)	66.0–2.7 (2.85–2.7)
Space group	*C*2
Unit-cell parameters	
a (Å)	137.16
b (Å)	159.15
c (Å)	73.31
β (°)	117.89
Unique reflections	37859
Average redundancy	6.3 (6.1)
Completeness (%)	100 (100)
$R_{\rm merge}$† (%)	13.1 (30.7)
Mean $I/\sigma(I)$	6.8 (3.8)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith intensity measurement of reflection hkl and $\langle I(hkl)\rangle$ is its weighted average.

Samples of PchP were equilibrated against 100 m*M* NaCl, 30 m*M* sodium acetate/acetic acid pH 5.0 plus 10 m*M* $MgCl_2$ when appropriate and were centrifuged at 8 800*g*, 12 200*g* and 23 500*g* at 293 K. Radial scans at 280 nm were taken at 12, 14 and 16 h. The three scans were identical (equilibrium conditions were reached). The weight-average molecular mass (MW) of PchP was determined using the program *HETEROANALYSIS* v.1.1.2 (Cole & Lary, 2003) with the partial specific volume of PchP set to 0.7345 at 293 K as calculated from its amino-acid composition with the program *SEDNTERP* (Laue *et al.*, 1992; Hayes *et al.*, 2003).

2.4. Crystallization screening and optimization

Sitting-drop crystallization experiments were initially set up at 291 K in Innovaplate SD-2 microplates using a NanoDrop robot (Innovadyne Technologies Inc.) with the following commercially available crystallization screens: Crystal Screen, Crystal Screen 2 (Hampton Research) and JCSG+ Suite (Qiagen). The initial drops consisted of 0.25 µl protein solution (10 mg ml^{-1}) in 200 m*M* NaCl, 10 m*M* Tris–HCl pH 8.0 and 0.25 µl well solution and were equilibrated against 500 µl well solution. Condition No. 95 (0.1 *M* bis-tris pH 5.5, 0.2 *M* $MgCl_2$, 25% PEG 3350) from the JCSG+ Suite (Qiagen) produced clustered plate-like microcrystals (data not shown). For further optimization, the pH of the protein solution was adjusted to 5.5 (see §2) and the same condition was tested in microbatch using 60-well HLA plates (Nalge Nunc International), mixing 1 µl protein solution (10 mg ml^{-1}) in 200 m*M* NaCl, 20 m*M* bis-tris pH 5.5 with 2 µl precipitant solution. The plates were covered with vaseline oil and stored at 291 K. Favourable conditions were identified that produced crystals with maximum dimensions of 0.6 × 0.4 × 0.3 mm that diffracted X-rays to 2.7 Å resolution. Various additives and detergents (Hampton Research) were tested around these conditions, but none of them led to improvement of the crystals.

2.5. X-ray diffraction data collection and analysis

Cryoprotection conditions were optimized prior to data collection. Different concentrations of both ethylene glycol and glycerol were tested. For the best results, crystals were mounted in a fibre loop and cryoprotected by a quick soak in 0.1 *M* bis-tris pH 5.5, 0.2 *M* $MgCl_2$, 25% PEG 3350, 15% glycerol. Diffraction data were collected on a MAR345 detector using Cu $K\alpha$ X-rays generated by an in-house MicroStar rotating-anode diffractometer (Bruker AXS Inc.) equipped with a double-mirror focusing system and operated at 45 kV and 60 mA. The crystal-to-detector distance was set to 150 mm, with $\Delta\varphi = 1^\circ$ and 300 s per image. The maximum resolution reached was 2.7 Å. Diffraction data were processed and scaled using *MOSFLM* (Leslie, 1992) and *SCALA* from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

The purified protein prepared for crystallization was analyzed by size-exclusion chromatography (Fig. 1*a*) and SDS–PAGE (Fig. 1*b*). Sample purity was assessed by SDS–PAGE analysis and SEC and the protein appeared to be at least 95% pure and free of aggregates. The peak eluting at 34.9 min in the SEC chromatogram was typical of a particle of ∼60 kDa, compared with the prominent protein band at around 37 kDa on SDS–PAGE, suggesting that native 6×His-PchP (theoretical MW of 39.4 kDa) is a dimer under nonreducing conditions. This was further confirmed by sedimentation-equilibrium experiments (see §2), which also indicated a dimeric nature of the sample, as suggested by Beassoni *et al.* (2010) considering the elementary hydrodynamics of PchP.

The purification method used here together with careful optimization of the initial protein pH to 5.5 and the buffer composition to maintain the NaCl concentration above 200 m*M* proved to be crucial to obtaining the best diffraction-quality crystals. Crystals of PchP appeared during the screening process using condition No. 95 of the JCSG+ Suite (Qiagen) and had a well defined morphology after 48 h (Fig. 2). They belonged to the monoclinic space group *C*2, with unit-cell parameters a = 137.16, b = 159.15, c = 73.31 Å, β = 117.89°. The merged data set was 100.0% complete to 2.7 Å resolution, with an $R_{\rm merge}$ of 13.1% and a mean $I/\sigma(I)$ of 6.8 (Table 1). Neither the use of additives (Additive Screen from Hampton Research) nor of different experimental setups (hanging drop or microbatch) improved the crystal growth.

