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FOR THE RECORD

Crystallization of the Aα subunit of protein phosphatase 2A

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Abstract: The Aα subunit of human protein phosphatase 2A forms crystals in space group $P2_1$ with cell dimensions a = 104.0, b = 174.9, c = 168.2 Å, and β angle = 90.2°. At cryogenic temperatures, the crystals diffracted to a resolution limit of ~3.0 Å. Based on the unit cell dimensions and a calculated molecular mass of 65,277 Da, the Matthews coefficient suggests eight molecules per asymmetric unit. Two native data sets were collected to a nominal resolution of 3.0 Å and merged to provide a set that is 93% complete, with R_{sym} of 9.9%.

Keywords: cryocooling; crystallization; protein phosphatase 2A; X-ray crystallography

Protein phosphatase 2A (PP2A), the most abundant serine/threonine-specific phosphatase in mammals, is composed of three subunits, A, B, and C. The 36-kDa catalytic C subunit and the 65-kDa regulatory A subunit form the core enzyme to which one of several regulatory B subunits ranging in molecular mass from 54 to 130 kDa is bound (Cohen, 1989; Mumby & Walter, 1993; Mayer-Jaekel & Hemmings, 1994, for reviews). PP2A plays an important role in many cellular processes, including cell division (Lee, 1995, for review), development (Mayer-Jaekel & Hemmings, 1995), and signal transduction (Mumby, 1995, for review). It is also involved in carcinogenesis and forms complexes with tumor antigens encoded by the small DNA tumor viruses SV40 and polyoma (Walter & Mumby, 1993, for review). The A subunit polypeptide consists of 15 nonidentical repeats of 38–43 amino acids in length (Walter et al., 1989; Hemmings et al., 1990). In the trimeric form, the B subunit binds to repeats 1–10 at the N terminus and the C subunit to repeats 11–15 at the C terminus. Tumor antigens compete with the B subunit for binding to the N-terminal region (Ruediger et al., 1992, 1994). A model of the A subunit has been proposed based on the fact that it is rod-shaped and has a helix content of greater than 80%. In addition, helical wheel projections demonstrated that each repeat could give rise to two amphipathic α helices that form a pair. We proposed that 15 adjacent pairs interact to form a rod-shaped molecule that provides a basis for B and C subunit association and interaction. Short loops between adjacent helices were proposed to be involved in B and C subunit binding. Extensive analysis of subunit interaction by site-directed mutagenesis provided strong support for the model (Ruediger et al., 1992, 1994). However, definitive proof will come from the elucidation of the three-dimensional structure of the A subunit. In this report, we describe the conditions for crystallizing the A subunit and the properties of the crystals.

Methods used to express and purify recombinant A subunit have been described (Kamibayashi et al., 1992) and were followed with modifications to increase yield and purity. Insect cells (Sf9) in TMN-FH media (JRH Biosciences) were infected with recombinant baculovirus containing full-length human PP2A-Aα cDNA. Fourteen-hundred milliliters of Sf9 cells, grown to a density of 1×10^6/mL, were infected (Summers & Smith, 1987) and harvested 70 h post infection by centrifugation at 2,500 × g for 7 min. They were stored frozen at −70 °C until use. After thawing, the cells were resuspended in 33 mL buffer D (10% glycerol, 50 mM Tris-HCl, pH 7.4, 10 mM dithiothreitol (DTT), 1 mM EDTA, 0.1% sodium azide, 1 μg/mL aprotinin, leupeptin, and pepstatin A, and 0.4 mM Pefabloc SC) containing 0.1 M NaCl. They were homogenized by 20 strokes with a 30-mL teflon mortar and pestle. The homogenate was centrifuged for 25 min at 134,000 × g and the resulting supernatant was filtered and fractionated by FPLC on EAH Sepharose 4B, a weak anion exchanger, using an XK 26/20 column. Elution was performed by applying a linear gradient from 0.1 to 1.2 M NaCl in buffer D at 0.8 mL/min. Fractions corresponding to 0.19–0.28 M NaCl were pooled, diluted fourfold in buffer D, and applied to a Mono Q HR 10/10 column. A single major peak eluted with a linear gradient from 0 to 0.5 M NaCl in buffer D at 2.0 mL/min. Fractions corresponding to 0.15–0.17 M NaCl were pooled, concentrated with a Centricon 30 (Amicon) to a

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volume of less than 2 mL, and applied to a Superdex 200 HR 16/60 column. The protein was eluted with buffer D containing 0.15 M NaCl at 0.5 mL/min. The peak fractions were pooled and dialyzed overnight against 10 mM NaCl, 10 mM Tris-HCl, pH 7.4, 10 mM DTT, 1 mM EDTA, 0.1% sodium azide. The dialyzed protein was concentrated with a Centricon 30 to 14.5 mg/mL. All operations were conducted at 4 °C. The typical yield was 6–10 mg of A subunit per 1.5×10^9 cells. The purity as determined by SDS-PAGE was higher than 95%. Inductance-coupled plasma mass spectrometry analysis indicated no bound metals (Ron LaBorde, Scripps Institute of Oceanography).

