Protein Expression and Purification 98 (2014) 32–37

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Expression and purification of the aortic amyloid polypeptide medin

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ARTICLE INFO

Article history:
Received 6 December 2013
and in revised form 12 February 2014
Available online 3 March 2014

Keywords:
Amyloid
Fibril
SUMO
pOPINS
Solid-state NMR

ABSTRACT

The 50-amino acid protein medin is the main fibrillar component of human aortic medial amyloid (AMA), the most common form of localised amyloid which affects 97% of Caucasians over the age of 50. Structural models for several amyloid assemblies, including the Alzheimer's amyloid-β peptides, have been defined from solid-state nuclear magnetic resonance (SSNMR) measurements on ^{13}C- and ^{15}N-labelled protein fibrils. SSNMR-derived structural information on fibrillar medin is scant, however, because studies to date have been restricted to limited measurements on site-specifically labelled protein prepared by solid-phase synthesis. Here we report a procedure for the expression of a SUMO-medin fusion protein in *Escherichia coli* and IMAC purification yielding pure, uniformly ^{13}C,^{15}N-labelled medin in quantities required for SSNMR analysis. Thioflavin T fluorescence and dynamic light scattering measurements and transmission electron microscopy analysis confirm that recombinant medin assembles into amyloid-like fibrils over a 48-h period. The first ^{13}C and ^{15}N SSNMR spectra obtained for uniformly-labelled fibrils indicate that medin adopts a predominantly β-sheet conformation with some unstructured elements, and provide the basis for further, more detailed structural investigations.

Introduction

Over 30 proteins and peptides polymerise via soluble oligomeric intermediates into insoluble amyloid or amyloid-like fibrils associated histopathologically with human disorders including Alzheimer's, Parkinson's and type II diabetes [1,2]. Despite considerable research into the cellular and molecular basis of amyloidoses, there is no clear consensus as to the contribution of amyloid to disease or how amyloid pathogenicity is influenced by the structure of the protein assemblies at the molecular level. There is substantial evidence that the pre-fibrillar aggregates of amyloidogenic proteins and peptides are cytotoxic [3–7], and the mature fibrillar deposits detected in tissue by immunochemical staining may consequently be a benign end-point of the assembly process [8,9]. Further research is required to elucidate the nature and cytotoxic mechanism of these species at the molecular level in order to guide the development of targeted therapies. Solid-state nuclear magnetic resonance (SSNMR)[1] of ^{13}C- and ^{15}N-labelled protein fibrils has played a valuable role in these ongoing research efforts and has been utilised to define structural models for several amyloid assemblies, including the Alzheimer's amyloid-β peptides, the human islet amyloid polypeptide and β2-microglobulin [10–16].

Aortic medial amyloid (AMA) is the most common form of localised amyloid identified to date, affecting 97% of Caucasians over the age of 50 [17]. It occurs predominately in the medial layer of the aorta and may have a role in vascular pathologies such as thoracic aneurysm and dissection [18]. The major protein component of AMA is fibrillar medin (AMed), a cleavage product of the glycoprotein lactadherin [19]. AMed was first isolated from cadaveric tissue of three patients with extensive medial amyloid deposits, the isolated proteins had a ragged N-terminus but the main protein component consisted of the 50 amino acid polypeptide medin (Fig. 1A) [17,19]. The 8 C-terminal residues of medin are important for aggregation [20] and a peptide corresponding to residues 42–49 (Med_{42-49}) forms needle-like aggregates composed of parallel hydrogen-bonded β-strands aligned in-register [11]. SSNMR analysis of selectively ^{13}C-labelled medin and Med_{42-49} fibrils prepared by solid-phase synthesis indicates differences in the packing arrangements of the C-terminal amino acid side-chains (7). Very little else is known about the molecular architecture of medin fibrils, however, and a more detailed structural analysis of full-length medin using SSNMR will require extensively or uniformly ^{13}C and ^{15}N labelled protein. To date, however, no detailed procedure has been published for the production of medin in

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[1] *Abbreviations used:* SSNMR, solid-state nuclear magnetic resonance; AMA, aortic medial amyloid; IMAC, immobilised metal affinity chromatography; DAB, diaminobenzidine tetrahydrochloride; TEM, transmission electron microscopy; TEV, tobacco etch virus; DLS, dynamic light scattering.

http://dx.doi.org/10.1016/j.pep.2014.02.009
1046-5928/

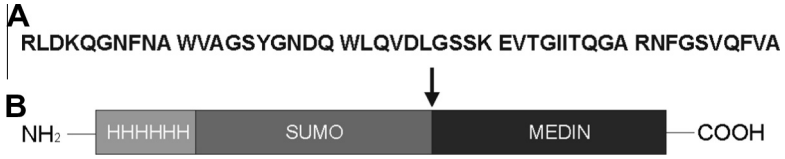

Fig. 1. Amino acid sequence of medin (A) and schematic of pOPINS construct, black arrow indicates SUMO protease cleavage site (B).

sufficient quantities to permit structural investigations. Here, we initially employed a pMal vector construct commonly used for expression in *Escherichia coli* of amyloidogenic peptides fused with maltose binding protein, but this approach was aborted because of difficulties encountered with the proteolytic cleavage of medin after purification by affinity chromatography. We report here that the successful expression and purification of medin is achieved using a pOPINS vector to express the protein fused with an N-His_6-SUMO tag. The Alzheimer's $A\beta_{1-42}$ polypeptide was previously expressed and purified with a SUMO tag [21], but the utility of this approach for the production of other amyloidogenic proteins has until now not been demonstrated. The preparative procedure described yielded up to 6 mg of fibrillar protein per litre of growth media. We present the first ^{13}C and ^{15}N SSNMR spectra of uniformly ^{13}C ^{15}N-labelled medin fibrils. This protocol will allow further characterisation of medin using SSNMR and other biophysical techniques.

