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Crystallization and preliminary X-ray diffraction analysis of tau protein microtubule-binding motifs in complex with Tau5 and DC25 antibody Fab fragments

The Alzheimer's disease-associated protein tau is an intrinsically disordered protein with no preferred structure in solution. Under physiological conditions, tau binds to microtubules and regulates their dynamics, whereas during the development of neurodegeneration tau dissociates from microtubules, misfolds and creates highly insoluble deposits. To elucidate the determinants of tau-protein misfolding, tau peptides from microtubule-binding motifs were crystallized in complexes with Fab fragments of specific monoclonal antibodies. The crystals diffracted to 1.69 Å resolution and gave complete data sets using a synchrotron X-ray source. Molecular replacement was used to solve the phase problem.

1. Introduction

Tau protein, which is the major component of neurofibrillary pathology in Alzheimer's disease and other tauopathies, is a typical representative of intrinsically disordered proteins (IDPs; Kovacech *et al.*, 2010). Like other IDPs, tau protein is extremely flexible under physiological conditions. It exists as a conformational ensemble populated with transient and confined structural motifs (Dyson & Wright, 2005). The tau molecule oscillates from one state to another owing to very low energy barriers (Tomba, 2005). IDPs have important functions in cellular processes; however, many of them are involved in the pathogenesis of protein-conformational diseases (Skrabana, Sevcik *et al.*, 2006; Skrabana, Skrabanova *et al.*, 2006). As a general rule, the frequency of aggregation-nucleating regions is almost three times lower in IDPs than in globular proteins (Linding *et al.*, 2004); however, it seems that under conditions comprising pathological post-translational modifications the extended character of IDP molecules facilitates their assembly (Skrabana, Sevcik *et al.*, 2006; Skrabana, Skrabanova *et al.*, 2006).

In the human brain, six tau-protein isoforms are generated by alternative splicing of the tau gene (Goedert *et al.*, 1989). The tau molecule can be divided into a predominantly acidic N-terminal projection domain, with or without one or two highly acidic inserts, followed by a basic proline-rich region and a C-terminal microtubule-binding domain with three or four microtubule-binding repeats followed by a C-terminal tail containing both acidic and basic subregions. Under physiological conditions tau is an axonal microtubule-associated protein. In the course of disease tau dissociates from microtubules, mis-sorts into the somatodendritic compartment and creates highly insoluble paired helical filaments (PHFs) which survive neuronal death and form deposits in the brain tissue (Kovacech *et al.*, 2010). The loss of axonal connections and the toxic effect of filamentary tau result in severe neurodegeneration (Novak *et al.*, 2011; Zilka *et al.*, 2006). The overall structural switch from the conformational ensemble of physiological tau, which is competent to bind and stabilize microtubules, to the pathological assembled fibrillar form is intimately connected with the structure of the microtubule-binding tau-protein domains.

It is difficult to obtain direct insight into the atomic structure of the physiological conformations of the IDP tau and/or its assembled form in Alzheimer's paired helical filaments, as neither of these can be prepared in a form that is amenable to X-ray crystallography, while

the NMR technique confers prevalently time-averaged structural data. However, using monoclonal antibodies (mAbs) as surrogate tau protein binding partners (Sevcik *et al.*, 2009; Skrabana *et al.*, 2010), the structure of the PHF C-terminal fragment has been determined by X-ray crystallography (Csóková *et al.*, 2006; Sevcik *et al.*, 2007). In the present work, mAb Tau5 (epitope Pro218–Lys225, which precedes the tubulin-binding hotspot Lys225–Thr231; LoPresti *et al.*, 1995; Porzig *et al.*, 2007) and mAb DC25 (epitope Lys347–Lys353, which lies in the fourth microtubule-binding repeat of tau in the proximity of the motif Lys370–Asn380, which strongly interacts with microtubules; Mukrasch *et al.*, 2007) were used to prepare crystals, allowing structural elucidation of physiologically and pathologically important tubulin-interaction hotspots on the tau-protein molecule.

