Crystallization and preliminary neutron diffraction experiment of human farnesyl pyrophosphate synthase complexed with risedronate

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Received 16 December 2013
Accepted 21 February 2014

Nitrogen-containing bisphosphonates (N-BPs), such as risedronate and zoledronate, are currently used as a clinical drug for bone-resorption diseases and are potent inhibitors of farnesyl pyrophosphate synthase (FPPS). X-ray crystallographic analyses of FPPS with N-BPs have revealed that N-BPs bind to FPPS with three magnesium ions and several water molecules. To understand the structural characteristics of N-BPs bound to FPPS, including H atoms and hydration by water, neutron diffraction studies were initiated using BIODIFF at the Heinz Maier-Leibnitz Zentrum (MLZ). FPPS–risedronate complex crystals of approximate dimensions $2.8 \times 2.5 \times 1.5$ mm (~ 3.5 mm³) were obtained by repeated macro-seeding. Monochromatic neutron diffraction data were collected to 2.4 Å resolution with 98.4% overall completeness. Here, the first successful neutron data collection from FPPS in complex with N-BPs is reported.

1. Introduction

Farnesyl pyrophosphate synthase (FPPS) is a key enzyme in isoprenoid biosynthesis which supplies sesquiterpene precursors for several classes of essential metabolites including sterols, dolichols, ubiquinones and carotenoids as well as substrates for farnesylation and geranylgeranylation of proteins (Szkopińska & Plochocka, 2005; Glomset *et al.*, 1990). FPPS, a 41 kDa Mg²⁺-dependent homodimeric enzyme, occupies the first branching point of the mevalonate pathway and catalyzes the sequential elongation of dimethylallyl pyrophosphate (DMAPP) to geranyl pyrophosphate (GPP) and then to farnesyl pyrophosphate (FPP) *via* sequential condensation of isopentenyl pyrophosphate (IPP) units. Consequently, FPPS also modulates the post-translational prenylation of the Ras family of GTPases that are essential for cell survival (Berndt *et al.*, 2011).

Nitrogen-containing bisphosphonates (N-BPs) block excessive bone resorption by inhibiting FPPS in osteoclasts, thus inhibiting the mevalonate pathway, blocking the prenylation of GTPase signalling protein and causing osteoclast apoptosis (Rogers, 2003). Currently, N-BPs such as zoledronate and risedronate are the only class of clinically validated drugs that target human FPPS (Rondeau *et al.*, 2006; Kavanagh *et al.*, 2006). Recent clinical investigations have provided evidence for multiple mechanisms that mediate N-BP-induced antitumour effects, including apoptotic cell death, cell-cycle disruption and $\gamma\delta$ T-cell activation (Mitrofan *et al.*, 2010; Mönkkönen *et al.*, 2006). N-BP-induced inhibition of cell proliferation and apoptosis has been reported in prostate cancer (Naoue *et al.*, 2010), breast cancer (Gnant *et al.*, 2009) and multiple myeloma cells (Almubarak *et al.*, 2011). The apoptotic effects of N-BPs have been correlated with the intracellular accumulation of IPP as a consequence of FPPS inhibition (Mitrofan *et al.*, 2010; Mönkkönen *et al.*, 2006). Furthermore, N-BPs have also been tested as potential drugs against protozoan pathogens, and infection by trypanosomatids can be arrested by N-BPs at IC₅₀ concentrations from low micromolar to nanomolar (Montalvetti *et al.*, 2001; Gabelli *et al.*, 2006; Martin *et al.*, 2001). Although N-BPs are expected to become drugs for various cancers and parasitic diseases, the structural features and physico-chemical properties of N-BP drugs limit their therapeutic use to bone-related diseases, owing to their high affinity for bone tissue, poor oral bioavailability and low cell-membrane permeability

Table 1

Neutron diffraction data statistics.

Values in parentheses are for the highest resolution shell.