Analysis of the Matthews coefficient (Matthews, 1968) of the crystals suggests that there are four molecules in the asymmetric unit and that the crystals have a solvent content of approximately 45% (Matthews coefficient of 2.24 Å^3 Da^{-1}). We investigated the local symmetry relating the units in the asymmetric unit using the program *POLARRFN* (Kabsch *et al.*, 1976) from the *CCP*4 package. Several self-rotation functions were computed in the resolution range 15–3 Å,

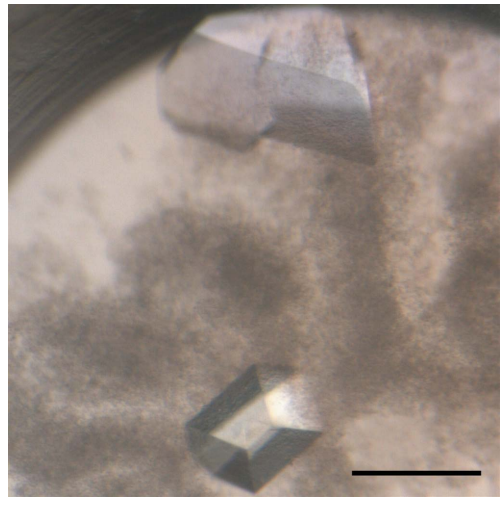

Figure 2
Crystals of PchP grown by the microbatch method. The crystals were obtained using condition No. 95 (0.1 *M* bis-tris pH 5.5, 0.2 *M* $MgCl_2$, 25% PEG 3350) of the JCSG+ Suite screening kit (Qiagen) at 291 K. The scale bar indicates 0.3 mm.

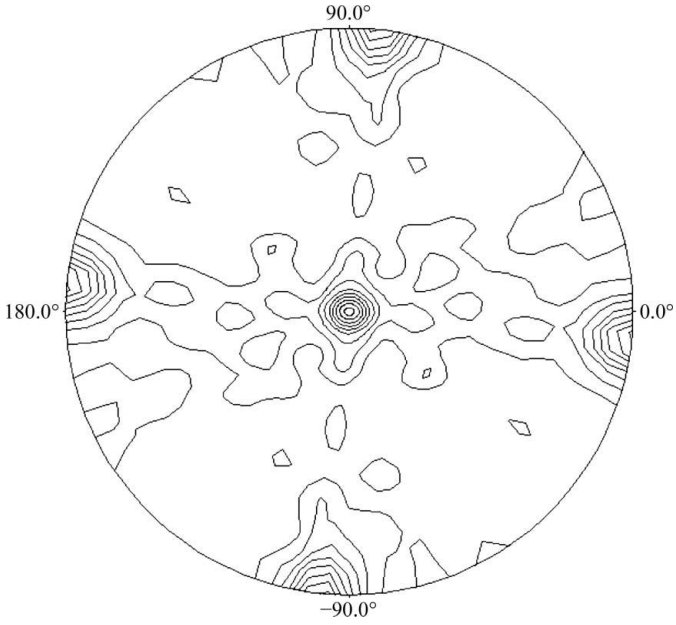

Figure 3
Plot of the self-rotation function of PchP crystals, using data between 15.0 and 2.7 Å resolution and a 25.0 Å radius of integration in the $\kappa = 180^\circ$ section. The view is down the *b* axis. The peaks highlighted show a noncrystallographic twofold axis perpendicular to the crystallographic *b* axis.

with Patterson vectors with a radius of integration of 20–40 Å. Analysis of the self-rotation peaks revealed the presence of a non-crystallographic twofold-symmetry axis perpendicular to the *b* axis. This leads to the generation of a third axis perpendicular to the previous axes. This is consistent with the presence of a tetramer with local symmetry belonging to point group 222. This suggested the presence of an orthorhombic higher order crystal symmetry, which was discarded after careful analysis of the reflection intensities. A stereographic projection ($\kappa = 180^\circ$ section) of the self-rotation is shown in Fig. 3. This appears to contradict the previously reported characterization of the molecule (Beassoni *et al.*, 2010) and the data presented here. Therefore, the oligomeric state of the molecule will be elucidated when the structure is solved following the present protocol, which has the advantage of being simpler and faster than that described previously (Beassoni *et al.*, 2010), in addition to producing soluble protein as opposed to inclusion bodies, and leads to good-quality diffracting crystals. Since the PDB does not contain a suitable model that could be exploited to solve the PchP structure by molecular replacement, our efforts are now focused on the preparation of selenomethionine-substituted protein and the measurement of synchrotron diffraction at a suitable wavelength to phase the structure.

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