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Mercury-induced crystallization and SAD phasing of the human Fe65-PTB1 domain

Jens Radzimanowski,[a] Stéphanie Ravaud,[a] Konrad Beyreuther,[b] Irmgard Sinning[a] and Klemens Wild[a]*

[a]Heidelberg University Biochemistry Center, INF328, D-69120 Heidelberg, Germany, and [b]Center for Molecular Biology, University Heidelberg, INF282, D-69120 Heidelberg, Germany

Correspondence e-mail: klemens.wild@bzh.uni-heidelberg.de

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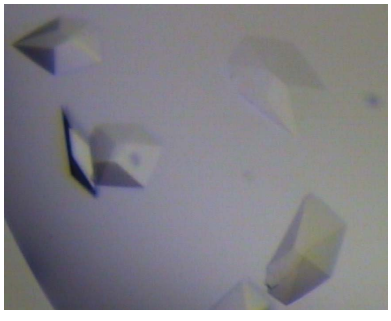


Fe65 is a three-domain neuronal adaptor protein involved in brain development and amyloid precursor protein (APP) signalling. The phosphotyrosine-binding domain 1 (PTB1) of human Fe65 has been cloned, overexpressed, purified and crystallized using the hanging-drop vapour-diffusion method. Native crystals belong to the space group $R3$ and diffract to 2.6 Å resolution. This crystal form suffered from high thermal B factors and pseudo-symmetry, resulting in a bisection of the c axis. Co-crystallization with a mercury compound under similar conditions induced an orthorhombic crystal form in the space group $P2_12_12_1$ diffracting to 2.2 Å resolution. SAD phases have been computed to the diffraction limit at the wavelength of maximum absorption (L_{III} edge).

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder that afflicts an increasing part of our aging population (see the review by Mattson, 2004). The amyloid plaques, the main diagnostic alteration within the brain of affected patients, mainly contain the amyloid-β peptide (Aβ). This peptide arises from subsequent proteolytic processing of the amyloid precursor protein (APP), an integral type I transmembrane protein present in many cell types including neurons and glia cells, by the aspartyl proteinase β-secretase and the large γ-secretase complex (see the review by Selkoe, 2001). However, the physiological role of APP and its processing are still poorly understood.

In recent years, several interaction partners of APP have been identified that bind *via* their phosphotyrosine-binding domains (PTB) to the conserved YENTPY sequence motif located in the intracellular domain of APP (AICD). Of special interest among the APP-binding proteins is Fe65, a protein predominantly expressed in the brain where it plays a critical role in brain development and APP signalling (Guenette *et al.*, 2006). Fe65 is a multidomain protein composed of three protein–protein interaction domains, one WW domain and two C-terminal PTB domains (PTB1 and PTB2). The WW domain recognizes polyproline sequences in several proteins, including c-Abl tyrosine kinase (Zambrano *et al.*, 2001) and the mammalian homolog of the *Drosophila* protein enabled (Mena) (Ermekova *et al.*, 1997) while the PTB2 domain interacts with the C-terminus of the APP and its homologs APLP1 (APP-like protein1) and APLP2 (Bressler *et al.*, 1996; Borg *et al.*, 1996; Fiore *et al.*, 1995; Guenette *et al.*, 1996; Trommsdorff *et al.*, 1998). The PTB1 domain was shown to bind to the histone acetyltransferase Tip60 (Cao & Sudhof, 2001), the transcription factor CP2/LSF/LBP1 (Zambrano *et al.*, 1998) and to the cytoplasmic domains of the low-density lipoprotein receptor-related protein (LRP-1) (Kounnas *et al.*, 1995; Kinoshita *et al.*, 2003; Pietrzik *et al.*, 2004) and the ApoEr2 receptor (Hoe *et al.*, 2006).

Here we report the cloning, overexpression, purification, crystallization and the preliminary crystallographic analysis of the PTB1 domain of human Fe65. Since attempts to calculate phases by the molecular replacement method using known PTB domain structures were unsuccessful, we co-crystallized the protein with different heavy metal compounds. Crystals obtained by co-crystallization with methyl mercurychloride showed a different space group compared with

native crystals and diffracted to a higher resolution. Single anomalous dispersion (SAD) phases were measured to a resolution of 2.2 Å.

