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Crystallization and preliminary crystallographic analysis of p40^{phox}, a regulatory subunit of NADPH oxidase

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p40^{phox} is a cytosolic component of the phagocyte NADPH oxidase, which is responsible for production of the superoxide that kills invasive microorganisms. Full-length p40^{phox} was expressed in *Escherichia coli*, purified and crystallized by the sitting-drop vapour-diffusion method at 293 K using polyethylene glycol 20 000 as a precipitant. Diffraction data were collected to 3.0 Å resolution at 100 K using synchrotron radiation. The crystal belongs to space group $C222_1$, with unit-cell parameters a = 146.27, b = 189.81, c = 79.88 Å. This crystal was estimated to contain two or three protein molecules per asymmetric unit from the acceptable range of volume-to-weight ratio values.

1. Introduction

The phagocyte NADPH oxidase plays an important role in killing invasive microorganisms by generating superoxide. NADPH oxidase is a multisubunit enzyme comprised of a membrane-bound cytochrome b_{558} (p22^{phox} and gp91^{phox}), Rac and three cytosolic regulatory subunits, p47^{phox}, p67^{phox} and p40^{phox} (Babior, 1999; Sumimoto *et al.*, 2005). In the resting state, the cytosolic subunits Rac and cytochrome b_{558} exist separately. When activated, the cytosolic subunits and Rac are tethered to the membrane, where they associate with cytochrome b_{558} and form the active NADPH oxidase complex (Babior *et al.*, 2002; Vignais, 2002; Sumimoto *et al.*, 2005).

p40^{phox} is a multidomain protein of 39 030 Da, consisting of PX, SH3 and PB1 domains (Wientjes *et al.*, 1993; Ponting, 1996; Ito *et al.*, 2001). The PX domain of p40^{phox} specifically interacts with phosphatidylinositol 3-phosphate [PtdIns(3)P; Ago *et al.*, 2001] and the PB1 domain of p40^{phox} interacts with that of p67^{phox} (Nakamura *et al.*, 1998). It is known that p40^{phox} enhances membrane translocation of p67^{phox} and p47^{phox} in stimulated cells, thereby facilitating superoxide generation (Kuribayashi *et al.*, 2002). The binding ability of the PX domain of p40^{phox} to PtdIns(3)P is thought to play an important role in this process. Although the structures of each of the domains of p40^{phox} have already been determined (Bravo *et al.*, 2001; Wilson *et al.*, 2003; Massenet *et al.*, 2005), its full-length structure has not been reported so far. In this report, we describe the expression, purification and crystallization of full-length p40^{phox}.

2. Protein expression, purification and crystallization

Full-length (residues 1–339) human p40^{phox} was expressed in *Escherichia coli* as described previously (Hashida *et al.*, 2004). Briefly, full-length p40^{phox} was cloned between the *Nco*I and *Eco*RI sites of pProEX HTb to express p40^{phox} as a 6×His-tagged protein. The plasmid pT-Trx was a generous gift from Dr S. Ishii (Laboratory of Molecular Genetics, The Institute of Physical and Chemical Research, RIKEN, Japan; Yasukawa *et al.*, 1995). These plasmids were co-transformed in *E. coli* BL21 (DE3) and overexpressed. The cells were disrupted by sonication at 277 K in PBS with 0.5 m*M* AEBSF. The protein was applied onto an Ni–NTA column (Qiagen) and was washed with 25 m*M* Tris–HCl pH 7.4, 500 m*M* NaCl and 5 m*M* imidazole. The bound protein was eluted with 25 m*M* Tris–HCl pH 7.4, 500 m*M* NaCl and 250 m*M* imidazole. Fractions containing proteins were purified on a Superdex 75 gel-filtration column (GE

Table 1
Data-collection statistics.

Values in parentheses refer to the highest resolution shell (3.11–3.00 Å).

Resolution range (Å)	50.0–3.00
Observed reflections	161611
Unique reflections	22612
Completeness (%)	99.5 (98.5)
$R_{\rm merge}(I)$†	0.071 (0.479)
$I/\sigma(I)$	20.3 (3.7)

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

Healthcare) and eluted with 25 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl. The amino-terminal His tag of p40^{phox} was removed by incubation with TEV protease for 12 h at 298 K. This solution was applied onto a SourceQ column (GE Healthcare) and eluted using a gradient of 0–100 m*M* NaCl in running buffer (20 m*M* Tris–HCl pH 8.0). Further purification was carried out on a Superdex 75 gel-filtration column eluted with 25 m*M* Tris–HCl pH 8.0 and 150 m*M* NaCl. After addition of dithiothreitol (DTT) to a final concentration of 40 m*M* to prevent protein oxidization, the purified protein was concentrated to about 10 mg ml^{-1} for crystallization screening.