2. Materials and methods

2.1. Fab production

The monoclonal antibodies Tau5 (LoPresti *et al.*, 1995) and DC25 (Axon Neuroscience, Bratislava, Slovakia) were isolated from serum-free hybridoma supernatants by affinity chromatography on a 5 ml HiTrap Protein G column (GE Healthcare, Freiburg, Germany). The Fab fragments of Tau5 and DC25 were prepared by digestion of the full-length antibody with 1 mg ml⁻¹ papain in the presence of 0.01 M cysteine and 0.01 M EDTA at a papain:antibody weight ratio of 1:8. The digestion was monitored by size-exclusion chromatography on a Superdex 200 HR 10/30 column (GE Healthcare) and was stopped after 100 min by addition of 0.03 M iodoacetamide (Sigma–Aldrich, St Louis, Missouri, USA). After buffer exchange on a 5 ml HiTrap Desalting column (GE Healthcare) to PBS (0.137 M NaCl, 0.0027 M KCl, 0.01 M Na₂HPO₄, 0.002 M KH₂PO₄ pH 7.4) supplemented with 3 M NaCl, the digestion mixture was loaded onto a HiTrap Protein A column (GE Healthcare) equilibrated with the same buffer; the Fab fragment appeared in the flowthrough separated from the Fc fragment. For further purification of the Fab fragment from residual papain, the flowthrough was loaded in the same buffer onto a 5 ml HiTrap Protein G column (GE Healthcare), washed with two column volumes and eluted with 0.1 M glycine pH 2.7. Final polishing of the Fab fragment was performed on a HiLoad Superdex 16/60 column (GE Healthcare) equilibrated in 0.01 M Tris–HCl pH 7.2, 0.05 M NaCl (Tris–N buffer). The Fab fragments were concentrated to 15–20 mg ml⁻¹ by ultrafiltration (3 kDa cutoff; Millipore, Billerica, Massachusetts, USA) and stored in Tris–N buffer at 277 K.

2.2. Crystallization

For cocrystallization of complexes, the tau peptides were freshly dissolved in Tris–N buffer before the preparation of crystallization drops and were mixed with the Fab fragment in a 1.5:1 molar ratio before the addition of the precipitant. All necessary dilutions were performed in Tris–N buffer. The following peptides were used for complex preparation: tau201–230 (GSPGTPGSRSRTPSLPTPPPK-KVAVVR, 95% purity; EzBioLab, Carmel, Indiana, USA; numbering is according to the longest neuronal tau isoform tau40; *cf.* Goedert *et al.*, 1989), Ptau201–230 (pT217; GSPGTPGSRSRTPSLPpTPPTRE-PKKVAVVR, 95.64% purity; Antagene, Lyon, France), Ptau341–370 (pS356; SEKLDFKDRVQSKIGpSLDNITHVPGGGNKK, 95.64% purity; CASLO Lab, Lyngby, Denmark). Crystallization screening of Tau5 Fab complexes and apo Tau5 Fab was performed at 295 K by the vapour-diffusion technique in sitting drops set up manually in an MRC 96-well crystallization plate (Molecular Dimensions, Suffolk, England). Nanolitre sitting-drop dispensing was performed as described in Skrabana *et al.* (2012). Briefly, 100 µl precipitant solution

was pipetted into the reservoir of each well; 0.35 µl precipitant solution was then transferred into the sitting-drop platforms using a handheld motorized eight-channel pipette. Subsequently, 0.5 µl protein solution was pipetted by a motorized single-channel pipette using a repetitive pipetting mode. During plate assembly, the pipetted drops were protected against evaporation by using a home-made sliding cover similar to that described previously (Biertümpfel *et al.*, 2005). The final concentration of Tau5 Fab after equilibration was 10 mg ml⁻¹ in a 0.35 µl drop. The JCSG+, PACT premier (Molecular Dimensions) and Crystal Screen HT (Hampton Research, Aliso Viejo, California, USA) crystallization screens were used. Crystals suitable for data collection were obtained directly from the initial screening conditions.