2. Material and methods

2.1. Cloning and protein expression

A gene fragment containing residues 366–505 of human Fe65 was amplified from a cDNA of the full-length protein by the polymerase chain reaction (PCR) with the primers 5′-GTACCCATGGGG-ATCAAGTGTTTCGC-3′ (forward primer) and 5′-GTACCTC-GAGTTCGGCCATGATC-3′ (backward primer) and cloned into the *Nco*I and *Xho*I sites of the pET24d vector (Novagen). The construct was verified by sequencing and contains two additional residues (Leu, Glu) and a hexa-histidine tag at the C-terminus of the protein due to the cloning strategy. The recombinant protein was overexpressed in BL21 (DE3) *Escherichia coli* cells. The cells were grown at 310 K in LB (lysogeny broth) medium containing kanamycin (30 mg l^{-1}). Protein expression was induced by 1 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG) when the OD_{600} reached ∼0.6 and took place overnight at 293 K. Cells were harvested by centrifugation for 20 min at 5000*g* and 277 K, and frozen at 193 K.

2.2. Purification

Cell pellets were resuspended in 10 ml lysis buffer per gram of cells and passed through an M1-10L Microfluidizer (Microfluidics). The lysis buffer contained 300 m*M* NaCl, 50 m*M* Tris pH 8.0, 10 m*M* imidazole and 0.02%(*v*/*v*) 1-thioglycerol. The cell lysate was centrifuged at 125 000*g* at 277 K and the supernatant was applied to a 1 ml His-Trap HP column (GE Healthcare). The column was washed with ten column volumes lysis buffer containing 30 m*M* imidazole and the protein was eluted in lysis buffer containing 300 m*M* imidazole. The protein was further purified on an S75 26/60 size exclusion column (GE Healthcare) equilibrated in 150 m*M* NaCl, 10 m*M* Tris pH 8.0 and 0.02%(*v*/*v*) 1-thioglycerol. Prior to the crystallization experiments, the protein was concentrated using an Amicon Ultracel-10K (Millipore) to a final concentration of 20 mg ml^{-1} in the size exclusion column buffer. The purity of the protein was checked by Coomassie-stained polyacrylamide gel electrophoresis (SDS–PAGE). The protein was either immediately used for crystallization trials or shock frozen in liquid nitrogen and stored at 193 K.

2.3. Dynamic light scattering

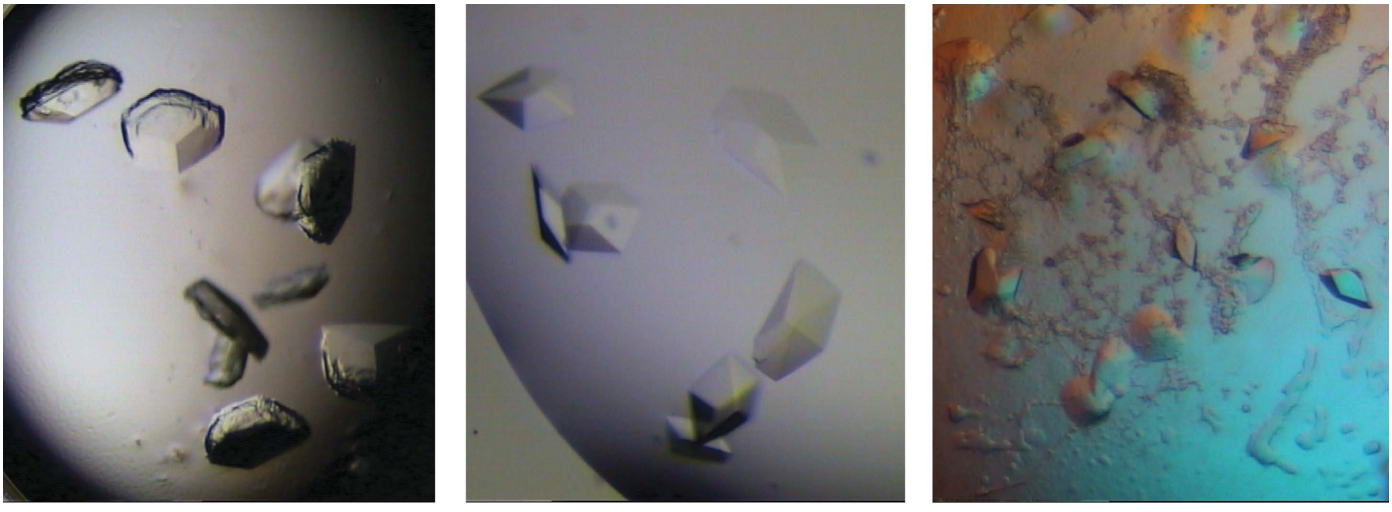
Dynamic light scattering (DLS) analyses of Fe65-PTB1 were performed using a Dynapro instrument (Protein Solutions) by injecting 12 µl of 1–2 mg ml^{-1} protein solutions in a buffer containing 10 m*M* HEPES pH 7.5, 150 m*M* NaCl and 0.02%(*v*/*v*) 1-thioglycerol. Measurements were performed at temperatures of 277, 285 and 293 K.