Materials and methods

Vector and cloning

Initially the medin gene was prepared synthetically and inserted into a pMalC2X vector by GenScript (U.S.A.). In order to improve the yield and facilitate purification of the target protein, the medin gene was sub-cloned into pOPINS vector using the In-Fusion™ method [22]. The pOPIN suite of vectors developed at the Oxford Protein Production Facility can be used in conjunction with the In-Fusion™ PCR cloning system (Clontech) [22]. This method is a ligase independent method that is quicker and more efficient than many sub-cloning techniques and the pOPIN suite provides several options for fusion partners and cleavage sites. The pOPINS vector is derived from the pET 28a vector and comprises a N-His_6-SUMO tag with kanamycin resistance (Fig. 1B). The resultant vector was sequenced to confirm the insertion of medin (GATC).

Expression of medin

The pOPINS-medin plasmid was transformed into *E. coli* BL21 (DE3) competent cells (Top 10, NEB). For expression of unlabelled medin a colony was then selected and grown in 50 ml of Luria Broth (LB) containing kanamycin at a concentration of 35 μg/ml overnight at 37 °C. This culture was then harvested by centrifugation at 3000×*g* and used to inoculate 2 L of LB. The culture was incubated at 37 °C until an $O.D._{600}$ = 0.8–1 was reached. Expression of the fusion protein was induced by the addition of isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 1 mM and the cell growth allowed to continue for a further 16 h at 20 °C. Cells were harvested by centrifugation at 3000×*g* for 20 min at 4 °C. This process generated on average a 12 g/L of wet cell pellet in LB and 9 g/L in minimal media. Cell pellets were resuspended in 40 ml of buffer A (20 mM Tris, 0.5 M NaCl, pH. 7.4) and frozen at −20 °C in preparation for lysis and purification.

Expression of $^{15}N/^{13}C$ labelled medin proceeded as described above except that the 50 ml overnight starter culture grown in LB was resuspended in 2 L of M9 minimal medium containing 2 g of $^{15}NH_4Cl$ and 8 g of ^{13}C-glucose.

Cell lysis and purification

The SUMO-medin fusion (18,163 Da) protein can be isolated from crude extract with the use of affinity chromatography. The presence of the His_6-tag enables the use of immobilised metal affinity chromatography (IMAC), using resin functionalised with Ni^{2+} ions. The bound protein is then eluted with imidazole, which competes with the histidines to chelate the Ni^{2+}.

The resuspended cells (from 1 L of culture) in 40 ml buffer A were subjected to a single freeze–thaw cycle prior to 2 French press cycles in the presence of DNase at a final concentration of 20 ng/ml and a cOmplete EDTA-free™ protease inhibitor cocktail tablet (Roche Applied Sciences). The lysed cells were then centrifuged at 19,000×*g* to remove the cell debris and the supernatant filtered through a 0.22 μm Acrodisc™ filter. A 5 ml His-Trap FF ™ column (GE Healthcare) was equilibrated with 5 column volumes (CV) of buffer A and 5 CV of buffer B (20 mM Tris, 0.5 M NaCl, 0.5 M imidazole) followed by a further 5 CV of buffer A at 4 ml/min and the supernatant loaded using an AKTA purification system at 1 ml/min (GE Healthcare). The fusion protein was eluted from the column with a step-wise gradient, with the target protein eluting at 0.2 M imidazole concentration. The fusion protein was collected and desalted using a 26/10 desalting column (GE Healthcare) to remove the imidazole.

Tag removal

The His_6-SUMO tag was removed by incubation with SUMO protease I at 30 °C for 2 h as per the manufacturer's instructions (Tebu-bio) to yield the cleaved tag (12,372 Da) and medin (5430 Da). Following cleavage, a second IMAC purification step was performed to remove the cleaved His_6-SUMO tag and the His_6-SUMO protease. The cleavage mixture was passed through a 5 ml Ni^{2+} NTA column and the flow through containing medin collected. Medin was collected in the flow-through and prepared for aggregation and NMR studies.

Gel electrophoresis

The expression and purification of medin was analysed by SDS–PAGE using the Laemmli method [23]. Analysis was carried out using 15% Tris-Tricine gels [24] in a Bio-Rad gel electrophoresis system. 5 μl protein samples were added to 5 μl of 2× sample buffer, boiled for 5 min and loaded onto the gel. All gels were run at 200 V for 60 min, stained with Coomassie brilliant blue G-250 and de-stained with H_2O, methanol, and acetic acid in a ratio of 50/40/10 (v/v/v).

Western blot analysis

Polyclonal antibodies against two epitopes of medin, corresponding to residues 1–10 and 19–31, were raised in rabbit and purified by GenicBio (Hong Kong). Anti-rabbit IgG horseradish