Initial crystallization screening of the complex of DC25 Fab with Ptau341–370 and of apo DC25 Fab was performed by the vapour-diffusion technique at 295 K in hanging drops on EasyXtal (Qiagen, Venlo, Netherlands) and VDX (Hampton Research) plates, unless stated otherwise. The drops were prepared by mixing 0.75 µl protein solution and 0.75 µl precipitant solution. Initial crystallization conditions were screened using commercial screens including Structure Screens 1 and 2, Clear Strategy Screen II HT-96 (Molecular Dimensions), Index and PEG/Ion (Hampton Research). The final concentration of DC25 Fab after equilibration was either 10 or 5 mg ml⁻¹. Conditions that gave crystallization hits were further optimized, mostly by varying the concentration of the protein and the precipitant.

2.3. Peptide detection

Crystals were fished out from the drop using a nylon loop, washed twice in the precipitant solution and dissolved in a small volume of PBS. The Tau5 Fab complex crystal was analysed after data collection. The antibody Fab fragment was precipitated using acetonitrile (final concentration 75%; Sigma–Aldrich) and separated by centrifugation at 10 000g for 10 min at room temperature, leaving the soluble peptide in the supernatant. The supernatant was subsequently dried and the resulting pellet was dissolved in 10% acetonitrile. A Waters Quattro Premier XE triple quadrupole mass spectrometer (Waters, Milford, Massachusetts, USA) coupled to an Acquity UPLC system and a Bruker Amazon ETD ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany) coupled to a Dionex Ultimate 3000 nanoHPLC system were used for detection. Peptides separated on C18 media were detected by MS/MS using the specific decay of the parent ion to up to three daughter ions. For development of the LC-MS/MS protocol, a standard solution of the pure peptide was used.

2.4. Diffraction data collection and processing

Crystals cryoprotected with Paratone-N or by sequential cryoprotection using 20% glucose and Paratone-N as an internal and an external cryoprotectant, respectively (Alcorn & Juers, 2010), were mounted in nylon loops (Hampton Research). Mounted crystals were flash-cooled in liquid nitrogen. Diffraction data were collected at 100 K using a synchrotron source and the unit-cell content was estimated using the *Matthews Probability Calculator* (Kantardjiev & Rupp, 2003). Data were indexed and integrated with *XDS* (Kabsch, 2010), merged and scaled with *SCALA* (Evans, 2006) and the space group was determined using *POINTLESS* (Evans, 2006). Phases were obtained by molecular replacement with the structure of the MN423 Fab fragment (PDB entry 311o; Skrabana *et al.*, 2010) as a model using *Phaser* (McCoy *et al.*, 2007). The last three tools are part of the *CCP4* suite (Winn *et al.*, 2011).

Figure 1

(a) SDS–PAGE of purified Tau5 (lane 1) and DC25 (lane 2) Fab fragments together with molecular-weight markers (labelled in kDa). (b) Crystals of the Tau5 Fab fragment in space group $C2$. (c) Crystals of the DC25 Fab fragment in space group $P2_12_12_1$. (d) Crystal of the complex of Tau5 with tau201–230. (e) Crystals of the complex of Tau5 with Ptau201–230 (pT217). (f) Nondiffracting crystals of DC25 Fab with Ptau341–370 (pS356). The scale bar represents 200 μm in (b), (c), (e) and (f) and 50 μm in (d).

