Crystallization and X-ray diffraction analysis of a putative bacterial class I labdane-related diterpene synthase

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Labdane-related diterpenoids are natural products with potential pharmaceutical applications that are rarely found in bacteria. Here, a putative class I labdane-related diterpene synthase (LrdC) identified by genome mining in a streptomycete was successfully crystallized using the microbatch method. Crystals of the LrdC enzyme were obtained in a holo form with its natural cofactor Mg^{2+} (LrdC- Mg^{2+}) and in complex with inorganic pyrophosphate (PP_i) (LrdC- Mg^{2+} - PP_i). Crystals of native LrdC- Mg^{2+} diffracted to 2.50 Å resolution and belonged to the trigonal space group $P3_221$, with unit-cell parameters $a = b = 107.1$, $c = 89.2$ Å. Crystals of the LrdC- Mg^{2+} - PP_i complex grown in the same conditions as the native enzyme with PEG 8000 diffracted to 2.36 Å resolution and also belonged to the trigonal space group $P3_221$. Crystals of the LrdC- Mg^{2+} - PP_i complex grown in a second crystallization condition with PEG 3350 diffracted to 2.57 Å resolution and belonged to the monoclinic space group $P2_1$, with unit-cell parameters $a = 49.9$, $b = 104.1$, $c = 66.5$ Å, $\beta = 111.4^\circ$. The structure was determined by the single-wavelength anomalous dispersion (SAD) technique using the osmium signal from a potassium hexachloroosmate (IV) derivative.

1. Introduction

Recent advances in genomics have shown that terpenoid-biosynthesis genes are widely distributed in bacteria (Cane & Ikeda, 2012). However, compared with the presence and production of other terpenoids with a different number of isoprene (C_5) units, from which they are biosynthetically derived, bacterial diterpenoids (C_{20}) are relatively uncommon (Yamada *et al.*, 2015). As a consequence of their structural and stereochemical diversity, diterpenoids exhibit a broad range of biological activities, including antibiotic, fungicidal, anti-inflammatory and tumorstatic activities, amongst others (Rasoamiaranjanahary *et al.*, 2003; Toyomasu *et al.*, 2007; De Boer & Leeuwen, 2012; Janke *et al.*, 2014). Diterpenoids have been divided into several groups on the basis of biosynthetic hydrocarbon ring construction (Peters, 2010). One of these groups are the labdane-related diterpenoids (LRDs), which are natural products defined as minimally containing the fused bicyclic hydrocarbon structure found in the labdane family of diterpenoids. It is well known that the biosynthesis of LRDs is initiated by the cyclization of geranylgeranyl pyrophosphate (GGPP) to a specific stereoisomer of labdadienyl/copalyl pyrophosphate (CPP) in a reaction catalyzed by class II

diterpene synthases. This CPP bicyclic structural core is then rearranged and dephosphorylated by class I diterpene synthases. Finally, tailoring enzymes such as cytochromes P450 produce more complex and structurally diverse molecules (Cyr *et al.*, 2007; Peters, 2010). In actinobacteria, class I and II labdane-related diterpene synthases, GGPP synthase and cytochromes P450 genes are frequently arranged in the same gene cluster (Meguro *et al.*, 2013).