Crystals were grown by the vapor diffusion method in Cryschem sitting drop plates (Charles Supper Co., Natick Massachusetts). Initially, crystals were obtained overnight at 22 °C as rectangular spears in drops with 10% PEG 4000, 133 mM imidazole-malate buffer (Im-Ma), pH 5.4, 50 mM NaCl, 0.7% 2-methyl-2,4-pentanediol, and A subunit at 3.3 mg/mL over a reservoir of 30% PEG 4000, 178 mM Im-Ma, pH 5.4, and 86 mM NaCl. These crystals were used as seeds to optimize crystallization conditions. Under the final conditions, equal volumes of protein at 14.5 mg/mL and reservoir consisting of 15% PEG 3350, 125 mM tetramethylene sulfone, 75 mM NaCl, 10 mM $CaCl_2$, 10 mM DTT in 200 mM Im-Ma buffer, pH 5.55, were mixed and microseeded at 4 °C. Crystals appeared within 4 h of seeding. They were grown for two days, then washed three times in reservoir solution and used to macroseed 10 μL of protein and reservoir mixed equally with the PEG in the reservoir reduced to 11%. These crystals grew up to a maximum size of 0.3 × 0.4 × 2.0 mm within two weeks.

Initial inspection of the crystals was done at 4 °C on a Xuong-Hamlin Multiwire Area Detector using graphite monochromatized Cu Kα radiation produced by a Rigaku RU200 generator at 50 kV, 120 mA (Hamlin, 1985; Xuong et al., 1985). The crystals diffracted to around 6 Å, but radiation damage was severe. After 8–10 h, there were no discernible diffraction spots. Cryocooling effectively eliminated radiation damage and also increased the limiting resolution to around 4.5 Å with rotating anode Cu Kα X-rays (Hope, 1990). Prior to cooling, the crystals were soaked for 5 min each in 5, 10, and 15% ethylene glycol in 13% PEG reservoir (v/v). They were subsequently immersed in liquid nitrogen and transferred to the diffractometer using cryogenic stainless steel transfer tongs (Hope et al., 1995). Data to 4.7 Å were collected on area detectors with a modified Siemens LT2 low-temperature machine (120 K). Cooling caused the unit cell constants to shrink by 2.5–5%; the unit cell volume decreased by 9.8%.

Native data sets were collected using synchrotron radiation at Stanford Synchrotron Radiation Laboratory (SSRL) beamline 7-1 and National Synchrotron Light Source, Brookhaven National Laboratory (BNL) beamline X12B (Table 1). Synchrotron radiation at SSRL increased the average resolution of cryocooled crystals from 4.7 to 3.0 Å. Due to limited beamtime availability, data to only 3.8 Å were collected. Later, at BNL X12B, data were collected on a crystal measuring 0.25 × 0.25 × 1.0 mm with a MAR Research imaging plate system in 30-cm format with a crystal to detector distance of 350 mm, $\Delta\phi = 0.75°$ oscillations and 120-s exposures. Data were collected through 360° in ϕ. Crystals were mounted in loops and cryocooled using the liquid propane technique (Hope et al., 1989). Crystals were kept at 93 K with an Oxford Cryosystems Cryostream. A prominent ice ring was observed at approximately 3.2 Å. Images were integrated with Denzo and reduced using SCALEPACK (Otwinowski, 1993). The crystals belong to the space group $P2_1$ with cell dimensions $a = 104.0$, $b = 174.9$, $c = 168.2$ Å, and $\beta = 90.2°$. Space group symmetry and cell constants were initially determined using an Enraf-Nonius precession camera. The cell exhibits pseudo twofold symmetry along the a and c axes, which, coupled with a β angle near 90° and poor diffraction along the c axis, made the data initially appear to be consistent with a $P2_12_12$ space group. One of the expected noncrystallographic twofold axes is visible in the self-rotation function. A Matthews coefficient of 3.17 $Å^3$/Da suggests eight proteins (M_r = 65,277) per asymmetric unit, with solvent content of 61% (Matthews, 1968). A search for suitable heavy-atom derivatives is now in progress.

Table 1. *Native A subunit diffraction statistics*

Crystal	d_{min}[a] (Å)	Total observations/ unique reflections	Completeness (%)	R_{sym}[b]
BNL X12B	3.0	188,109/ 93,490	81.2	0.076
SSRL 7-1	3.8	140,079/ 50,115	78.7	0.064
Merged	3.0	328,188/ 107,076	93.0	0.099

[a] Minimum Bragg spacing with $\langle I \rangle / \sigma = 1.8$.

[b] $R_{sym} = \dfrac{\sum |I_{obs} - I_{avg}|}{\sum I_{avg}}$.

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