3. Results and discussion

3.1. Crystallization of Fab fragments and their complexes with tau peptides

Multistep affinity purification of the Tau5 and DC25 Fab fragments yielded proteins of high purity (Fig. 1a). Crystallization of the Tau5 Fab fragment alone and in complex with tau peptides was performed by the vapour-diffusion technique in nanolitre-sized sitting drops, which were manually loaded onto 96-well microplates using a newly developed method (Skrabana *et al.*, 2012). Remarkably, nearly all of the crystals that appeared were of excellent optical quality and the reported diffraction data were obtained using crystals fished out directly from the microplate screening drop. Tau5 Fab crystals were obtained after two weeks from JCSG+ Screen condition H8 (25% PEG 3350, 0.1 *M* bis-Tris pH 5.5, 0.2 *M* NaCl; Fig. 1b). Additional crystallization hits were obtained in 0.15 *M* potassium bromide, 30% PEG MME 2000 (JCSG+ condition G10), 0.2 *M* ammonium sulfate, 0.1 *M* bis-Tris pH 5.5, 25% PEG 3350 (JCSG+ condition H7), 0.2 *M* magnesium chloride, 0.1 *M* bis-Tris pH 5.5, 25% PEG 3350 (JCSG+ condition H11), 0.2 *M* magnesium chloride, 0.1 *M* MES pH 6.0, 20% PEG 6000 (PACT premier condition B10) and 0.1 *M* MMT buffer pH 5.0, 25% PEG 1500 (PACT premier condition D2). Crystals of average dimensions $0.2 \times 0.1 \times 0.05$ mm were fished out from six-month-old drops, cryoprotected with Paratone-N and flash-cooled in liquid nitrogen.

For the Tau5 Fab in complex with tau201–230, a single crystal with approximate dimensions $0.03 \times 0.01 \times 0.01$ mm grew after three months in 30% PEG 4000, 0.1 *M* Tris pH 8.5, 0.2 *M* lithium sulfate (condition B5 of Crystal Screen HT; Fig. 1d). Another crystal of the

Tau5 Fab in complex with Ptau201–230 (pT217) grew after one month using 0.02 *M* sodium/potassium phosphate, 20% PEG 3350 (PACT premier condition E10; Fig. 1e). The crystals were fished out from four-month-old drops, cryoprotected with Paratone-N and flash-cooled in liquid nitrogen.

Screening for crystallization conditions of the DC25 Fab fragment and its complex with tau phosphopeptide was performed by the vapour-diffusion technique in 0.75 μl hanging drops. No promising hits were obtained using commercial screens and the DC25 Fab fragment was eventually crystallized using ‘in-house’ formulated conditions consisting of 0.1 *M* imidazole buffer pH 7.0 with 0.01 *M* zinc sulfate or 0.01 *M* zinc chloride and 20% PEG 3350, similar to those used previously for crystallization of the MN423 Fab fragment (Skrabana *et al.*, 2010; Fig. 1c). Needle and rod-shaped crystals grew in 5–10 d. The crystals for diffraction experiments were cooled from five-month-old drops and cryoprotected sequentially using glucose and Paratone-N.

Crystallization of DC25 Fab in complex with Ptau341–370 (pS356) was attempted using more than 350 conditions. Likely crystalline forms (Fig. 1f) appeared in conditions containing various PEGs as precipitant (Supplementary Table 1[†]). For optimization of the crystallization of the DC25 Fab complex, 0.2 *M* sodium acetate, 0.2 *M* magnesium acetate and combinations of 0.1 *M* sodium cacodylate buffer pH 6.5 with 0.2 *M* magnesium acetate, of 0.1 *M* imidazole buffer pH 7.0 with 0.01 *M* zinc sulfate or 0.01 *M* zinc chloride, and of 0.01 *M* zinc sulfate with 0.2 *M* magnesium acetate or 0.2 *M* sodium

[†] Supplementary material has been deposited in the IUCr electronic archive (Reference: EN5500).

acetate were chosen. As a precipitant, PEG 3350 was adopted at a concentration varying from 10 to 20%(w/v). However, we were not able to substantially increase the crystal quality; none of the crystals obtained diffracted at the synchrotron source.