Recently, we have used genome mining to identify a putative labdane-related diterpenoid cluster of five genes (*lrdABCDE*) encoded in the genome sequence of *Streptomyces* sp. K155, a streptomycete isolated from the soil of Valle de Chalco, State of Mexico by our research group (unpublished work). Sequence analysis of this gene cluster predicts five enzymes: a GGPP synthase (LrdA), class II (LrdB) and class I (LrdC) labdane-related diterpene synthases and two cytochromes P450 (LrdD and LrdE). The *LrdABCDE* enzymes exhibit a low level (<30%) of average sequence identity compared with other enzymes of bacterial diterpene clusters that have previously been reported (Meguro *et al.*, 2013), suggesting a novel LRD biosynthetic gene cluster. As mentioned above, class I diterpene synthases play a key role in the production of structurally diverse LRD molecules. Therefore, among the five enzymes of the *lrdABCDE* gene cluster, we have focused our attention on the LrdC enzyme. Sequence analysis reveals that LrdC shares 100% sequence identity with a hypothetical protein from *Streptomyces* sp. (NCBI accession No. WP_019525557.1) and contains the typical DDXXE motif and the NSE triad ND(L/I/V)-XSXXE (Table 1) that have been found in class I terpene synthases (Christianson, 2006; Yamada *et al.*, 2015). Both motifs together bind the trinuclear Mg^{2+} cluster, which triggers the departure of the substrate pyrophosphate leaving group to generate inorganic pyrophosphate (PP_i) and a highly reactive allylic carbocation that can undergo rapid isomerization and cyclization (Christianson, 2006). X-ray crystallographic studies of this family of enzymes indicate that structural changes occur upon the binding of the trinuclear Mg^{2+} cluster and PP_i between the open (apo) and closed (substrate or ligand-bound) conformations (Aaron *et al.*, 2010; Baer *et al.*, 2014). Specifically, in the closed conformation an induced-fit mechanism ensures that carbocation formation only takes place when the active site is firmly locked and not accessible to solvent (Baer *et al.*, 2014). The crystal structure of the epizizaene synthase (EIZS), a sesquiterpene cyclase from *S. coelicolor* A3(2), bound to Mg^{2+} - PP_i -BTAC (benzyltriethylammonium cation) has been determined (Aaron *et al.*, 2010). As a hydrophobic cation, BTAC mimics the carbocation intermediate proposed for the EIZS-catalyzed cyclization mechanism (Aaron *et al.*, 2010). However, it has been shown that only the Mg^{2+} - PP_i complex is sufficient to obtain a closed conformation in terpene synthases (Baer *et al.*, 2014).

Currently, structural information on bacterial class I diterpene synthases is limited to CotB2 from *S. melanosporofaciens* (PDB entry 4omg; Janke *et al.*, 2014), which shares 26% sequence identity with LrdC and catalyzes the direct cyclization of GGPP to cyclooctat-9-en-7-ol, a diterpene compound

Table 1

Macromolecule-production information.

Source organism	<i>Streptomyces</i> sp. K155
DNA source	Synthetic DNA
Cloning vector	pBSK-TrpC
Expression vector	pET-22b(+)
Expression host	<i>E. coli</i> BL21(DE3)pLysS
Complete amino-acid sequence of the construct produced†	MTD TDDGGTMLPLPDF TATFPPEFPAGPHSERTE-HRLLDWLEEHPLPSAKAKAVLVNITSHGASR-TFTPADADLLFAELLWLTAFFDDVHAENG-VGGPAALVDRASELMVLAGGNPPRAMSPFPA-VLHDLARFRARASAAAYHRLAASLRDTLMAL-VWEAHVAKPEGVALATYLAMRPHTVFIKTIT-AAGEILLGYELDTQRALAAVRNLETAVANLAGWINDLASEREMQRGRGQPLSLPTLLHARHG-GTIEEAFTRASSMCENEA AVARRGITHLAHAS-PNALT AHARALEDITRSFIWHTSHARYQGIRP-NRGSTSSPARSLQH HHHHHH

† The sequence from pET-22b(+) is underlined.

unrelated to labdane which is the precursor of the anti-inflammatory drug ciclooctatin (Janke *et al.*, 2014). Furthermore, structural information on bacterial class I LRD synthases is limited to ent-kaurene synthase from *Bradyrhizobium japonicum* (BjKS; PDB entry 4w4r; Liu *et al.*, 2014), which shares 23% sequence identity with LrdC. Therefore, structural studies of LrdC enzyme in a holo form with its natural cofactor Mg^{2+} (LrdC- Mg^{2+}) and in a closed conformation in complex with PP_i (LrdC- Mg^{2+} - PP_i) will offer an opportunity to gain insights into this particular LRD biosynthetic pathway and the reaction mechanism of this family of enzymes.

