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An allosteric transition trapped in an intermediate state of a new kinesin-inhibitor complex

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Abbreviations used: STLC, S-trityl-L-cysteine; ATP, Adenosine triphosphate; ADP, Adenosine diphosphate; AMPPNP, 5'-adenylyl- β,γ -imidodiphosphate; FRET, Fluorescence resonance energy transfer; RNAi, RNA interference; Mg^{2+} , Magnesium ion; PIPES, Piperazine-1,4-bis(2-ethanesulfonic acid); PDB, Protein Data Bank; DTT, Dithiothreitol; EGTA; Ethylene glycol tetraacetic acid; Å, angstrom; cryoEM, cryo-electron microscopy.

ABSTRACT

Human kinesin Eg5 plays an essential role in mitosis by separating duplicated centrosomes and establishing the bipolar spindle. Eg5 is an interesting drug target for the development of cancer chemotherapy, with 7 inhibitors already in clinical trials. Here, we report the crystal structure of the Eg5 motor domain complexed with a potent antimetabolic inhibitor S-trityl-L-cysteine to 2.0 Å resolution. The Eg5-STLC complex crystallises in space group $P3_2$ with 3 molecules per asymmetric unit. Two of the molecules reveal the final inhibitor-bound state of Eg5, whereby Loop L5 has swung downwards to close the inhibitor-binding pocket, helix $\alpha 4$ has rotated by about 15° and the neck-linker has adopted a docked conformation. The third molecule, however, revealed an unprecedented intermediate state, whereby local changes at the inhibitor-binding pocket have not propagated to structural changes at the switch II cluster and neck-linker. This provides structural evidence for the sequence of drug-induced conformational changes.

INTRODUCTION

Human Eg5 is a member of the Kinesin-5 subfamily and is responsible for the formation of the bipolar mitotic spindle [1]. Current models suggest that the Eg5 homotetramer separates the duplicated centrosomes by pushing antiparallel microtubules apart [2, 3]. The inhibition of Eg5 by RNAi, specific inhibitors, or immunodepletion causes cells to arrest in mitosis with monoastrial spindles. [1, 4, 5]. Prolonged mitotic arrest then leads to the activation of the mitotic checkpoint and subsequent apoptotic cell death in certain cancer cell lines. Thus, Eg5 is an interesting candidate for drug development in cancer chemotherapy.

Cell-based and *in vitro* assays have led to the discovery of a series of inhibitors specific to Eg5. S-trityl-L-cysteine (STLC) was previously identified as a new, potent Eg5 inhibitor [6] and subsequent work revealed that STLC is a reversible, tight-binding inhibitor that does not compete with either ATP or microtubules [7], but slows down ADP release. In recent years, several crystal structures of Eg5 in complex with

inhibitors have surfaced. When compared with the native structure of Eg5 without inhibitor [8], these crystal structures reveal several important conformational changes at key structural elements upon inhibitor binding. These conformational changes, which are observed in virtually all the inhibitor-bound Eg5 motor domain crystal structures, include the downward swing of the Loop L5 to close the inhibitor-binding pocket and also a shift in switch I [9]. In addition, helix $\alpha 4$ of the switch II cluster rotates by about 20° , on the plane of the Eg5 motor-microtubule interface, as shown by cryoEM reconstruction of the motor-microtubule complex [10]. This new position of the switch II cluster is also described as the “permissive” conformation, as this rearrangement opens up space for the neck-linker to dock onto the motor domain [11].

A conformational change in the neck-linker, which links the motor domain to the stalk domain, is believed to be pivotal in producing movement for the molecular motor. This highly conserved neck-linker is inherently flexible and mobile. In the ADP-bound conventional kinesin structure, the neck-linker is observed to be disordered [12] and undocked [13], but becomes ordered and docked in the microtubule-bound kinesin-AMPPNP (slowly-hydrolyzable ATP analogue) structure [14]. Similarly, in the ADP-bound Eg5 motor domain native structure, the neck-linker is ordered, undocked and perpendicular to the motor domain [8]. To date, all inhibitor-bound Eg5 motor domain structures reveal a docked neck-linker conformation, parallel to the motor domain.

From static images of the native structure and the final inhibitor-bound state, it was unclear, how these conformational changes were induced by inhibitor binding and what the sequence of these conformational changes was. Studies using FRET (fluorescence resonance energy transfer) have been carried out to gain insight into the intermediate transition states. Kinetic studies by Maliga *et al.* propose a model in which, following Monastrol binding to Eg5 motor domain, the downward swing of Loop L5 occurs first, followed by conformational changes in the switch II cluster, which allows the neck-linker to dock [15].