3.2. Determination of the peptides in complex crystals

To provide independent evidence of the crystallization of complexes, we performed careful mass-spectrometric analyses of the obtained crystals. Peptide fractions prepared from dissolved crystals were subjected to reverse-phase chromatographic separation with MS/MS detection. The analytical conditions were identified using a peptide standard solution. Peptide peaks with corresponding retention volumes and daughter-ion spectra compositions were unequivocally identified (Fig. 2). To exclude the possibility of peptide adsorption on the crystal surface, we performed a quantitation of the amount of peptide in the Tau5 Fab fragment–tau201–230 complex

crystal. Considering the crystal volume of $(3 \pm 1) \times 10^{15} \text{ \AA}^3$ and the unit-cell volume of $4.85 \times 10^5 \text{ \AA}^3$, we obtained an estimation of the number of Fab molecules and of the corresponding peptide amount ($32 \pm 11 \text{ pg}$) in the crystal. This value corresponds within the margins of error to the peptide amount detected in LC/MS analyses (22 pg).

3.3. Diffraction data characterization and preliminary analysis

A data set for the Tau5 Fab fragment was collected from a crystal separated from a cluster of plates grown in 25% PEG 3350, 0.1 M bis-Tris pH 5.5, 0.2 M NaCl. A monoclinic crystal diffracting to 1.69 Å resolution belonged to space group *C2*, with unit-cell parameters $a = 129.39$, $b = 50.43$, $c = 75.18 \text{ \AA}$, $\beta = 115.36^\circ$. As the beam intensity varied during data collection, some of the data images had a low signal-to-noise ratio which worsened the overall statistics; therefore, we processed high-resolution data from only half of the recorded images. The resulting $R_{\text{r.i.m.}}$ of the processed data set was 13.9%. For

Figure 2 Mass-spectrometric analyses of the peptides in the complex crystals. (a–c) Tau201–230 peptide in a cocrystal with the Tau5 Fab fragment. (d–f) Ptau341–370 (pS356) in a cocrystal with the DC25 Fab fragment. (a) Parent spectra of pure tau201–230 peptide ($M_r = 3112.6$); the peak at m/z 779.28 corresponds to a fourfold-charged peptide and the peak at m/z 1038.73 corresponds to a threefold-charged peptide. (b) Daughter spectra: fragmentation (MS^2) of the ion with m/z 779.28. (c) Chromatograms of crystal sample (black), pure peptide (6 pg injected; magenta) and blank injection (blue). (d) Mass-scan spectra of pure tau341–370 (pS356) peptide ($M_r = 3347$); the peak at m/z 558.6 corresponds to a sixfold-charged peptide, the peak at m/z 670.2 corresponds to a fivefold-charged peptide and the peak at m/z 837.67 corresponds to a fourfold-charged peptide. (e) Daughter spectra of the m/z 670.2 parent ion; the fragments used for MRM detection are encircled. (f) Chromatograms of the pure tau341–370 (pS356) peptide, the peptide isolated from a cocrystal with the DC25 Fab fragment and a negative control: an irrelevant crystal soaked in tau341–370 (pS356) peptide solution and processed identically as for the cocrystal.

Table 1

Data collection and processing.

Values in parentheses are for the last resolution shell.