2. Materials and methods

2.1. Gene subcloning, protein expression and purification

The nucleotide sequence encoding the LrdC enzyme from *Streptomyces* sp. K155 was codon-optimized for expression in *Escherichia coli* and the synthetic gene was prepared by Epoch Life Science Inc., Texas, USA. The synthetic *lrdC* gene was then subcloned into the NdeI and XhoI sites of the pET-22b(+) expression vector (Novagen), which contains a C-terminal His₆-tag sequence, resulting in plasmid pLrdC. The identity of the resulting construct was confirmed by DNA sequencing. Electrocompetent *E. coli* BL21(DE3)pLysS cells were transformed with pLrdC and grown on Luria-Bertani (LB) agar plates containing 100 $\mu\text{g ml}^{-1}$ ampicillin at 310 K. A sample of a liquid culture of exponentially growing BL21(DE3)pLysS/pLrdC was resuspended in a sterile solution of 40% (v/v) glycerol and maintained at 203 K. For LrdC expression, aliquots of the frozen cell stock were thawed, cultured for 12 h at 310 K in 100 ml LB medium containing 100 $\mu\text{g ml}^{-1}$ ampicillin and used to inoculate 1 l 2 $\times$ YT medium at an initial optical density at 600 nm (OD_{600}) of 0.05 at 310 K and 200 rev min⁻¹. When an OD_{600} of 0.6 was reached, the temperature was reduced to 295 K and recombinant LrdC expression was induced by the addition of IPTG (final concentration of 0.1 mM) to the medium. The culture was then incubated for 10 h with shaking at 150 rev min⁻¹. The cells were harvested by centrifugation (7500g, 10 min,

277 K) and resuspended in 50 ml buffer *A* [50 mM sodium phosphate pH 7.5, 500 mM NaCl, 10%(v/v) glycerol, 10 mM β -mercaptoethanol] with 20 mM imidazole containing Complete Protease Inhibitor Cocktail Mini Tablet EDTA-free (Roche Molecular Biochemicals) and DNase ($1 \mu\text{g ml}^{-1}$). The cell suspension was sonicated on ice and the resulting cell debris was removed by centrifugation (19 000g, 35 min, 277 K). The supernatant containing His₆-tagged LrdC was loaded onto a 5 ml Ni²⁺-chelating HisTrap HP column (GE Healthcare) pre-equilibrated with buffer *A* and containing 20 mM imidazole. The column was then washed with ten bed volumes of buffer *A* with 100 mM imidazole in order to remove contaminants. Bound LrdC enzyme was eluted with 300 mM imidazole in buffer *A* at a flow rate of 2 ml min^{-1} and analyzed by SDS-PAGE with Coomassie staining. The 300 mM imidazole eluate containing the LrdC enzyme was collected, concentrated and dialyzed against several volumes of buffer *B* [50 mM HEPES–NaOH pH 7.5, 150 mM NaCl, 10%(v/v) glycerol, 10 mM MgCl₂, 10 mM β -mercaptoethanol] in an ultrafiltration cell (Amicon Ultracel filter, 30 kDa molecular-weight cutoff). This pool was loaded onto a 120 ml HiLoad 16/600 Superdex 75 pg column (GE Healthcare) pre-equilibrated with buffer *B*. LrdC was then eluted with the same buffer at a flow rate of 1.0 ml min^{-1} (Fig. 1). Fractions containing LrdC were pooled, dialyzed against several volumes of buffer *C* [20 mM HEPES–NaOH pH 7.5, 100 mM NaCl, 10%(v/v) glycerol, 2 mM MgCl₂, 5 mM DTT] and concentrated to 20 mg ml^{-1} as estimated using an extinction coefficient of $34.8 \text{ mM}^{-1} \text{ cm}^{-1}$ at 280 nm as calculated using the *Protein Calculator* v.3.4 software (<http://www.scripps.edu/~cdputnam/protcalc.html>). Macromolecule-production information is summarized in Table 1.

2.2. Protein crystallization

Crystallization conditions were initially screened at 277 and 291 K by the microbatch method using screening kits from

Figure 1
Size-exclusion chromatography profile of the LrdC enzyme. Inset, Coomassie-stained SDS-PAGE gel (12%) of purified LrdC after size-exclusion chromatography. Lane 1, molecular-weight markers (Thermo Fisher Scientific). Lane 2, purified LrdC.

Hampton Research (Crystal Screen, Crystal Screen 2, Crystal Screen Cryo, Crystal Screen Lite, Index, MembFac and PEG/Ion) and from Emerald Bio (Wizard I, II, III and IV). Protein droplets consisting of $1 \mu\text{l}$ LrdC and $1 \mu\text{l}$ reservoir solution were prepared and covered with paraffin oil using a Mosquito LCP crystallization robot (TTP Labtech). Native LrdC-Mg²⁺ crystals in different habits were obtained in several crystallization conditions, but they diffracted poorly at a synchrotron-radiation source. In an effort to obtain crystals suitable for X-ray diffraction, co-crystallization trials of native LrdC-Mg²⁺ enzyme with tetramethylammonium (TMA) chloride and PP_i were performed using the same procedure as described above. However, we named the latter crystals of LrdC in the closed conformation the LrdC-Mg²⁺–PP_i complex, since after the structure determination of LrdC no electron density was observed for TMA cations at the active site. Diffraction-quality crystals of the LrdC-Mg²⁺–PP_i complex (Fig. 2) were obtained over a period of one week in condition No. 18 of Crystal Screen Cryo, consisting of 0.16 M magnesium acetate tetrahydrate, 0.08 M sodium cacodylate trihydrate pH 6.5, 16%(w/v) PEG 8000, 20%(v/v) glycerol (Fig. 2*a*) and condition No. 25 of PEG/Ion, consisting of 0.2 M magnesium acetate tetrahydrate, 20%(w/v) PEG 3350 (Fig. 2*b*). Since no convincing hit was obtained in the initial crystallization trials

