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Short communication

Crystal structure of the C-terminal domain of tubulin-binding cofactor C from *Leishmania major*

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ABSTRACT

Tubulin-binding cofactor C stimulates GTPase activity and contributes to the release of the heterodimeric α/β-tubulin from a super-complex of tubulin monomers and two ancillary cofactors. We have determined the 2.2 Å resolution crystal structure of the C-terminal domain of tubulin-binding cofactor C from *Leishmania major* based on single wavelength anomalous dispersion measurements targeting a selenomethionine derivative. Although previously predicted to consist of two domains the structure is best described as a single domain dominated by a right-handed β-helix of five turns that form a triangular prism. One face of the prism is covered by the C-terminal residues leaving another face solvent exposed. Comparisons with an orthologous human GTPase activating protein match key residues involved in binding nucleotide and identify the face of the β-helix fold likely involved in interacting with the β-tubulin:GTP complex.

The cytoskeleton of trypanosomatids is highly enriched in microtubules. They form a network of subpellicular network beneath the cell membrane and maintain the overall parasite shape. Microtubules are abundant in the flagellum, an organelle important for pathogenicity as well as motility, for attaching the parasite to the salivary gland of the insect vector prior to the infection of a new host and also contributing to the evasion of the host immune system [1,2]. The folding and polymerization of α- and β-tubulin subunits is carefully regulated to ensure their correct tertiary structure and to prevent spontaneous aggregation or premature polymerization [3]. The assembly process involves distinct stages, each influenced by several chaperones or cofactors [4,5]. After a tubulin polypeptide is produced it is captured by prefoldin [6] then passed to the T-complex polypeptide 1 complex [7] where the folding is essentially completed. The tubulin-binding cofactors (TBC) are then involved in heterodimer assembly and polymerization [4,5,8]. The proteins involved are highly conserved across species and we are exploiting trypanosomatids as the model system to help dissect the contributions that they make to microtubule assembly [9–11].

There are five TBCs, termed A–E. TBCB and E bind α-tubulin whilst A and D interact with β-tubulin, to deliver each tubulin subunit into a super-complex comprising the α/β-tubulin heterodimer and cofactors D and E [8]. TBCC is involved in the final stage activation of GTP hydrolysis by β-tubulin, promoting release of the α/β-tubulin heterodimer from the super-complex protein assembly that can then proceed to polymerization [8,12,13]. This TBCC cofactor is a polypeptide of about 340 amino acids, located in the centrosome and predicted to form three distinct domains [14]. The N-terminal domain of the human protein has been characterized by nuclear magnetic resonance spectroscopy (NMR) and structures deposited in the Protein Data Bank (PDB) [14]. This N-terminal domain carries a flexible and unstructured N-terminus, that interacts with tubulin, and this leads into a bundle of three α-helices [14]. The fold is similar to that of TBCA [11]. NMR structures of a truncated C-terminal fragment of human TBCC, consisting of 179 amino acids are deposited in the PDB (code 2yuh, unpublished). This protein domain shares a sequence identity of approximately 20% with the corresponding domain of the trypanosomatid proteins.

Here we concentrate on TBCC from *L. major* (*Lm*TBCC, Uniprot code Q4Q1A3). We describe the crystallographic analysis of the C-terminal domain of *Lm*TBCC-C. Recombinant forms of the full-length protein from *Trypanosoma brucei* and *L. major* (335 amino acids, approximate mass 36.8 kDa) were prepared but proved recalcitrant to structural studies. In particular, speedy degradation of the

Abbreviations: ARL3, ADPribosylation factor-like protein 3; CARP, cyclase-associated proteins and X-linked retinitis pigmentosa 2 gene products; TEV, tobacco etch virus; TBC, tubulin-binding cofactor; LmTBCC, TBCC from *Leishmania major*; LmTBCC-C, C-terminal domain of the *L. major* protein; PEG 2000 MME, polyethylene glycol monomethyl ether; NMR, nuclear magnetic resonance spectroscopy; PDB, protein data bank; RP2, retinitis pigmentosa 2 protein; SAD, single-wavelength anomalous dispersion; SeMet, selenomethionine.

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Table 1
Crystallographic details of *Lm*TBCC-C.

Data collection	
Wavelength (Å)	0.97907
Space group	$P2_1$
Cell dimensions	
a, b, c (Å)a, b, g (°)	37.6 93.2 48.390.0 108.4 90.0
Resolution (Å)	46.6–2.2 (2.27–2.20)[a]
R_{merge}/<I/sI>	10.2 (23.9)/17.5 (6.8)
CC ½[b]	0.998 (0.967)
Completeness (%)/multiplicity	98.3 (86.8)/11.9 (7.1)
Anomalous completeness	97.7 (82.5)
Anomalous multiplicity	6.0 (3.4)
Wilson B($Å^2$)	14.6
Refinement	
No. reflections (total/Rfree)	15026 (792)
Rwork/Rfree	15.8/19.7
No. atoms protein/water	2697/300
B-factors($Å^2$)	
Protein (chainA/chain B)	19.8/20.1
Water	28.4
r.m.s. Deviations	
Bond lengths (Å)/angles (°)	0.010/1.326
Ramachandran distribution (%)[c]	
Favored/outliers	98.2/0

The gene fragment encoding residues 152–355 of *Lm*TBC-C from *Leishmania major* strain Friedlin identified in GeneDB (LmjF.36.3160, [21]) was amplified from genomic DNA using PCR. To permit the use of selenomethionine for phase determination a single mutation, Leu223Met was introduced (Quikchange mutagenesis, Stratagene). The gene was cloned into a modified pET15b plasmid to encode an N-terminal His-tag followed by a tobacco etch virus (TEV) protease cleavage site. The resulting vector was transformed into *Escherichia coli* B834 (DE3), and cells grown in Selenomethionine Medium (Molecular Dimensions, UK), expression induced with 1 mM IPTG at an OD_{600} 0.6 and growth continued at room temperature for 16 hours. Cells were harvested by centrifugation and resuspended in 50 mM Tris–HCl pH 7.5, 250 mM NaBr, 20 mM imidazole before storage at −20 °C.

