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Crystallization and preliminary X-ray diffraction analysis of two peptides from Alzheimer PHF in complex with the MN423 antibody Fab fragment

The major constituent of the Alzheimer's disease paired helical filaments (PHF) core is the intrinsically disordered protein (IDP) tau. Globular binding partners, e.g. monoclonal antibodies, can stabilize the fold of disordered tau in complexes. A previously published structure of a proteolytically generated tau fragment in a complex with the PHF-specific monoclonal antibody MN423 revealed a turn-like structure of the PHF core C-terminus [Sevcik *et al.* (2007). *FEBS Lett.* **581**, 5872–5878]. To examine the structures of longer better-defined PHF segments, crystals of the MN423 Fab fragment were grown in the presence of two synthetic peptides derived from the PHF core C-terminus. For each, X-ray diffraction data were collected at 100 K at a synchrotron source and initial phases were obtained by molecular replacement.

1. Introduction

In the process of protein crystallization, disordered regions in proteins suppress the regular ordering of protein molecules required for crystal nucleation and growth. Therefore, the presence of disordered sequences is considered to be an obstacle to the successful preparation of well diffracting protein crystals (Derewenda, 2004). Several strategies have been developed to remove disordered regions before crystallization, including deletion of disordered sequences at the gene level, limited proteolysis before or during the crystallization process and entropy reduction of the protein surface by site-directed mutagenesis (Derewenda, 2010; Makabe *et al.*, 2006). These strategies have been useful for structure determination of globular proteins because the removal of disorder-prone termini or flexible surface loops generally does not influence protein activity (Kwong *et al.*, 1999). However, the strategy of disordered-region removal is not advisable when the function of the protein depends upon its disordered character. Such proteins, designated intrinsically disordered proteins (IDPs), have recently been recognized as an indispensable component of cellular processes, with a surprisingly high incidence in the functional proteome (Uversky & Dunker, 2010). IDPs have no preferred three-dimensional structure and exist in solution as an ensemble of interconverting conformers (Mittag & Forman-Kay, 2007). IDPs may create functional contacts with their cellular partners undergoing binding-coupled folding (Dyson & Wright, 2005). The structures of the bound states of IDPs are of the greatest importance owing to their involvement in signalling and development and also in the pathogenesis of several protein-misfolding diseases, namely neurodegeneration (Skrabana, Sevcik *et al.*, 2006; Tompa, 2005; Uversky *et al.*, 2008). Several examples of IDP stabilization after complex formation have been documented by X-ray crystallography. Examples include the crystallization of disordered inhibitor 2 with protein phosphatase 1 (Hurley *et al.*, 2007), the complex of the inhibitor calpastatin with its target protease calpain (Hanna *et al.*, 2008) and the toxin–disordered antitoxin pair SpoIIISA–SpoIISB (Florek *et al.*, 2011).

It has also been hypothesized that by using monoclonal antibodies that specifically recognize distinct IDP conformers it may be possible to obtain crystalline forms of their complexes and to solve their structures by diffraction methods. Monoclonal antibodies could serve

as surrogate binding partners with two functions: they will induce the functional structure of IDPs and facilitate crystal contacts (Sevcik *et al.*, 2009; Koide, 2009). The VHH fragment of a single-domain camelid antibody was used to crystallize the unstructured bacterial addition antidote protein MazE. The nanobody stabilizes the physiologically relevant protein conformation and mediates all of the crystal lattice-stabilizing interactions (Loris *et al.*, 2003). A nanobody raised against unstructured α -synuclein, which binds to its C-terminus, was crystallized with its C-terminal nonapeptide (De Genst *et al.*, 2010). Recently, the C-terminal domain of mouse cellular prion protein has been crystallized with an antibody Fab fragment (Baral *et al.*, 2011).

