crystallization communications

Crystallization and preliminary crystallographic studies of the catalytic subunits of human pyruvate dehydrogenase phosphatase isoforms 1 and 2

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Pyruvate dehydrogenase phosphatase (PDP) is a mitochondrial serine phosphatase that activates phosphorylated pyruvate dehydrogenase complex by dephosphorylation. In humans, two PDP isoforms (1 and 2) have been identified. PDP1 is composed of a catalytic subunit (PDP1c) and a regulatory subunit (PDP1r), whereas PDP2 consists of only a catalytic subunit (PDP2c). Both PDP1c and PDP2c have been crystallized individually and complete X-ray diffraction data sets have been collected to 2.45 and 2.0 Å resolution, respectively. The PDP1c crystals belonged to space group $P4_12_12$ or $P4_32_12$, with unit-cell parameters $a = b = 65.1$, $c = 216.1$ Å. The asymmetric unit is expected to contain one molecule, with a Matthews coefficient V_M of 2.56 Å^3 Da^{-1}. The PDP2c crystals belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 53.6$, $b = 69.1$, $c = 109.7$ Å. The asymmetric unit is expected to contain one molecule, with a Matthews coefficient V_M of 1.91 Å^3 Da^{-1}.

1. Introduction

The mitochondrial pyruvate dehydrogenase complex (PDC) is a macromolecular complex that catalyzes the oxidative decarboxylation of pyruvate to produce acetyl-CoA and NADH, linking glycolysis to the Krebs cycle and lipogenic pathways (Harris *et al.*, 2002). Since the decarboxylation reaction is irreversible, PDC plays the role of a rate-limiting enzyme in glucose metabolism. PDC activity is tightly regulated by reversible phosphorylation in mammalian cells (Harris *et al.*, 2002; Reed *et al.*, 1985; Holness & Sugden, 2003). Pyruvate dehydrogenase kinase (PDK) inactivates PDC by phosphorylation of the E1p component of PDC, whereas pyruvate dehydrogenase phosphatase (PDP) restores PDC activity by dephosphorylation of phospho-E1p. Starvation and diabetes cause a decrease in PDP expression with a reciprocal increase in PDK expression to inactivate PDC for glucose conservation (Wu *et al.*, 2000; Huang *et al.*, 2003), which is an unfavourable condition for type 2 diabetes. In humans, many disease-causing mutations in PDC components have been reported to be associated with lactic acidosis (Robinson, 2001; Hengeveld & de Kok, 2002). Recently, two PDP mutations associated with diminished or abolished PDP activity in cells have been shown to result in PDC deficiency and death in one case (Maj *et al.*, 2005; Cameron *et al.*, 2009).

To date, two PDP (1 and 2) isoforms have been identified in mammalian mitochondria (Huang *et al.*, 1998). PDP1 is a heterodimer consisting of subunits with molecular weights of 97 and 50 kDa. The smaller subunit (PDP1c) has phosphatase activity, whereas the larger subunit is an FAD-containing regulatory protein (PDP1r; Teague *et al.*, 1982). Since native PDP2 has not been isolated to date, it is not known whether the PDP2 catalytic subunit (PDP2c) requires a regulatory subunit. PDP1c and PDP2c share a sequence identity of 53% and both belong to the Mg^{2+}-dependent protein phosphatase M1A (PPM1A; formally PP2C) family (Huang *et al.*, 1998). PDP1c, but not PDP2c, binds to a lipoyl-bearing domain of the E2p component of PDC in a Ca^{2+}-dependent manner, resulting in a tenfold enhancement of PDP1c activity (Pettit *et al.*, 1982; Maj *et al.*, 2006; Turkan *et al.*, 2002). Co-localization with the E1p substrate is

apparently the primary factor in the Ca^{2+}-dependent stimulation of PDP1c activity. A crystal structure of rat PDP1c alone has recently been determined (Vassylyev & Symersky, 2007). The structure revealed that PDP1c has a very similar structure to that of PPM1A. However, the locations of the Ca^{2+}-binding and lipoyl domain-binding sites are still unclear. On the other hand, PDP2c binds to neither Ca^{2+} ion nor the lipoyl domain, despite its high sequence identity to PDP1c (Huang *et al.*, 1998; Karpova *et al.*, 2003). Since PDP2c has not been crystallized, the molecular basis for its inability to interact with the lipoyl domain is not known.

In order to obtain structural insights into the different characteristics of human PDP1c and PDP2c, both proteins have been crystallized as the first step towards solving their structures.