The crystallization of p40^{phox} was performed using the sitting-drop vapour-diffusion method at 293 K. In each trial, a sitting drop of 1 µl purified protein solution was mixed with 1 µl reservoir solution and equilibrated against 100 µl reservoir solution. Initial screening was performed using Crystal Screen, Crystal Screen 2 (Hampton Research) and Wizard I and II (Emerald Biostructures), but no crystals were obtained. However, gelatinous precipitates, which are known to be a solid phase of the protein close to crystals (Bergfors, 2001), were observed in drops where sodium cacodylate was used as a buffer. Since sodium cacodylate seemed to be important, it was added to a final concentration of 10 m*M* to the purified p40^{phox} solution as an additive and grid screening (PEG 20 000 concentrations of 5, 10, 15, 20, 25, 30% *versus* a pH range of 3.6 to 9.6) was performed. Small crystals of p40^{phox} were obtained in reservoir solution containing 5% PEG 20 000, 200 m*M* Bis-Tris–HCl pH 6.5. To obtain larger crystals, a microseeding technique was applied. Small crystals were transferred into an Eppendorf tube containing a Seed Bead (Hampton Research) and 50 µl stabilization buffer (8% PEG 8000, 100 m*M* sodium citrate pH 6.4, 10 m*M* sodium cacodylate, 10 m*M* DTT) and were vortexed to produce microseeds. PEG 8000 was used in the stabilization solution instead of PEG 20 000 because it was less viscous and easier to handle. The sample was then briefly centrifuged and serially diluted (tenfold to 10^8-fold) in the same stabilization buffer. Microseeds were introduced by adding 0.2 µl of each diluted solution to 2 µl of newly prepared drops (consisting of 5% PEG 20 000, 200 m*M* Bis-Tris–HCl pH 6.5). Crystals appeared at a dilution rate of 10^3-fold to 10^4-fold and grew in 5 d to maximal dimensions of 0.3 × 0.2 × 0.2 mm (Fig. 1).

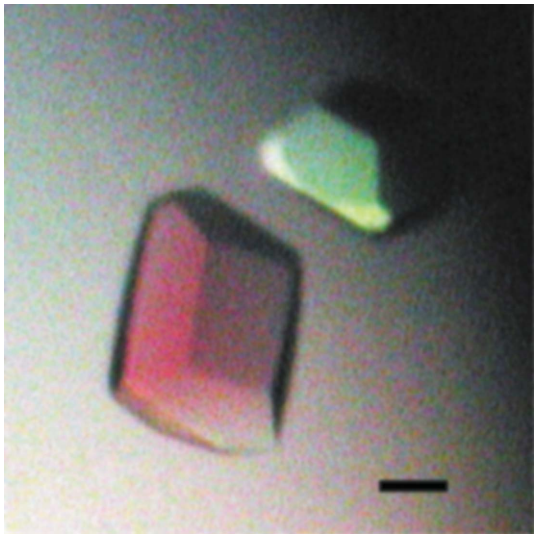

Figure 1
Crystals of p40^{phox}. The black scale bar is 100 µm in length.

3. Data collection and processing

Crystals were immersed in a cryoprotectant (25% glycerol, 200 m*M* acetate pH 5.6, 5% DMSO) for several seconds and then flash-cooled and maintained under nitrogen gas at 100 K during data collection. Diffraction data were collected using an ADSC Quantum 210 charge-coupled device detector on beamline PF-AR NW12 at the Photon Factory (Tsukuba, Japan). Data collection was performed with a total oscillation range of 180° with a step of 1.0° for each exposure time of 10 s and a wavelength of 1.00 Å. Diffraction data were processed using the *HKL*-2000 program suite (Otwinowski & Minor, 1997). The data-collection statistics are summarized in Table 1. The crystals belong to space group $C222_1$, with unit-cell parameters a = 146.27, b = 189.81, c = 79.88 Å. The crystal diffracted to 3 Å, after which the resolution limit abruptly declined. The acceptable range of the volume-to-weight ratio ($V_{\rm M}$) values (Matthews, 1968) indicates that the crystal contains two ($V_{\rm M}$ = 3.54 Å^3 Da^{-1}) or three ($V_{\rm M}$ = 2.36 Å^3 Da^{-1}) protein molecules per asymmetric unit. A self-rotation function revealed no obvious NCS twofold or threefold axes. The structures of the three domains (PX, SH3 and PB1 domains) of p40^{phox} have already been reported. Therefore, phase determination by the molecular-replacement method using these structures is now in progress.

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