In this study, we report the crystal structure of an unprecedented intermediate state of the Eg5-STLC complex, which provides structural evidence for the sequence of structural changes that occur upon inhibitor binding.

RESULTS & DISCUSSION

Overall structure

The crystal structure of the Eg5-STLC complex was solved at a resolution of 2.0 Å. Data collection and refinement statistics are shown in Table 1. The Eg5-STLC complex crystallises with three molecules (subsequently named A, B and C) in the asymmetric unit. The final model comprises of residues Asn18 to Asn271 and Asn287 to Glu364 for molecules A (Fig 1A) and B; and Lys17 to Asn271 and Asn289 to Pro363 for molecule C. Loop L11 is missing as in most other kinesin structures. All three molecules have one molecule of Mg^{2+} ADP bound in the nucleotide-binding pocket with the magnesium coordinated octahedrally with the β -phosphate, the oxygen (OG1) of the hydroxyl side chain of Thr112 and four water molecules. The inhibitor-binding pocket, bordered by helix $\alpha 2$, Loop L5 and helix $\alpha 3$, is occupied by the inhibitor STLC, which has a very well-defined electron density (Fig 1B).

Interactions in the inhibitor-binding pocket

The conformation of STLC and the interactions between STLC and Eg5 are virtually identical in all three molecules. The three phenyl rings in STLC, which represent the hydrophobic part of the inhibitor, are buried in the allosteric site and display several hydrophobic interactions with the alkyl side chains of Glu215, Glu116 and Arg119 (Fig 2). We also observed edge-to-face (T-shape) interaction between the first phenyl group of STLC and the phenyl ring of Trp127; C—H--- π interaction between the first phenyl group of STLC and the pyrrolidine ring of Pro137; offset stacked π - π interaction between the second phenyl group and Tyr211; and C—H--- π interaction between the third phenyl group and the side chain of Leu214 [16]. On the other hand, the hydrophilic part of the inhibitor, namely the cysteine moiety, is exposed to the solvent and we observed several hydrogen bond interactions: the amino group of the cysteine moiety with the main chain carbonyl oxygen of Gly117, the side chain oxygen (OE1) of Glu116, and a structural water molecule; an oxygen (OXT) of the carboxyl group of the cysteine moiety with one of the side chain amino groups (NH1) of Arg221 and four structural water molecules; and the other oxygen of the carboxyl group with one structural water molecule. (All structural water molecules mentioned are conserved in all three molecules of the asymmetric unit) Thus, our crystal structure reveals several important interactions in the inhibitor-binding pocket and helps to corroborate the mutagenesis studies, conducted by Brier *et al.* [17], which identified the residues that are essential for the inhibition of Eg5 by STLC.

Conformational changes at the inhibitor-binding pocket

The three Eg5-STLC molecules in the asymmetric unit reveal a variety of structural differences throughout the molecule, when compared with the native structure of the Eg5 motor domain [8] in the absence of any bound inhibitor. The most obvious proximal changes occur in the inhibitor-binding pocket. In all three molecules, we observed that Arg119 and Tyr211 move outwards, while Trp127 of the Loop L5 region (Gly117 to Ala133) swings downwards, by about 7 Å, from a solvent-exposed region to form an aromatic edge-to-edge π -interaction with the phenol ring of Tyr211 (Fig 2, Movie 1). These conformational changes are consistent with those observed in the Eg5-Monastrol complex [9]. These conformational changes also support the induced-fit model of the binding of the inhibitor to the Eg5 motor domain: Arg119 and Tyr211 move outwards to accommodate the inhibitor, while Trp127 swings downwards to close the entrance of the induced-fit pocket and forms a snug inhibitor-binding pocket. We also observed that residues Pro121 to Glu124 of molecule A are displaced by a maximum of 2 Å away from the core of the motor domain, as compared to that of molecules B and C. Upon close examination, we saw that crystal contacts between these residues in molecule A and a symmetry-related molecule could have caused this small displacement.