Alzheimer's disease (AD) and related dementias are characterized by the accumulation of intrinsically disordered tau protein in the misfolded form of neurofibrillary tangles with the morphological structural unit of a paired helical filament (PHF; Mandelkow *et al.*, 2007; Novak *et al.*, 1993; Skrabana, Skrabanova-Khuebachova *et al.*, 2006; Wischik *et al.*, 1988). The key question in tauopathy pathogenesis is which structural changes of tau are responsible for its pathological transformation. Recently, we used monoclonal antibody MN423 recognizing the conformational epitope on the core of PHF for structure determination of the PHF core C-terminus (Sevcik *et al.*, 2007; Skrabana *et al.*, 2010). In this work, we describe the preparation and characterization of crystals of the MN423 Fab fragment grown in the presence of two new tau peptides from the C-terminus of the PHF core as a prerequisite for a more detailed structural investigation of the Alzheimer PHF.

2. Materials and methods

2.1. Preparation of MN423 Fab fragment

All chemicals were purchased from Sigma–Aldrich unless stated otherwise. Monoclonal antibody MN423 (IgG2b) was obtained by immunization of mice with a preparation of tau protein from Alzheimer's disease brain tissue (Novak *et al.*, 1989). Intact monoclonal antibody was produced in serum-free medium. After purification on a 5 ml Protein A Sepharose column (GE Healthcare), the Fab fragment was produced by partial digestion with papain (papaya latex papain, Roche) and purified as described previously (Csokova *et al.*, 2006). Papain at a final concentration of 2 mg ml⁻¹ was activated with 0.05 M cysteine in 0.1 M sodium acetate pH 5.5 supplemented with 0.003 M EDTA at 310 K for 30 min. Cysteine was removed on an HT desalting column (GE Healthcare) equilibrated with PBS (0.137 M NaCl, 0.0027 M KCl, 0.01 M Na₂HPO₄, 0.002 M KH₂PO₄ pH 7.4) supplemented with 0.003 M EDTA. Activated papain solution was subsequently mixed with MN423 in a 1:20(w:w)

ratio. The final mAb concentration in the digestion mixture was 4 mg ml⁻¹. Hydrolysis was allowed to continue for about 2 h at 310 K. The Fab was purified from the digestion mixture using cation-exchange and size-exclusion chromatography. After digestion, the mixture was loaded onto a 1 ml Mono S column (GE Healthcare) equilibrated with 0.05 M PIPES pH 6.9 and the Fab fragment was eluted using a 0–30% gradient of 1 M NaCl. The final purification step was performed on a Sephacryl 200 HR 16/60 column (GE Healthcare) in 0.01 M Tris pH 7.2, 0.05 M NaCl (Tris–N buffer). The Fab fragment was concentrated to 20 mg ml⁻¹ in Tris–N buffer by ultrafiltration and stored at 277 K.

2.2. Crystallization of tau-peptide complexes

Crystallization was performed by the vapour-diffusion technique using 0.5–1 µl hanging drops in EasyXtal plates (Qiagen). The drops were prepared by mixing equal volumes of protein and precipitant solution. The synthetic tau peptides DHGAE and AKAKTDHGAE with acetylated N-termini (>95% purity; Thermo Electron; Fig. 1) were dissolved in fresh Tris–N buffer before the preparation of the crystallization drops. The tau peptides were mixed with the Fab fragment before the addition of precipitant. A 2–80-fold molar excess of peptides was used with a 4–15 mg ml⁻¹ Fab concentration. Initial crystallization screening was performed using an in-house-formulated PEG 3350 grid screen in two buffers, 0.1 M imidazole pH 7.0 supplemented with 0.01 M zinc sulfate and 0.1 M HEPES pH 7.6, both of which were derived from previously found crystallization conditions for apo-form MN423 (Skrabana *et al.*, 2010). Crystallization was performed at 280 and 294 K. The obtained crystallization conditions were optimized and seeding techniques were used to improve the crystal quality. For the preparation of the crystals used in diffraction data collection, the pentapeptide DHGAE was dissolved to 1.6 mg ml⁻¹ as a stock solution in Tris–N buffer and mixed in a 1:3 ratio with Fab stock solution, giving final pentapeptide and Fab concentrations of 0.4 and 15 mg ml⁻¹, respectively. The decapeptide AKAKTDHGAE was dissolved to 0.6 mg ml⁻¹ as a stock solution; after mixing it in a 1:3 ratio with Fab stock solution, the final decapeptide and Fab concentrations were 0.15 and 15 mg ml⁻¹, respectively.