2.4. Crystallization

Initial crystallization trials were performed using the Nextal screen formulations (Qiagen) and the sitting-drop diffusion technique in a 96-well plate (Axygen) set up with a Janus robot (PerkinElmer). The protein was mixed with reservoir in a 1:1 ratio (2 µl droplets). After optimization, crystals suitable for X-ray diffraction studies were obtained by the hanging-drop technique. Protein solution and reservoir were mixed here in a 2:1 ratio (3 µl droplets). Co-crystallization experiments with various heavy metal compounds (gold, lead, europium, platinum, uranium and mercury) were performed to obtain experimental phases. For co-crystallization, 1 µl of protein solution was mixed with 0.8 µl of the reservoir and 0.2 µl of the respective heavy metal solutions (10 m*M* stocks in water). All crystals were flash cooled in liquid nitrogen prior to diffraction experiments and after rapid soaking in reservoir supplemented by 20%(*v*/*v*) ethylene glycol.

2.5. Data collection and SAD phasing

Diffraction data on the native crystals were collected at beamline ID14-eh2 at the European Synchrotron Radiation Facility (ESRF) at cryogenic temperatures (100 K) on an ADSC Q4 CCD detector. Data were processed with *MOSFLM* and scaled with *SCALA* from the *CCP4* program suite (Collaborative Computational Project, Number 4, 1994). For the molecular replacement method, we used *PHASER* (McCoy, 2007). Data for the mercury derivative were collected at 100 K at the wavelength-tunable ESRF beamline ID23-1 equipped with an ADSC Q315r CCD detector. SAD phasing was carried out by



(*a*) (*b*) (*c*)

Figure 1
Crystals of Fe65-PTB1. (*a*) Unrefined crystals with 2-propanol as an additive of native Fe65-PTB1 in the trigonal space group *R*3. (*b*) Refined crystals with ethylene glycol as an additive. (*c*) Crystals of mercury-derivatized Fe65-PTB1 in the orthorhombic space group $P2_12_12_1$. The crystals were typically ∼200 µm in their longest dimension.

Table 1
Data collection statistics.

Crystal form	Native (short axis)	Native (long axis)	Mercury derivative
Wavelength (Å)	0.9793	1.0723	1.0073
Space group	*H*3	*H*3	$P2_12_12_1$
Unit-cell parameters (Å)	$a = b = 146.71$, $c = 39.66$	$a = b = 145.91$, $c = 79.05$	$a = 43.76$, $b = 79.57$, $c = 83.97$
Total number of reflections	96604	56208	96368
Number of unique reflections	9764 (1413)	15431 (2260)	15508 (2208)
Resolution (Å)	25–2.6 (2.74–2.6)	72.9–2.8 (2.95–2.8)	83.9–2.2 (2.32–2.2)
Completeness (%)	99.9 (100)	99.8 (99.9)	100 (100)
$\langle I/\sigma_I \rangle$	16.3 (2.7)	14.2 (3.2)	19.2 (4.6)
Redundancy	5.7 (5.7)	3.6 (3.7)	6.2 (6.4)
Molecules per AU	2	4	2
$R_{\rm merge}$ (%)†	7.8 (46.9)	8.9 (34.7)	6.7 (34.7)
$R_{\rm anom}$ (%)‡			6.7 (16.9)
Anomalous completeness			99.9 (100)
Anomalous multiplicity			3.3 (3.4)
Mean figure of merit (f.o.m.)			0.56

† $R_{\rm merge} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i |I_i(hkl)| \times 100\%$. ‡ $R_{\rm anom}$ as defined in program *SCALA* (Collaborative Computational Project, Number 4, 1994).

the automated search routine *AUTOSOL* from the *PHENIX* (Python-based Hierarchical Environment for Integrated Xtallography) program suite (Adams *et al.*, 2002).