P-loop, helix α 3 and switch I

Another important region in the Eg5 motor domain is the catalytic site or nucleotide-binding pocket. In the native and STLC-bound structures, Mg^{2+} ADP occupies the active site through binding of the α - and β -phosphates to the P-loop (residues Gln106 to Gly110). The overlay of molecules A, B and C with the native structure showed no differences in conformation of the P-loop. We then investigated the helix α 3. In all three molecules, the apex of the helix α 3 is shifted towards the solvent by a maximum of about 2 Å at Glu209, when compared to the native structure (Fig 3B,

Movie 2). This is probably to accommodate the inhibitor, since several residues of the helix $\alpha 3$ participate in the formation of the inhibitor-binding pocket. Following helix $\alpha 3$ is switch I (residues Asn229 to His236), which is thought to be a γ -phosphate sensor. We observed that the conformation of all three molecules are identical, but they differ from that of the native structure in that the Loop L9 is shifted towards the solvent by about 6 Å and displays an additional one-turn of a helix. The hydroxyl group of Tyr231, which is originally solvent-exposed in the native structure, moves by about 12 Å into the hydrophobic environment of the protein.

Intermediate state of switch II cluster and neck-linker

Conformational changes at and around the inhibitor-binding pocket are thought to be translated to conformational changes at the switch II cluster (helix $\alpha 4$, Loop L12, and helix $\alpha 5$) and the neck-linker region at the back of the Eg5 motor domain (Fig 3C, Movie 3). We overlaid molecules A, B and C, using the main chain C_{α} atoms from residues 19 to 363, and found that molecules A and B were almost identical with a root mean square deviation of 0.33 Å. However, we observed a larger mean deviation of 1.32 Å when we overlaid molecules A and B with C. Upon closer examination of molecule C, we believe that we have trapped an intermediate state of inhibitor-bound Eg5, never seen before in any kinesin crystal structures. Henceforth, the structures of molecules A and B, which have similar conformations, will be referred to as the final inhibitor-bound state, while the structure of molecule C will be referred to as the intermediate state. The occurrence of this intermediate state, in particular the structural differences at the switch II cluster and the neck-linker, is not caused by crystal contacts with the symmetry-related molecule and is not a crystallographic artifact (Supplementary figure 1). The B-factors for the switch II cluster and neck-linker region, though higher than the average B-factor of the entire molecule, are consistent throughout molecules A, B and C. In fact, trapping of structural intermediates is possible in flexible regions of the kinesin motor in the absence of microtubules, because such structural changes would require low energy input [8]. We observed a clear progression of the switch II cluster as it rotates about 15° from the native structure to the intermediate state and eventually to the final inhibitor-bound state. In fact, this progression can be seen as two distinct movements: first an anti-clockwise rotation of approximately 10° about the origin around residue Leu293, then a similar rotation of approximately 5° about an origin beyond the apex of helix $\alpha 4$. The rearrangement of the switch II cluster opens up space for the neck-linker to adopt a docked position in the final inhibitor-bound state. In both the native and intermediate structures, the neck-linker, which connects the motor to the stalk domain of Eg5, is in an ordered, albeit undocked position, perpendicular to the helix $\alpha 6$. The neck-linker eventually moves by about 32 Å to adopt a docked position, parallel to helix $\alpha 6$ in the final inhibitor-bound state. Thus, the structural intermediate found in molecule C reflects a dynamic switch II cluster and a flexible neck-linker region. Such flexibility in the neck-linker serves to emphasize its role in force generation and motility.

Pathway of structural changes

Although several crystal structures of Eg5 in complex with inhibitors have been deposited in the Protein Data Bank, all of them exhibit the same conformation and structural changes representative of the final state of the inhibitor-bound Eg5. As the crystal structures are static images, it remains unclear how these structural changes come about and the sequence of events leading to the final inhibitor-bound state. Studies using FRET have been carried out to gain insight into the intermediate

transition states [15, 18]. By monitoring the quenching of Trp127 by Monastrol binding to Eg5 and using FRET, between mant-ADP at the active site and an Oregon green fluorophore attached to an introduced cysteine near the end of the neck-linker, Maliga *et al.* observed drug-induced biphasic conformational change of the Eg5 motor domain [15]. The first rapid phase involves conformational changes at Trp127 and other residues in the inhibitor-binding site. A much slower subsequent conformation change then results in the docking of the neck-linker onto the motor domain. The conformational changes observed in the intermediate state is consistent with that expected after initial inhibitor binding – significant local changes at the inhibitor-binding pocket – but before the docking of the neck-linker. Significantly, the changes in the intermediate state are moving in the direction of the final state. Complete conversion only occurs in the final inhibitor-bound state, where the neck-linker becomes docked. Therefore, we propose that the binding of STLC to Eg5 causes drug-induced structural transition in 3 steps: first, Loop L5 swings downwards to close the inhibitor-binding pocket, which then translates to a rearrangement of the switch II cluster to adopt a “permissive” conformation, and finally the neck-linker docks onto the motor domain.