Figure 2
Crystals of the LrdC-Mg²⁺–PP_i complex obtained using (a) 0.16 M magnesium acetate tetrahydrate, 0.08 M sodium cacodylate trihydrate pH 6.5, 16%(w/v) PEG 8000, 20%(v/v) glycerol and (b) 0.2 M magnesium acetate tetrahydrate, 20%(w/v) PEG 3350. The photographs were taken with different magnifications. The scale bar corresponds to 200 μm.

Table 2
Crystallization.

	LrdC-Mg ²⁺ , native	LrdC-Mg ²⁺ -PP _i complex	
		Space group <i>P</i> 3 ₂ 21	Space group <i>P</i> 2 ₁
Method	Microbatch	Microbatch	Microbatch
Plate type	96-well plates	96-well plates	96-well plates
Temperature (K)	277	277	277
Protein concentration (mg ml ⁻¹)	20	20	20
Buffer composition of protein solution	20 mM HEPES-NaOH pH 7.5, 100 mM NaCl, 10%(v/v) glycerol, 2 mM MgCl ₂ , 5 mM DTT	20 mM HEPES-NaOH pH 7.5, 100 mM NaCl, 10%(v/v) glycerol, 2 mM MgCl ₂ , 5 mM DTT, 10 mM TMA, 10 mM PP _i	20 mM HEPES-NaOH pH 7.5, 100 mM NaCl, 10%(v/v) glycerol, 2 mM MgCl ₂ , 5 mM DTT, 10 mM TMA, 10 mM PP _i
Composition of reservoir solution	0.16 M magnesium acetate tetrahydrate, 0.08 M sodium cacodylate trihydrate pH 6.5, 16%(w/v) PEG 8000, 20%(v/v) glycerol	0.16 M magnesium acetate tetrahydrate, 0.08 M sodium cacodylate trihydrate pH 6.5, 16%(w/v) PEG 8000, 20%(v/v) glycerol	0.2 M magnesium acetate tetrahydrate, 20%(w/v) PEG 3350
Volume and ratio of drop	2 µl: 1 µl protein solution and 1 µl reservoir solution	2 µl: 1 µl protein solution and 1 µl reservoir solution	2 µl: 1 µl protein solution and 1 µl reservoir solution

for native LrdC-Mg²⁺, crystals of the LrdC-Mg²⁺-PP_i complex (Fig. 2*a*) were also used as seeds in order to obtain LrdC crystals in a holo form without PP_i. Therefore, using the streak-microseeding technique, crystal nuclei were transferred using a horse-tail hair into droplets consisting of 1 µl LrdC and 1 µl reservoir solution prepared using the microbatch method. Small crystals of native LrdC-Mg²⁺ were observed in 30 min, but they required one week to reach approximate dimensions and a similar crystalline habit to the crystals of the LrdC-Mg²⁺-PP_i complex (Fig. 2*a*). In addition, osmium-derivative crystals were prepared by soaking crystals of the LrdC-Mg²⁺-PP_i complex (Fig. 2*a*) with 1 mM potassium hexachloro-osmate(IV) for 24 h at 277 K. Crystallization information is summarized in Table 2.