Thawed cells were lysed using French Press at 16 kpsi and lysate was clarified by centrifugation at 37,500 × *g* for 30 min at 4 °C. Soluble supernatant was filtered (0.2 μm) and loaded onto a 5 mL HisTrap HP column (GE Healthcare) pre-equilibrated with 50 mM Tris–HCl, 250 mM NaBr pH 7.5 for an initial affinity chromatography capture step. Elution of *Lm*TBCC-C was performed by applying an imidazole gradient with the target protein eluting at approximately 140 mM. The product was treated with TEV protease at 30 °C for 2 h. Dialysis at room temperature, to remove excess imidazole, was followed by reverse affinity chromatography prior to a final purification step with size exclusion chromatography using a calibrated Superdex 200 26/60 gel filtration column and the equilibration buffer. The protein eluted with an estimated mass of 20 kDa, which corresponds to that expected for a monomeric sample (20.4 kDa). The sample was pooled, buffer exchanged into 10 mM Tris–HCl, 100 mM NaBr pH 7.5 and concentrated using a centrifugal concentrator (10 kDa cutoff, Sartorius) prior to crystallization. The protein concentration was determined by measurement of absorbance at 280 nm and an estimated extinction coefficient 38,680 M^{-1} cm^{-1} [22]. Poor quality crystals were produced at 18 °C by the hanging drop vapor diffusion method using 0.75 μL of protein solution at a concentration of 7 mg mL^{-1}, mixed with 0.75 μL of reservoir containing 100 mM MES (4-morpholineethanesulfonic acid) pH 6.5, 25–30 % PEG 2000 MME (polyethylene glycol monomethyl ether). A crystal was placed into an Eppendorf tube with 100 μL of reservoir and a small nylon ball was added before vortexing for 30 seconds to create a micro-crystal suspension. Fresh conditions were prepared with 100 mM MES pH 6.7, 22% PEG 2000 MME in the reservoir, and protein solution as before but at a reduced concentration of 3 mg mL^{-1}. A cryo-loop was used to streak the micro-seed suspension into the conditions and the plates stored at 18 °C. Well formed needles (40 × 40 × 500 mm) appeared in several days. Single-wavelength anomalous dispersion (SAD) data were measured from a single crystal at −170 °C on beam line I24 of the Diamond Light Source with a Pilatus 6 M detector. A helical data collection protocol to minimize radiation damage was used. Data were indexed and integrated using XDS [23] and scaled using AIMLESS [24]. The structure was solved via SAD-phasing using Phenix AutoSolv [25]. Two molecules of *Lm*TBCC-C constitute the asymmetric unit and each contains two SeMet residues. These four Se positions were identified and provided an initial figure-of-merit 0.44. The density modification step yielded an improved figure-of-merit of 0.69 and was followed by automated model building to produce a partial model consisting of 311 residues giving an R/R_{free} of 24.2%/29.1% and a map-model correlation coefficient of 0.79. The model was then completed with the graphics software COOT [26]. Refinement was performed in REFMAC5 [27] utilizing Translation/Libration/Screw refinement [28], and alternated with rounds of electron and difference density map inspection and model manipulation together with ligand incorporation using COOT, and the incorporation of waters and alternate conformer side chains. Non-crystallographic symmetry restraints were not employed. MOLPROBITY [29] was used to investigate model geometry in combination with the validation tools provided in COOT.

[a] Values in parenthesis are for the highest resolution shell.
[b] Pearson correlation coefficient [30].
[c] Calculated using the Molprobity server (http://molprobity.biochem.duke.edu).

polypeptides was noted. Limited proteolysis of the *L. major* protein, using trypsin, followed by mass spectrometry finger printing matched to the identification of a C-terminal fragment. A recombinant form of this domain, comprising residues 152–355 with a Leu223Met mutation was prepared (*Lm*TBCC-C) to allow the production of selenomethionine (SeMet) derivative protein. The strategy behind the mutation was to enhance the chances of obtaining a good anomalous dispersion signal by placement of selenium into the hydrophobic core of the protein fold at a position unlikely to influence the structure. Sequence comparisons (not shown) indicated that at positions corresponding to 223 a leucine, isoleucine (as in human TBCC PDB code 2yuh) or methionine is observed. This form was crystallized and the structure determined at 2.2 Å resolution by exploiting the anomalous dispersion X-ray scattering properties of selenium. Crystallographic details are presented in Table 1 and the coordinates and structure-factor data have been deposited in the PDB with accession code 5aj8.

Two polypeptides constitute the asymmetric unit and in each there are two segments, residues 155–157 and 326–355, which could not be modeled due to disorder. Non-crystallographic symmetry was not restrained during the refinement and it is noteworthy that the two molecules adopt a similar structure with an r.m.s.d for all atoms of just 0.77 Å between residues 158–325, reducing to just 0.18 Å when only the main chain atoms are considered. It is therefore only necessary to detail one molecule.

The N-terminal segment, residues 158–242, forms a right-handed parallel β-helix barrel consisting of five coils or layers. The helical barrel is shaped as an approximate triangular prism,