Neutron source	BIODIFF, FRM II
No. of images	349
Wavelength (Å)	3.99
Space group	$P4_12_12$
Unit-cell parameters (Å)	$a = b = 111.9, c = 72.6$
Resolution (Å)	2.4 (2.49–2.40)
Observed reflections	69884
Unique reflections	18415 (1795)
Mean $I/\sigma(I)$	8.2 (1.8)
Completeness (%)	98.4 (98.0)
$R_{\text{merge}}^{\dagger}$ (%)	10.7 (61.2)
$R_{\text{p.i.m.}}^{\ddagger}$ (%)	5.8 (37.1)
Multiplicity	3.8 (2.7)
Wilson-plot B factor (Å ²)	35.4

$$\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}, \quad \ddagger R_{\text{p.i.m.}} = \frac{\sum_{hkl} [1/\langle I(hkl) \rangle - 1]}{\sum_i [1/I_i(hkl) - 1]}^{1/2}$$

(Dunford, 2010). The discovery of non-N-BP inhibitors of FPPS for the treatment of bone diseases and cancers is therefore a current goal.

N-BPs bind to the DMAPP/GPP ligand pocket of FPPS and several water molecules are known to be involved in the interaction between N-BPs and FPPS (Kavanagh *et al.*, 2006; Rondeau *et al.*, 2006). These water molecules connect between N-BPs and FPPS by forming several hydrogen bonds and cancelling the electrical charges and polarities of the phosphate groups of N-BPs. However, the X-ray crystal structures of FPPS complexed with N-BPs did not reveal a detailed hydrogen-bond network including H atoms owing to their limited resolution (~ 1.8 Å; Kavanagh *et al.*, 2006; Rondeau *et al.*, 2006). In some cases of structure-based drug design, the role of the water molecule and its influence on computational approaches are critical considerations for determining success or failure (de Beer *et al.*, 2010). We consider that neutron protein crystallography is suitable to elucidate the roles of the water molecule, because hydrogen and its isotope deuterium can be more easily located using neutrons (Niimura & Bau, 2008; Yokoyama *et al.*, 2012; Chen *et al.*, 2012). Here, we report the crystallization and preliminary neutron diffraction experiments of human FPPS complexed with risedronate. This represents the first study of a target protein of N-BPs using neutron crystallographic analysis.

2. Materials and methods

2.1. Protein preparation

A synthesized DNA encoding human FPPS residues 8–353 with codon optimization was purchased from Takara Bio (Supporting Information¹). FPPS was subcloned into the *NdeI*–*XhoI* sites of the pET-22b(+) vector (Novagen). FPPS protein was expressed in *Escherichia coli* BL21(DE3) cells. The transformed cells were grown at 310 K with agitation at 250 rev min^{−1} in Luria Broth medium containing 50 µg ml^{−1} ampicillin for 2–3 h to an OD₆₀₀ of 0.55–0.65. At this stage, protein expression was induced overnight at 291 K with 0.25 mM isopropyl β-D-1-thiogalactopyranoside. The cells were harvested by centrifugation (4000 rev min^{−1}) for 10 min at 277 K. The cell pellets were resuspended in lysis buffer consisting of 20 mM Tris–HCl pH 8.0, 250 mM NaCl, 30 mM imidazole and stored at 243 K. The pellets were thawed at room temperature and lysed using sonication on ice. The sonicated cell debris was then centrifuged (12 000 rev min^{−1}, 277 K, 60 min) and the resulting supernatant was

mixed with Ni–NTA agarose beads (Qiagen) pre-equilibrated with lysis buffer. The column was washed with 10 column volumes (CV) of lysis buffer and the protein was eluted with 3 CV of the elution buffer consisting of 20 mM Tris–HCl pH 8.0, 250 mM NaCl, 200 mM imidazole. The elution fraction was analyzed by SDS–PAGE. The desired protein fractions were dialyzed against gel-filtration buffer consisting of 20 mM Tris–HCl pH 8.0, 150 mM NaCl. The dialyzed sample was concentrated using 10 kDa molecular-weight cutoff Amicon Ultra centrifugal filters (Millipore). The protein sample was then subjected to a HiPrep 26/60 Sephacryl S-200 column equilibrated with gel-filtration buffer (GE Life Sciences). The purity of the protein was judged by SDS–PAGE analysis and the total yield was ~ 50 mg per litre of cell culture.