2. Materials and methods

2.1. Expression and purification of PDP1c and PDP2c proteins

Expression plasmids for N-terminally His_6-tagged mature forms of human PDP1c (residues 71–537) and PDP2c (residues 67–529) in pET28 vector (EMD Chemicals, Gibbstown, New Jersey, USA) were kindly supplied by Dr Brian Robinson, University of Toronto, Canada. The expressed proteins from these plasmids have 21 residues including the His_6 tag (MGSSHHHHHHSSGLVPRGSHM) at the N-terminus of the mature sequences of PDP1c and PDP2c. For PDP1c preparation, the original plasmid was used without modifications. For PDP2c expression, the *Nhe*I–*Xho*I fragment coding the mature PDP2c sequence in the parent plasmid was subcloned into the pSUMO (Small Ubiquitin MOdifier) vector (Lifesensors, Malvern, Philadelphia, USA). In the resulting vector, the protein is linked to an N-terminally His_6-tagged SUMO protein moiety. A tobacco etch virus (TEV) protease recognition sequence (ENLYFQ↓AS, with the arrow showing the cleavage site) was engineered into the linker region. The primer sequences used for PCR amplification were TC-AA**GCTAGC**TCAACAGAGGAAGATGATTTTC (forward, *Nhe*I site in bold) and GGTG**CTCGAG**TAGGATGG (reverse, *Xho*I site in bold). The sequence of the resulting plasmids was confirmed by DNA sequencing.

The His_6-tagged PDP1c or His_6-tagged SUMO-PDP2c protein was expressed by co-transformation with pGroESL, which overexpresses the chaperonins GroEL and GroES in the *Escherichia coli* BL21 cell line. An initial culture was established by overnight incubation at 310 K in 50 ml LB medium. 1 l fresh LB medium was subsequently inoculated with 10 ml of the overnight culture and allowed to grow for 3.5 h. The incubation temperature was subsequently lowered to 301 K and maintained for 3 h, followed by 296 K for an additional 2 h. In the final incubation step, isopropyl β-D-1-thiogalactopyranoside was added to a final concentration of 0.5 mM and the cells were grown overnight. Harvested cells were ruptured on ice by sonication in a lysis buffer consisting of 100 mM potassium phosphate buffer pH 7.5, 500 mM NaCl, 0.1% Triton X-100, 0.1% Tween-20, 10% glycerol, 10 mM imidazole, 5 mM β-mercaptoethanol (BME), 0.5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride and 1 mg ml^{-1} lysozyme. The cell lysate was clarified by centrifugation and the supernatant was loaded onto an Ni–NTA column (Qiagen, Valencia, California, USA) at room temperature. After extensive washing with wash buffer (100 mM potassium phosphate pH 7.5, 500 mM NaCl, 10% glycerol, 20 mM imidazole and 5 mM BME), the proteins were eluted with an elution buffer consisting of the wash buffer and 250 mM imidazole. Further purification was conducted on a Superdex 200 column (GE Healthcare, Piscataway, New Jersey, USA) with a gel-filtration buffer consisting of 50 mM potassium phosphate pH 7.5, 100 mM KCl, 5% glycerol and 5 mM BME. Peak fractions were combined and the purified proteins in the gel-filtration buffer were concentrated by ultrafiltration for storage at 193 K (Fig. 1).

For crystallization of PDP1c, the N-terminally His_6-tagged PDP1c was used without removing the tag. For crystallization of PDP2c, the His_6-tagged SUMO moiety was removed as follows. His_6-SUMO-PDP2c and TEV protease were mixed in a mass ratio of 25:1 in digestion buffer (50 mM potassium phosphate pH 7.5, 250 mM KCl, 5% glycerol and 20 mM BME). The mixture was incubated at 277 K overnight with shaking. The completeness of the digestion was determined by SDS–PAGE. The digestion mixture was purified on an Ni–NTA column equilibrated with digestion buffer. The eluted fractions were concentrated and further purified on a Superdex 200 column as described above. The SUMO-free PDP2c contained two residual residues (AS) at the N-terminus of the mature sequence. The purified protein was concentrated to 15–20 mg ml^{-1} and stored in small aliquots at 193 K (Fig. 1).