	Tau5 complex	Tau5 Fab	DC25 Fab
Diffraction source	P14, PETRA III	X12, DORIS III	X12, DORIS III
Wavelength (Å)	1.2669	0.97004	0.97004
Temperature (K)	100	100	100
Detector	PILATUS 6M	MAR CCD, 225 mm	MAR CCD, 225 mm
Crystal-to-detector distance (mm)	160	170	260
Rotation range per image (°)	0.1	1	0.6
Total rotation range (°)	180	360	216
Exposure time per image (s)	3	120	120
Space group	C2	C2	P2 ₁ 2 ₁ 2 ₁
Unit-cell parameters			
<i>a</i> (Å)	124.61	129.39	36.35
<i>b</i> (Å)	59.61	50.43	93.46
<i>c</i> (Å)	70.90	75.18	127.22
$\alpha = \gamma$ (°)	90.00	90.00	90.00
β (°)	112.76	115.36	90.00
Mosaicity (°)	0.31	0.13	0.19
Resolution range (Å)	65.38–1.69 (1.78–1.69)	46.31–1.69 (1.79–1.69)	46.73–2.41 (2.54–2.41)
Total No. of reflections	170130 (24929)	174566 (23961)	102817 (12592)
No. of unique reflections	52194 (7618)	47537 (6723)	16976 (2342)
Completeness (%)	97.3 (97.7)	97.5 (95.5)	97.0 (94.4)
Multiplicity	3.3 (3.3)	3.7 (3.6)	6.1 (5.4)
$\langle I/\sigma(I) \rangle$	13.4 (1.8)	8.7 (1.6)	10.8 (1.8)
$R_{\text{r.i.m.}}^{\dagger}$ (%)	6.2 (78.2)	13.9 (94.1)	15.2 (115.2)
Overall <i>B</i> factor from Wilson plot (Å ²)	22.3	14.8	44.1
Matthews coefficient (Å ³ Da ^{−1})	2.37	2.32	2.25
Solvent content (%)	48.48	46.88	44.1
Monomers in asymmetric unit	1	1	1
Molecular replacement			
TF Z-score ‡	36.4	30.5	31.6
LLG §	1239	476	547
<i>R</i> factor (%)	51.8	53.3	52.5

$^{\dagger} R_{\text{r.i.m.}}$ (R_{meas}) is defined as $\sum_{hkl} \{N(hkl)/[N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$. ‡ The TF Z-score is a Z-score of the solution translation function. A TF Z-score value of higher than 8 indicates a definite molecular-replacement solution. § LLG (log-likelihood gain) is a parameter indicating how much better the model obtained is in comparison with a random model.

Tau5 Fab complexed with tau201–230, a data set was collected from a single crystal grown in 30% PEG 4000, 0.1 M Tris pH 8.5, 0.2 M lithium sulfate. The crystal diffracted to 1.69 Å resolution and belonged to the monoclinic space group C2, with unit-cell parameters $a = 124.61$, $b = 59.61$, $c = 70.9$ Å, $\beta = 112.76^\circ$. The crystal of Tau5 Fab in complex with Ptau201–230 (pS217) only diffracted to 15 Å resolution. The DC25 Fab fragment crystallized in 20% PEG 3350, 0.1 M imidazole pH 7.0, 0.01 M zinc chloride. The crystal used for data collection was separated from a cluster of rod-shaped crystals. The crystal diffracted to 2.41 Å resolution and belonged to the orthorhombic space group P2₁2₁2₁, with unit-cell parameters $a = 36.35$, $b = 93.46$, $c = 127.22$ Å. Crystals of the DC25 Fab in complex with tau peptide did not diffract. Complete statistics of diffraction data collection and processing are given in Table 1.

3.4. Molecular replacement

Molecular replacement as implemented in *Phaser* (McCoy *et al.*, 2007) was used to determine the phases, using the structure of the PHF tau-specific monoclonal antibody MN423 Fab fragment as a model (PDB entry 3110; Skrabana *et al.*, 2010). Initial attempts at molecular replacement using the whole model molecule as a search ensemble failed to give a solution, probably owing to unequal hinge

angles in the model and target structures. To overcome this, the variable and constant domains of MN423 Fab were split at Ser121 and Leu106 in the heavy and light chain, respectively, and used separately as two independent search ensembles in *Phaser*. A single molecular-replacement solution was found for all crystals (Table 1). Correct packing of the obtained structures was verified in *PyMOL* (v.1.5.0.1; Schrödinger LLC, Cambridge, Massachusetts, USA). Structure refinement is currently under way.

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