2.2. Crystallization

The macro-seeding method was used to obtain a large crystal suitable for neutron protein crystallography. Initially, a large-scale sitting-drop vapour-diffusion method was carried out to obtain the seed crystals. These crystals were obtained from 400 µl of protein solution consisting of 17 mg ml^{−1} FPPS, 5 mM MgCl₂, 2.0 mM risedronate, 0.5 M NaCl, 15 mM acetic acid-d₄, 35 mM sodium acetate-d₃ equilibrated against 5 ml reservoir solution consisting of 1 M NaCl, 30 mM acetic acid-d₄, 70 mM sodium acetate-d₃. At this stage, deuterated acetic acid and sodium acetate stock solutions were prepared in heavy water, whereas protein, NaCl, MgCl₂ and risedronate stock solutions were prepared in H₂O, because we found that this partially H/D-exchanged condition strongly suppresses nucleation. The seed crystals typically measured around 0.1 mm³. All reagents for the macro-seeding were prepared with heavy water and protein solution was exchanged for solution (10 mM Tris–HCl pD 8.0, 150 mM NaCl in D₂O). The seed crystals were soaked in a droplet (60 µl) consisting of 0.27 M NaCl, 13.3 mM acetic acid-d₄, 53.3 mM sodium acetate-d₃, 12 mg ml^{−1} FPPS, 1.3 mM risedronate, 3.3 mM MgCl₂. The droplet containing the seed crystal was then equilibrated against 1 ml reservoir solution consisting of 0.4 M NaCl, 20 mM acetic acid-d₄, 80 mM sodium acetate-d₃. Crystal growth stopped after 2–4 weeks and the soaked crystal was then transferred into fresh solution. This macro-seeding cycle was repeated at least ten times over 8 months. Finally, a crystal suitable for neutron protein crystallography was obtained with dimensions of 2.8 × 2.5 × 1.5 mm (~ 3.5 mm³).

Figure 1
Photograph of an H/D-exchanged large single FPPS crystal (2.8 × 2.5 × 1.5 mm).

¹ Supporting information has been deposited in the IUCr electronic archive (Reference: NO5042).

Figure 2

Monochromatic neutron diffraction image for the FPPS crystal collected at BIODIFF at FRM II.

2.3. Neutron diffraction data collection

Crystals were mounted in quartz capillaries with the reservoir solution to avoid dryness and then sealed with beeswax and Capillary Wax (Hampton Research). Full neutron diffraction data were collected with BIODIFF, a monochromatic diffractometer with a neutron imaging-plate system. The diffraction data set was collected at room temperature using a pyrolytic graphite monochromator (PG002) set at a wavelength of 3.99 Å. 264 frames (rotation mode) were recorded with a rotation range of 0.3° per frame and exposure times of 60 min (113 frames), 120 min (67 frames) and 240 min (84 frames). To collect the low-resolution Bragg reflections that were saturated on the longer exposures, 85 frames with a rotation range of 0.5° and an exposure time of 10 min were collected in addition. The collected data were indexed, integrated and scaled up to 2.4 Å resolution using *DENZO* (v1.96.2) and *SCALEPACK* (v2.3.6) (Otwinowski & Minor, 1997). The crystal data statistics are listed in Table 1.

3. Results and discussion

A bipyramid crystal with dimensions of $2.8 \times 2.5 \times 1.5$ mm (~ 3.5 mm³; Fig. 1) was obtained by repeated macro-seeding. The crystal sealed in a quartz capillary was subjected to neutron diffraction using BIODIFF at FRM II, TUM, Germany. The crystal belonged to space group $P4_12_12$, with unit-cell parameters $a = b = 111.9$, $c = 72.6$ Å, which are typical of the FPPS crystal structures deposited in the Protein Data Bank. The overall completeness of the neutron data set was 98.4% to 2.4 Å resolution, with an overall R_{merge}

of 10.7% (Table 1, Fig. 2). Whereas 33 X-ray structures of human FPPS have been deposited in the PDB, the neutron diffraction study of FPPS presented here is the first of its kind for any prenyl-transferase. Among deposited X-ray data, the maximum resolution is 1.8 Å (PDB entry 2qis; Structural Genomics Consortium, unpublished work) and their average is 2.2 Å. When these facts are taken into account, a 2.4 Å resolution limit of neutron data seems to be reasonable or very good. X-ray diffraction data at room temperature are supposed to be collected for joint X-ray and neutron structure refinement. We expect the X/N-joint refinement to reveal the protonation states, hydration and detailed hydrogen-bond network of riserisolate.

We acknowledge funding from a Grant-in-Aid for Young Scientists (B) (project No. 25860022).

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