2.2. Crystallization of PDP1c and PDP2c

For crystallization of PDP1c and PDP2c, the final concentrated protein solutions after the Superdex 200 gel filtration were used without a buffer change. Initial crystals were obtained by screening for crystallization conditions using a Phoenix RE crystallization robot (Rigaku, The Woodlands, Texas, USA) and Qiagen crystallization kits (Qiagen). Crystallization drops were set up on 96-well Intelli-Plates (Art Robbins, Sunnyvale, California, USA) by mixing 0.2 µl protein solution and 0.2 µl screening solution. The volume of the reservoir solutions was 50 µl. The plates were sealed with plastic film and stored at 293 K. The crystallization plates were observed with a Minstrel HP automated crystal-imaging system (Rigaku). Once initial crystallization conditions for PDP1c and PDP2c had been determined, optimization was carried out by the hanging-drop method with 24-well VDX plates (Hampton Research, Aliso Viejo, California, USA) at 293 K. The volumes of the crystallization drops and reservoir solution were 2 µl (1 µl protein solution plus 1 µl reservoir solution) and 1 ml, respectively.

To check the constitution of the PDP2c crystals, several PDP2c crystals of roughly 0.3 mm in size were removed from the crystallization drop and serially transferred into 5 µl reservoir solution ten times to wash the crystals. During washing, precipitate was removed
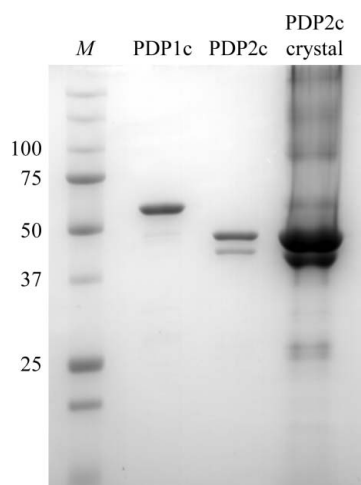

Figure 1
SDS–PAGE gel of purified PDP1c, PDP2c and dissolved PDP2c crystals. The gel was stained with Coomassie Brilliant Blue. Lane *M*, molecular-weight markers (kDa).

from the crystal surface as much as possible. Subsequently, the crystals were dissolved in SDS sample buffer, which was followed by electrophoresis.

2.3. X-ray diffraction data collection and processing

PDP1c and PDP2c crystals were serially transferred to cryo-protectant solutions containing the respective well solutions and increasing concentrations of glycerol (5–25% in 5% increments) for 1 h. These crystals were flash-frozen in liquid propane and stored in liquid nitrogen. Diffraction data were collected at 100 K using an ADSC Quantum 315 imaging detector on beamline 19ID in the Structural Biology Center at the Advanced Photon Source (Argonne, Illinois, USA). The beam size and wavelength were 0.1 × 0.1 mm and 0.9787 Å, respectively. For data collection from PDP1c crystals, the exposure time was 5 s and the oscillation angle was 0.5° for each image and a total of 200 images were collected to obtain a complete data set. For PDP2c crystals, the exposure time was 5 s and the oscillation angle was 0.3° for each image and a total of 400 images were collected. The data were processed using *HKL*-2000 (Otwinowski & Minor, 1997). Data-collection statistics are summarized in Table 1.

3. Results and discussion

His$_6$-tagged human PDP1c in the mature form was expressed in *E. coli* and was purified by metal-affinity chromatography and gel-filtration column chromatography (Fig. 1). The estimated final purity of PDP1c was >95%. PDP1c was soluble and stable at a concentration of 15 mg ml^{-1}. Crystallization screening produced small crystals from JCSG+ suite (Qiagen) condition No. 45 consisting of 100 m*M* HEPES pH 7.5, 200 m*M* MgCl$_2$ and 30% PEG 400. Crystals appeared from protein precipitation in 2 d and grew to 0.2 mm in a week (Fig. 2). Optimization of the crystal conditions by changing the pH or the concentration of protein or PEG 400 failed, suggesting that the initial conditions were optimal.

The expression level of His$_6$-tagged human PDP2c from the original pET28 plasmid was very low. Therefore, we constructed a new expression plasmid in which PDP2c was fused to a His$_6$-tagged SUMO protein at the N-terminus. This plasmid robustly improved the expression level. SUMO-PDP2c was expressed and purified using

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

	PDP1c	PDP2c
Wavelength (Å)	0.978	0.978
Resolution (Å)	2.45 (2.49–2.45)	2.0 (2.03–2.00)
No. of reflections	132449	125090
No. of unique reflections	17911	27044
Multiplicity	7.4 (5.5)	4.6 (4.3)
R_{merge}† (%)	9.0 (48.6)	5.8 (41.9)
Completeness (%)	99.3 (88.8)	95.0 (97.9)
Mean $I/\sigma(I)$	20.7 (2.3)	20.6 (3.3)
Space group	$P4_12_12$ or $P4_32_12$	$P2_12_12_1$

† $R_{merge} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the *i*th observation of reflection *hkl* and $\langle I(hkl)\rangle$ is the mean intensity for all observations *i* of reflection *hkl*.