The peptide content of the crystals was analysed as described in Cehlar *et al.* (2012). Briefly, the crystals were dissolved in a small volume of PBS, the Fab fragment was precipitated with acetonitrile (final concentration 75%) and the peptides were separated on a C18 column and detected by LC-MS/MS. For efficient retention of prevalently hydrophilic peptides, the mobile phase was supplemented with the ion-pairing reagent perfluorooctanoic acid at a concentration of 0.1%.

Figure 1

The epitope of antibody MN423 and the peptides used in this work. A subunit of the paired helical filaments core, tau297–391, comprises 95 amino acids from the longest human tau isoform, tau40 (tau1–441, above; I1 and I2 are amino-terminal inserts, R1, R2, R3 and R4 are microtubule-binding repeats and R' is a region of lower repeat homology). The black box above the scheme for tau297–391 highlights the sites that are required for immunoreactivity of the monoclonal antibody MN423. The sequences of the peptides used in the cocrystallization experiments are shown under the scheme for tau297–391.

Table 1

Data collection and processing.

	Putative MN423 Fab– pentapeptide complex	MN423 Fab– decapeptide complex
Diffraction source	X11, DORIS III	X11, DORIS III
Wavelength (Å)	0.8166	0.8166
Temperature (K)	100	100
Detector	MAR imaging-plate scanner	MAR imaging-plate scanner
Crystal-to-detector distance (mm)	280	245
Rotation range per image (°)	0.2	0.5
Total rotation range (°)	260	270
Exposure time per image (s)	10	15
Space group	$P2_1$	$P2_1$
Unit-cell parameters (Å, °)	$a = 78.44$, $b = 88.89$, $c = 145.16$, $\beta = 100.82$	$a = 80.82$, $b = 145.55$, $c = 83.86$, $\beta = 114.91$
Mosaicity (°)	1.5	1.0
Resolution range (Å)	20.00–2.95 (2.98–2.95)	20.00–2.70 (2.73–2.70)
Total No. of reflections	149658 (3910)	236041 (6620)
No. of unique reflections	36502 (931)	44536 (1298)
Completeness (%)	88.1 (68.5)	92.5 (81.9)
Multiplicity	4.1 (4.2)	5.3 (5.1)
$\langle I/\sigma(I) \rangle$	11.0 (2.5)	17.0 (2.3)
R_{int} (%)	10.2 (59.8)	7.1 (60.0)
Overall B factor from Wilson plot (Å ²)	69.7	58.2
Matthews coefficient (Å ³ Da ^{−1})	2.59	2.31
Solvent content (%)	52.52	46.69
Monomers in asymmetric unit	4	4
$MOLREP$ score	0.44	0.58
$MOLREP$ R factor	0.56	0.51

 $\dagger R_{\text{int}}$ (R_{meas}) is defined as $\sum_{hkl} [N(hkl)/[N(hkl) - 1]]^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$.