2.3. Data collection and processing

Data collection from crystals of the LrdC-Mg²⁺-PP_i complex was performed on beamline X25 of the National Synchrotron Light Source (NSLS), Brookhaven National Laboratory (BNL), New York, USA using a PILATUS 6M detector. X-ray diffraction data for the LrdC-Mg²⁺-PP_i complex were collected from a single crystal (Fig. 2*a*) at $\lambda = 1.1000$ Å. The crystal-to-detector distance was maintained at 400 mm, with an oscillation range per image of 1° and an exposure time of 1.5 s. Since the crystallization condition contained cryoprotectants, the crystal was flash-cooled in liquid nitrogen and directly tested for X-ray diffraction. The crystal diffracted to a maximum resolution of 2.36 Å and belonged to the trigonal space group *P*3₂21 (Fig. 3*a*), with unit-cell parameters $a = b = 107.2$, $c = 89.0$ Å. A total of 495 927 reflections were integrated to a resolution of 2.36 Å and were merged to obtain 24 673 unique reflections with an overall R_{merge} of 0.062 and a completeness of 100.0%. The calculated Matthews coefficient (V_M) of 3.95 Å³ Da⁻¹ suggests the presence of one molecule in the asymmetric unit. A second data set ($\lambda = 1.1000$ Å) was collected from a crystal of the LrdC-Mg²⁺-PP_i complex grown in a different crystallization condition (Fig. 2*b*). The crystal-to-detector distance was maintained at 430 mm, with an oscillation range per image of

1° and an exposure time of 1.0 s. For data collection under cryogenic conditions, the crystal was briefly soaked in a cryoprotectant solution consisting of 20 mM HEPES-NaOH pH 7.5, 0.2 M magnesium acetate tetrahydrate, 30%(v/v) PEG 400. Subsequently, the crystal was loop-mounted and flash-cooled in a 100 K dry nitrogen stream. The crystal diffracted to a maximum resolution of 2.57 Å and belonged to the monoclinic space group *P*2₁ (Fig. 3*b*), with unit-cell parameters $a = 49.9$, $b = 104.1$, $c = 66.5$ Å, $\beta = 111.4^\circ$. Matthews coefficient calculations suggested that there were two molecules per asymmetric unit ($V_M = 2.16$ Å³ Da⁻¹). Data collection from crystals of native LrdC-Mg²⁺ and an osmium derivative (LrdC-Mg²⁺-PP_i-Os) was performed on beamline 19BM of the Advanced Photon Source (APS), Argonne National Laboratory (ANL), Chicago, Illinois, USA using an ADSC Quantum 210r detector. A data set ($\lambda = 0.9790$ Å) was collected to a resolution of 2.50 Å from a crystal of native LrdC-Mg²⁺; its unit-cell parameters were $a = b = 107.1$, $c = 89.2$ Å. All data sets were processed using *XDS* (Kabsch, 2010), *POINTLESS* (Evans, 2006) and *SCALA* from the *CCP4* suite (Winn *et al.*, 2011). For phase determination using the Os signal, a data set from a crystal of the osmium derivative was collected to 2.80 Å resolution at the wavelength corresponding to the L_{III} absorption maximum of osmium ($\lambda = 1.1404$ Å), which was determined *via* an X-ray fluorescence spectrum recorded before the diffraction experiments. The osmium-derivative crystals belonged to the trigonal space group *P*3₂21 or *P*3₁21 as suggested by *POINTLESS* (Evans, 2006). Data-collection statistics are summarized in Table 3.

3. Results and discussion

The putative class I labdane-related diterpene synthase LrdC from *Streptomyces* sp. K155 was successfully expressed in *E. coli* strain BL21(DE3)pLysS with a purification yield of ~80 mg LrdC from 1 l of culture. The LrdC enzyme consists of 333 residues (Table 1) with a theoretical molecular weight of 36.0 kDa; it was purified to homogeneity as assessed by size-exclusion chromatography and SDS-PAGE (Fig. 1). Attempts

Table 3
Data collection and processing.

Values in parentheses are for the outer shell.