a procedure similar to that used for PDP1c. After purification of SUMO-PDP2c, the SUMO moiety was removed by TEV protease digestion, which was followed by further purification by gel filtration to remove the cleaved SUMO moiety and TEV protease. The final PDP2c sample contained a smaller contaminant protein (about 42 kDa) which accounted for about 20% of the total protein (Fig. 1). As the smaller band was always present from the beginning of purification (data not shown), this contaminant is probably a degradation product of PDP2c caused by residual protease contamination. This SUMO-free PDP2c sample was concentrated to 20 mg ml^{-1} and initially crystallized in JCSG+ suite condition No. 30 consisting of 100 m*M* phosphate–citrate pH 4.2 and 40% PEG 300. The maximum crystal size was 0.05 mm. We optimized the conditions to improve the crystal size. The optimized condition was 100 m*M* sodium citrate pH 4.4 and 30% PEG 400. In this condition, PDP2c crystals grew to up to 0.3 mm in two weeks (Fig. 2). To check whether the crystals were formed by nondegraded PDP2c, the crystals were dissolved and analyzed by SDS–PAGE. As shown in Fig. 1, the dissolved crystal sample showed both the larger and the smaller bands, indicating that the crystals at least contained nondegraded PDP2c. However, it will not be completely clear whether the crystals really consist of both nondegraded and the probable degraded PDP2c until the structure has been solved.

Native data sets for the PDP1c and PDP2c crystals were collected on beamline 19ID in the Structural Biology Center at the Advanced
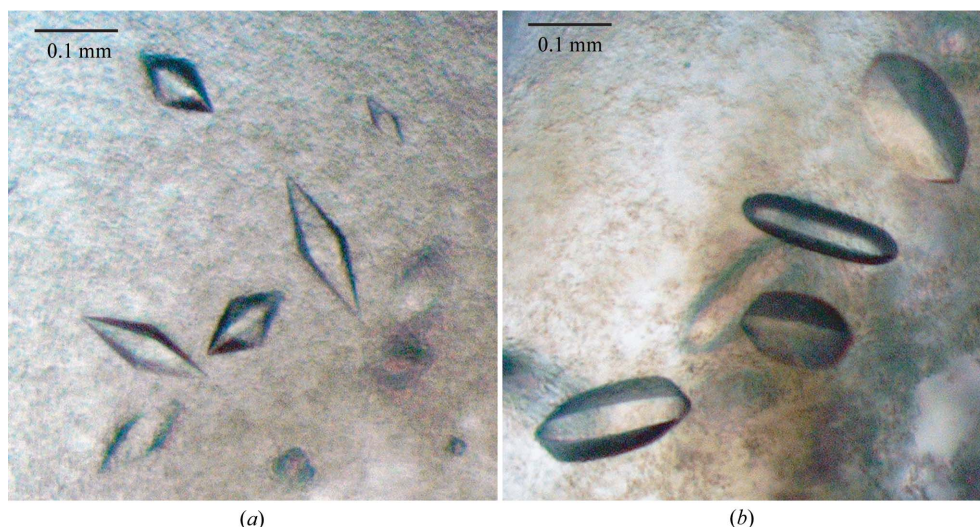

Figure 2
Crystals of (*a*) PDP1c and (*b*) PDP2c. The crystal dimensions are approximately 0.2 × 0.05 × 0.05 mm for PDP1c crystals and 0.3 × 0.1 × 0.1 mm for PDP2c crystals.

Photon Source (Argonne, Illinois, USA). The PDP1c crystals diffracted X-rays to 2.45 Å resolution. The crystals belonged to space group $P4_12_12$ or $P4_32_12$ (tetragonal system), with unit-cell parameters $a = b = 65.1$, $c = 216.1$ Å, $\alpha = \beta = \gamma = 90°$. The asymmetric unit is expected to contain one PDP1c molecule based on the value of the Matthews coefficient (2.56 $Å^3 Da^{-1}$). The PDP2c crystals diffracted to 2.0 Å resolution. The crystals belonged to space group $P2_12_12_1$ (orthorhombic system), with unit-cell parameters $a = 53.6$, $b = 69.1$, $c = 109.7$ Å, $\alpha = \beta = \gamma = 90°$. The asymmetric unit is expected to contain one PDP2c molecule based on the value of the Matthews coefficient (1.91 $Å^3 Da^{-1}$). The structure of rat PDP1c has previously been reported (PDB code 2pnq; Vassylyev & Symersky, 2007). The sequence identities between human PDP1c and rat PDP1c and between human PDP2c and rat PDP1c are 98% and 53%, respectively. Molecular replacement is being carried out using the rat PDP1c structure as a search model.

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