2.3. Collection of X-ray diffraction data, processing and structure solution

Crystals were mounted in nylon loops (Hampton Research), cryoprotected in Paratone-N (Hampton Research) and flash-cooled by immersion in liquid nitrogen. X-ray diffraction data were collected at 100 K using synchrotron radiation on EMBL/DESY beamline X11 (DORIS III, Hamburg, Germany) with a 0.8166 Å monochromatic fixed wavelength. For the crystal grown in the presence of the pentapeptide, a set of 1300 images was recorded with a 0.2° oscillation angle, an exposure time of 10 s per image and a crystal-to-detector distance of 280 mm. For the crystal grown in the presence of the decapeptide, a set of 540 images was recorded with a 0.5° oscillation angle, an exposure time of 15 s per image and a crystal-to-detector distance of 245 mm. Data sets were indexed, scaled and merged using the *HKL*-2000 package (Otwinowski & Minor, 1997), and unit-cell content analysis and data reduction were performed using tools from the *CCP4* suite v.6.02 (Winn *et al.*, 2011). For anisotropy correction,

the UCLA MBI Diffraction Anisotropy Server was used. Phases were obtained by molecular replacement with the structure of the MN423 Fab fragment (PDB entry 3l1o; Skrabana *et al.*, 2010) as a model using *MOLREP* (Vagin & Teplov, 2010) as implemented in the *CCP4* suite. Correct packing of the obtained solutions was verified in *PyMOL* (Schrödinger LLC). Data-collection and processing statistics are reported in Table 1.

3. Results and discussion

3.1. Cocrystallization of MN423 Fab fragment and tau peptides

The adopted purification procedure yielded MN423 antibody Fab fragment with greater than 95% homogeneity (Csóková *et al.*, 2006). Various strategies could be adopted to prepare crystals of the complexes, including soaking of crystals of the apo form with ligands, the use of ligands during protein purification and cocrystallization from a ligand–protein mixture (Hassell *et al.*, 2007; Urbanikova & Sevcik, 1998). Initially, we tried to prepare MN423 Fab–tau peptide complex crystals by in-drop soaking of well formed Fab crystals with the peptide. However, after adding the powdered peptide and its dissolution, the crystals of the Fab also dissolved, probably owing to binding of the peptide into the antibody combining site involved in crystal contacts in the MN423 apo-form crystal (Skrabana *et al.*, 2010). Therefore, the cocrystallization method was adopted using the optimized conditions used in crystallization of the MN423 apo form (Csóková *et al.*, 2006). Initial grid screening was performed in 7–17% PEG 3350 with an Fab concentration of 4–15 mg ml^{−1} using two buffers: (i) 0.1 M imidazole pH 7.0 supplemented with 0.01 M zinc sulfate and (ii) 0.1 M HEPES pH 7.6. Crystallization screening was performed at 280 and 294 K. Crystallization at 280 K and the presence of zinc ions produced long thin needle-like crystals, whereas at 294 K in 0.1 M HEPES pH 7.6 buffer three-dimensional crystals appeared after a few days. A crystal with dimensions of 0.2 × 0.1 × 0.07 mm (Fig. 2*a*) grown in the presence of the pentapeptide DHGAE was obtained by sequential macroseeding. Initially, approximately 0.15 µl of a suspension of small crystals grown using 9% PEG 3350, 0.1 M HEPES pH 7.6, 15 mg ml^{−1} Fab and a ninefold molar excess of pentapeptide were transferred into a pre-equilibrated 8% PEG 3350 drop with the same buffer, Fab and peptide concentrations. After 24 h, single crystals were transferred into a new pre-equilibrated drop with an identical composition. Crystals with dimensions of 0.3 × 0.1 × 0.1 mm were grown in the presence of the decapeptide AKAKTDHGAE in 11% PEG 3350, 0.1 M HEPES pH 7.6, 15 mg ml^{−1} Fab and a twofold molar excess of peptide after

Figure 2

Crystals of complexes of MN423 Fab with peptides derived from the PHF core C-terminus. (*a*) Putative complex with Ac-DHGAE. (*b*) Complex with Ac-AKAKTDHGAE. The scale bar represents 200 µm.

cross-macroseeded with crystals grown in the presence of pentapeptide under identical conditions (Fig. 2*b*). The seeds initially dissolved in the decapeptide drop and crystals of the putative decapeptide complex grew after 8 d.