	LrdC-Mg ²⁺ , native	LrdC-Mg ²⁺ -PP _i complex	LrdC-Mg ²⁺ -PP _i complex	LrdC-Mg ²⁺ -PP _i -Os, osmium SAD
Diffraction source	Beamline 19BM, APS	Beamline X25, NSLS	Beamline X25, NSLS	Beamline 19BM, APS
Wavelength (Å)	0.9790	1.1000	1.1000	1.1404
Temperature (K)	100	100	100	100
Detector	ADSC Q210r	PILATUS 6M	PILATUS 6M	ADSC Q210r
Crystal-to-detector distance (mm)	218	400	430	206
Rotation range per image (°)	1.0	1.0	1.0	1.0
Total rotation range (°)	240	360	360	240
Exposure time per image (s)	1.0	1.5	1.0	1.0
Space group	<i>P</i> 3 ₂ 21	<i>P</i> 3 ₂ 21	<i>P</i> 2 ₁	<i>P</i> 3 ₂ 21
<i>a</i> , <i>b</i> , <i>c</i> (Å)	107.1, 107.1, 89.2	107.2, 107.2, 89.0	49.9, 104.1, 66.5	106.7, 106.7, 90.4
α , β , γ (°)	90.0, 90.0, 120.0	90.0, 90.0, 120.0	90.0, 111.4, 90.0	90.0, 90.0, 120.0
Resolution range (Å)	32.6–2.50 (2.64–2.50)	35.1–2.36 (2.49–2.36)	34.7–2.57 (2.71–2.57)	34.9–2.80 (2.95–2.80)
Total No. of reflections	301962	495927	133102	216057
No. of unique reflections	20825 (3004)	24673 (3540)	20167 (2913)	15004 (2139)
Completeness (%)	99.9 (100.0)	100.0 (100.0)	99.8 (99.5)	100.0 (100.0)
Multiplicity	14.5 (14.8)	20.1 (20.3)	6.6 (6.2)	14.4 (14.7)
$\langle I/\sigma(I) \rangle$	25.6 (7.0)	31.6 (8.5)	23.0 (6.8)	27.0 (6.9)
<i>R</i> _{merge} † (%)	7.2 (38.7)	6.2 (39.4)	9.9 (39.8)	7.0 (38.5)
CC _{1/2} (%)	99.9 (97.1)	99.9 (98.0)	99.7 (94.2)	99.9 (96.9)
Anomalous completeness (%)				99.9 (100.0)
Anomalous multiplicity				7.5 (7.5)
FOM after density modification				0.67

† $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ and $\langle I(hkl) \rangle$ represent the diffraction-intensity values of the individual measurements and the corresponding mean values, respectively. The summation is over all unique measurements.

to co-crystallize the LrdC enzyme with TMA cations failed, probably owing to the low concentration in the crystallization drops or because of the small size of this cation compared with BTAC. However, diffraction-quality crystals of the LrdC enzyme in a holo form (LrdC-Mg²⁺) and in a closed conformation in complex with PP_i (LrdC-Mg²⁺-PP_i) were obtained

in two different crystallization conditions (Fig. 2). Complete data sets were collected for all crystals, as shown in Table 3.

The structure of the LrdC enzyme was determined by the single-wavelength anomalous dispersion (SAD) method using *AutoSol* from the *PHENIX* suite (Adams *et al.*, 2010). Two fully occupied and four partially occupied Os-atom sites were

Figure 3
X-ray diffraction patterns of (a) a crystal of the LrdC-Mg²⁺-PP_i complex grown in 0.16 *M* magnesium acetate tetrahydrate, 0.08 *M* sodium cacodylate trihydrate pH 6.5, 16% (w/v) PEG 8000, 20% (v/v) glycerol and (b) a crystal of the LrdC-Mg²⁺-PP_i complex grown in 0.2 *M* magnesium acetate tetrahydrate, 20% (w/v) PEG 3350. Data were measured on beamline X25 at BNL using a PILATUS 6M detector.

identified. The space-group ambiguity ($P3_221$ versus $P3_121$) and the correct enantiomorph of the structure were uniquely resolved by parallel phasing for the two alternatives using *AutoSol* from the *PHENIX* suite (Adams *et al.*, 2010). *AutoSol* unambiguously selected one of the possible enantiomorphs and indicated that the correct space group is $P3_221$, with a figure of merit (FOM) of 0.29. The phases were substantially improved during density modification to give a final FOM of 0.67. Initial construction of the LrdC model was carried out using *AutoBuild* from the *PHENIX* suite (Adams *et al.*, 2010). The resulting electron-density map was of sufficient quality to allow the tracing of ~80% of the main-chain atoms. The native LrdC-Mg²⁺ and both LrdC-Mg²⁺-PP_i complex structures were determined by molecular replacement using *Phaser* (McCoy *et al.*, 2007) with the osmium-derivative model as a search model, giving an average LLG of ~6000 for all structures. For native LrdC-Mg²⁺ and both LrdC-Mg²⁺-PP_i complex structures, Mg²⁺ atoms and PP_i were clearly visible in the expected locations. Refinement of all structures is currently under way using *PHENIX* (Adams *et al.*, 2010) and *Coot* (Emsley *et al.*, 2010).

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