MATERIAL AND METHODS

Protein expression and purification

The motor domain of human Eg5 (residues 1-368) was initially cloned into the vector pET28a [19]. It was then cut with restriction enzymes NcoI and XhoI (NEB) and cloned into a modified pProExHT expression plasmid (Invitrogen), yielding a Precision Protease-cleavable N-terminal hexahistidine-tagged protein. The plasmid was transformed into *E. coli* BL21 (DE3) pLysS (Invitrogen). Three liters of bacterial culture were grown at 37°C in TB (Terrific Broth) supplemented with 100 mg/l Ampicillin to an A_{600} of approximately 0.7 and induced overnight with 0.5 mM isopropyl β -D-thiogalactopyranoside (Melford). Harvested cells were resuspended in buffer A (50 mM PIPES pH 6.8, 2 mM $MgCl_2$, 250 mM NaCl, 20 mM Imidazole, 1 mM PMSF, 1 mM MgATP, and 2.5 mg lysozyme) and subjected to one cycle of freeze-thaw and sonication, before 1 mM DNaseI and 10 mM $MgCl_2$ were added.

Clear lysate was loaded onto a HisTrapFF column (GE Healthcare) equilibrated in buffer A. The resin was washed with buffer A containing 50 mM Imidazole and the proteins were eluted on a gradient of buffer A from 50 mM to 500 mM Imidazole. Fractions containing protein of interest were pooled and the buffer was exchanged for protease buffer (50 mM PIPES pH 6.8 and 250 mM NaCl). Precision Protease, 1 mM MgATP, and 2 mM $MgCl_2$ were added and reactions were incubated overnight at 4°C. Uncleaved proteins and protease were removed by NiNTA affinity chromatography. The cleaved protein was subjected to size exclusion chromatography on a Superdex75 column (GE Healthcare) equilibrated in gel filtration buffer (50 mM PIPES pH 6.8, 50 mM KCl, 1 mM DTT, 1 mM EGTA, and 1 mM $MgCl_2$). Fractions containing protein of interest were pooled and concentrated on an Amicon Ultra filtration device (Millipore) to a final concentration of approximately 20 mg/ml, frozen in liquid nitrogen, and stored at -80°C.

Crystallisation of the Eg5-STLC complex

Purified Eg5 was incubated with excess powdered STLC (Novabiochem) overnight at 4°C. Before setting up of crystal trays, the sample was centrifuged at 13,000 rpm for

5 min at 4°C to pellet undissolved inhibitor. Crystals of Eg5 with STLC appeared after 1 month in hanging drops by mixing 2.5 µl of protein-inhibitor complex (10 mg/ml) with 2.5 µl of reservoir solution containing 22% polyethylene glycol-3350, 0.15 M Sodium tartrate dibasic dihydrate, and 0.1 M MES pH 6.5 in VDX plates (Hampton Research) at 4°C. A block-shaped crystal with dimensions of approximately 0.1 x 0.1 x 0.3 mm was immersed in cryoprotectant solution (24% polyethylene glycol-3350, 0.18 M Sodium tartrate dibasic dihydrate, 0.12 M MES pH 6.5, 0.06 M KCl, and 20% erythritol) and flash frozen in liquid nitrogen.

Data collection and processing

Diffraction data were recorded at the European Synchrotron Radiation Facility (ESRF) on station ID23-1. The crystals belonged to space group $P3_2$. Data were processed using iMosflm [20] and SCALA from the CCP4 suite of programs [21]. The structure was solved by molecular replacement (MOLREP) using one molecule of Eg5 (PDB code 1X88) as a search model. Three subunits were positioned and refined with REFMAC5 [22]. The calculation of R_{free} used 5% of data. Electron-density and difference density maps all σ_A -weighted, were inspected and the model was improved using Coot [23]. The coordinates and the cif dictionary for the inhibitor molecule were calculated using Dundee PRODRG server [24]. Crystallographic statistics are given in Table 1. Model geometry was analysed using PROCHECK [25]. 96.49% (880) of the residues are in the preferred regions, 2.30% (21) are in the allowed regions, and 1.21% (11) are outliers as shown by the Ramachandran Plot. Figures are prepared using PyMOL [26].