We observed a peculiar role of zinc ions in Fab–peptide complex crystallization. It was found that the use of zinc as an additive markedly improved the crystal quality of the MN423 Fab fragment apo form (Csóková *et al.*, 2006). However, in the present study the addition of zinc impeded crystal formation of both tau-peptide complexes. Previously, we have shown that in the apo-form crystal the zinc ions facilitate crystal packing by tethering the neighbouring Fab molecules *via* surface-exposed aspartates in the CL and CH1 domains, improving the diffraction (Skrabana *et al.*, 2010). However, concomitantly, the antibody combining site became occluded by the protruding C-terminus of the heavy chain of the neighbouring molecule. As a consequence, Zn atoms can indirectly block the crystallization of MN423 complexes.

3.2. Crystal characterization and X-ray diffraction data collection

The MN423–DHGAE complex has a dissociation constant of 1.8 μM (Skrabana *et al.*, 2010) and the MN423–AKAKTDHGAE complex has only a slightly lower stability (Skrabana *et al.*, 2004); therefore, it could be deduced from the drop composition that the concentration of uncomplexed MN423 Fab fragment in the equilibrated drop was lower than 1/100 of the total Fab concentration. This indicated that the crystals that appeared were indeed crystals of the complex. We attempted to detect the peptides directly in the crystal by mass spectrometry. Peptide fractions prepared from dissolved crystals were subjected to reverse-phase chromatographic separation and MS/MS detection. We confirmed the presence of the decapeptide (Supplementary Fig. 1[†]); however, more crystalline material would be required for unequivocal detection of the pentapeptide, as the amount present in the examined crystal might be below the detection limit of the method (data not shown). In consequence, the crystals grown in the presence of pentapeptide should be considered to be putative pentapeptide–complex crystals. It is worth noting, however, that all four of the crystal forms that we have prepared with MN423, the two reported here, the complex reported previously (Sevcik *et al.*, 2007) and the uncomplexed form (Skrabana *et al.*, 2010), adopt unit cells with unique dimensions.

X-ray diffraction data were obtained using synchrotron radiation on EMBL/DESY beamline X11. All diffraction data were collected at 100 K. As the crystallization conditions did not contain an efficient cryoprotectant, optimization of cryoprotection was performed and Paratone-N was identified as a suitable cryoprotectant. The crystals of the putative pentapeptide complex and the decapeptide complex diffracted to 2.95 and 2.7 Å resolution, respectively (Table 1). The crystal of the putative pentapeptide complex exhibited relatively high mosaicity (1.5°); therefore, the data-collection strategy implied a small rotation range per image (0.2°). Both measured crystals exhibited diffraction anisotropy, which contributed to the low completeness of the last resolution shell observed in both data sets. The anisotropy was corrected using a web-based program (Strong *et al.*, 2006). Analysis of the unit cell determined a similar solvent content in both crystals (Table 1). Interestingly, the crystals contained four molecules in the asymmetric unit, which could lead to the refinement of four independent tau-peptide conformations in the antibody combining site.

[†] Supplementary material has been deposited in the IUCr electronic archive (Reference: EN5502).

3.3. Molecular replacement

For structure solution of the complexes of the tau peptides with the MN423 Fab fragment, we adopted the molecular-replacement method as implemented in the *MOLREP* software using the MN423 apo-form structure (PDB entry 3l1o; Skrabana *et al.*, 2010) as a search model. Initial attempts using the intact model molecule failed to give a correct solution, probably owing to the difference in the hinge angle in the model and target structures. In the next step, the MN423 Fab molecule was split at Ser121 and Leu106 into the heavy and light chain, respectively, separating the variable and constant domains of the Fab fragment. In the first run of *MOLREP*, the constant domain of the 3l1o structure was used as a model and the obtained solution was adopted as a fixed input structure in the second *MOLREP* run with the variable domain of the 3l1o structure as a model. The obtained solutions were checked for correct packing. Refinement is in progress for both complex structures.

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