3. Results

Expression of the Fe65-PTB1 domain resulted in a typical yield of about 40 mg l^{-1} of bacterial cell culture. The protein was purified by standard protocols as described above. Protein purity was greater than 95% as judged by Coomassie-stained SDS–PAGE with Fe65-PTB1 appearing as a single band at 17 kDa (data not shown). Mass spectroscopy and Western blot analysis using an anti-penta-His antibody (Qiagen) were used for protein identification. In DLS experiments the Fe65-PTB1 samples exhibited a monomodal distribution at all tested temperatures and the molecular weight was estimated to be around 20 kDa (data not shown).

An initial crystallization hit obtained from Nextal Classics Lite screen formulation condition No. 5 gave rise to irregular crystals which grew within 1 d in 10%(*w*/*v*) PEG 4000, 5%(*v*/*v*) 2-propanol and 100 m*M* HEPES pH 7.5 at 277 K (Fig. 1*a*). Optimized crystals grew within 1 d at 291 K in 10%(*w*/*v*) PEG 3350, 5%(*v*/*v*) ethylene glycol and 100 m*M* HEPES pH 7.5 (Fig. 1*b*). The mercury-derivative crystal used to determine phases was obtained by co-crystallization of Fe65-PTB1 with 1 m*M* methyl mercurychloride (CH_3HgCl) in 8%(*w*/*v*) PEG 3350, 5%(*v*/*v*) ethylene glycol and 100 m*M* HEPES pH 7.5 (Fig. 1*c*).

Native crystals of the Fe65-PTB1 domain belong to the trigonal space group *R*3. Table 1 lists the unit-cell parameters for the hexagonal setting (*H*3), which due to pseudo-symmetry can also be expressed in a cell with a halved *c* axis. The solvent content of these crystals is 50% with a Matthew's coefficient ($V_{\rm M}$) (Matthews, 1968) of 2.46 $Å^3$ Da^{-1}. The crystals contain two (short *c* axis) or four (long *c* axis) molecules per asymmetric unit and diffracted up to 2.6 Å using synchrotron radiation. Complete data statistics are presented in Table 1. Although the data were reasonable, all molecular replacement trials with known PTB domain structures failed. We therefore started to apply heavy-metal derivatization by co-crystallization of the native crystals. Interestingly, co-crystallization trials with the mercury compound methyl mercurychloride resulted in an unrelated orthorhombic crystal form ($P2_12_12_1$) under very similar conditions as observed for the native protein (two molecules per asymmetric unit, solvent content of 44%, $V_{\rm M}$ = 2.2 $Å^3$ Da^{-1}). These crystals had a much lower temperature factor according to Wilson plot statistics (40 $Å^2$ instead of 80 $Å^2$ for the native crystals) and diffracted up to 2.2 Å.

A fluorescence scan was taken around the mercury $L_{\rm III}$ absorption edge and diffraction data were collected at four wavelengths corresponding to the absorption peak (λ = 1.0073 Å), inflection point (λ = 1.0102 Å), low-energy remote (λ = 0.9951 Å) and high-energy remote (λ = 1.0121 Å). The first dataset collected at the peak with a maximum resolution of 2.2 Å was sufficient to calculate useful SAD phases. A total of six Hg atoms were detected in the asymmetric unit (three per molecule), with a mean figure of merit of 0.56.

In summary, the structure of the Fe65-PTB1 domain is important in helping to decode the structural network surrounding the APP. Both processing and signalling events depend on the interactions of APP with Fe65, and the PTB1 domain is the central compound in complexes with either the low-density lipoprotein receptor-related protein (LRP-1) or the histone acetyltransferase Tip60. The mercury-induced crystallization of this adaptor module highlights the usefulness of metal additives in improving significantly crystal quality to a point where structure determination is possible. We now expect the three-dimensional structure to be solvable by the SAD method using the data collected, and model building and refinement are currently in progress.

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