Since 3rd generation radiation was used for data collection, the crystal suffered slight radiation damage. The refined difference fourier map showed some negative density around the sulphur atom of STLC in all three molecules and also around the sulphur atoms of other residues at the surface. As sulphur atoms absorb more photons, at wavelength of about 1 Å, compared to other heavy atoms (C, O & N) present in the protein, the radiation damage is greater compared to that of the other atoms. Moreover, sulphur atoms that are more exposed (present at the surface) or are in close proximity to solvent water molecules experience more profound effect due to secondary radiation damage from the water molecules. [27, 28]

As the space group $P3_2$ is prone to crystal twinning, we carefully examined the cumulative distribution function in ctruncate and found no indication of twinning [29]. In addition, we submitted the data to the Merohedral Crystal Twinning Server [30], which also confirmed the absence of any significant twinning.

COORDINATE FILES

Coordinates and structure factors for the Eg5-STLC complex (PDB ID: 2wog) were deposited at the Protein Data Bank.

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TABLES

Table 1: Data collection and refinement statistics for the Eg5-STLC complex

	Eg5-STLC ¹
Unit cell dimensions	96.5, 96.5, 124.4,
a, b, c, γ ($\text{\AA}$, $^\circ$)	120
Space group	P3 ₂
Beamline / Detector	ID23-1 / Q315R
Molecules per asymmetric unit	3
Resolution range ($\text{\AA}$)	30 - 2.0
No. of unique reflections	87,598
Completeness (%)	99.9 (100.0) ²
Multiplicity	5.8
R _{sym} (%)	8.9 (40)
cl/ σ (I)	12.8 (4.0)
Wilson B ($\text{\AA}^2$) / DPI ³ ($\text{\AA}$)	27.2 / 0.14
Refinement statistics	
R _{work} / R _{free} (%)	15.9 / 21.8
Average B Factors	
Overall	35.9
Main Chain / Side Chain	33.6 / 37.7
No. of. ADP / Inhibitor / Water	3 / 3 / 1192
³ r.m.s.d. in bond length ($\text{\AA}$)	0.0127
r.m.s.d. in bond angle ($^\circ$)	1.61

¹ The S enantiomer was used for crystallization of the complex

² Values in parentheses pertain to the highest resolution shell

³ DPI: Diffraction-component precision index [31]

⁴ r.m.s.d. is the root-mean-square deviation from ideal geometry

FIGURE LEGENDS

Figure 1: Overall ternary structure of Eg5 motor domain in complex with Mg^{2+} ADP and STLC. Molecule A was used to generate these figures. **(A)** Stereoplot of the ternary ADP-Eg5-STLC complex with Mg^{2+} ADP (red) and STLC (green) shown as ball-and-stick models. **(B)** Electron density (contoured at $\sigma=1.00$, coloured in blue) for STLC bound in the inhibitor-binding pocket.

Figure 2: Stereoplot showing STLC in the inhibitor-binding pocket and important hydrophobic, aromatic and hydrogen bond (represented by broken lines) interactions (within 4 Å) between STLC and the Eg5 main chain and side chain molecules, and structural water molecules represented by red spheres. Molecule A was used to generate this figure.

Figure 3: Stereoplots showing the overlay of the intermediate (molecule C, pink) and final inhibitor-bound states (molecule B, blue) of the ternary ADP-Eg5-STLC complex against native Eg5 (PDB ID: 1II6, yellow). **(A)** Typical view of the inhibitor-binding pocket, showing the positions of Arg119, Trp127, Tyr211 and loop L5 before and after inhibitor-binding. The intermediate state (molecule C) is not shown because it has a similar conformation as the final inhibitor-bound state (molecule B). **(B)** Conformational changes in the helix $\alpha 3$ and Loop L9 of the switch I region upon inhibitor binding. The intermediate state (molecule C) is again not shown because it has a similar conformation as the final inhibitor-bound state (molecule B). **(C)** The switch II cluster (helix $\alpha 4$, Loop L12 and helix $\alpha 5$) and the neck-linker region, enlarged and shown as insets, undergo conformational changes from native to intermediate (molecule C) to final inhibitor-bound state (molecule B). Helix $\alpha 4$ rotates by about 15° to open up space for the ordered-to-ordered transition of the neck-linker, which adopts a docked conformation in the final inhibitor-bound state.