Protein Expression and Purification 67 (2009) 53–60

Contents lists available at ScienceDirect

Protein Expression and Purification

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

Bacterially expressed recombinant WD40 domain of human Apaf-1

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ARTICLE INFO

Article history:
Received 11 March 2009
and in revised form 10 April 2009
Available online 19 April 2009

Keywords:
Apoptosis
Apoptosome
Apoptotic protease activating factor-1
Apaf-1
WD40 domain

ABSTRACT

The apoptotic protease activating factor (Apaf-1) is a protein that binds to cytochrome *c*, and in the presence of dATP/ATP oligomerizes to assume the role of an adaptor platform for activating the caspase-9 zymogen. In order to study the biochemical and structural details of Apaf-1 function, we have generated an expression construct from pcDNA 3-Apaf-1XL for production of the WD40 domain (^{WD40}Apaf-1) in *Escherichia coli*. The WD40 domain expressed contains 825 amino acids in addition to an N-terminal His_6 tag derived from the cloning vector. The expressed protein is invariably localized in the inclusion body fraction of *E. coli*. A simple protocol involving Sephadex G100 chromatography developed for purifying the protein starting from inclusion bodies has allowed protein recovery in highly pure form. Basic fluorescence and CD spectra indicate that the refolded protein has extensive secondary and tertiary structures. Immunoprecipitation studies have provided qualitative information about the binding interaction of ^{WD40}Apaf-1 and cytochrome *c*. The binding interaction has been quantified by spectrophotometric titration of cytochrome *c* with recombinant ^{WD40}Apaf-1. The results demonstrate a weak binding for cytochrome *c* and ^{WD40}Apaf-1 interaction, the binding affinity being 390 nM. The analysis indicates a 2:1 or possibly even 3:1 stoichiometry for cytochrome *c* and ^{WD40}Apaf-1 binding interaction.

A key upstream event in the mitochondrial pathway of apoptosis is the cytochrome *c*-dependent activation of procaspase-9 so that the processed caspase-9 can propagate the death signal by triggering events leading to activation of downstream caspases [1,2]. Apaf-1[1] is the protein that mediates the initial event of caspase-9 activation [3,4]. By binding to cytochrome *c* translocated from mitochondria, Apaf-1 is thought to self-associate in the presence of dATP/ATP to produce a homo-multimer that contains at least 8 subunits of Apaf-1 [3]. This homo-multimeric complex in which cytochrome *c* and dATP are presumably still bound to each Apaf-1 subunit is generically dubbed the 'apoptosome' [3–6]—an assembly that recruits procaspase-9 and activates it into caspase-9 [3,4,7]. The initiating events and structural mechanism by which Apaf-1 multimerizes to assemble a functional apoptosome is inexplicably complex, and to a large extent the size and form of Apaf-1 itself generates the complexity. Apaf-1 contains a CED-4 homologous domain that straddles a *Ca*spase *R*ecruitment *D*omain (CARD) in the N-terminal region and a C-terminal WD40 domain containing 12 or 13 WD40 repeats depending on the isoform [3,4,8,9]. The oligomerization of Apaf-1, and gross structural and topological details of the apoptosome have been viewed by electron cryomicroscopy [10–12]. A theoretical model for Apaf-1 heptamer assembly has also been reported [13]. However, there exists an acute dearth of atomic-level structural descriptions of Apaf-1 and its multimeric complex, although NMR solution structure of the CARD domain [14] and a 2.2 Å crystal structure of an ADP-bound CARD and CED-4 homologous domains together [15] have been reported.

The WD40 domain at the C-terminal part accounts for approximately 66% of Apaf-1 primary sequence. The finding of at least 12 WD40 repeats in this domain aroused interest right from the seminal stage of Apaf-1 discovery [8]. WD-repeat proteins invariably assume a β-propeller fold and are thus structurally related, but their functions remain poorly understood [16,17]. Nonetheless, the large content of Asp in the WD40 domain of Apaf-1 together with the finding that Apaf-1 binds to cytochrome *c*, a basic protein rich in Lys, points to the possibility that Asp–Lys charge–charge interactions form the basis for Apaf-1 binding to cytochrome *c*. There is no definite structural support for this possibility except for the demonstration that ionic strength has a profound effect on the interaction [18]. In view of the importance and interest in the structure, binding epitope, and functional regulation of Apaf-1, it is imperative that recombinant WD40 domain is made available. Proteins the size of the WD40 domain (825 amino acid residues for the present study) are often difficult to produce in a bacterial overexpression system.

As a part of our structural and functional studies of proteins involved in mitochondrial apoptosis, we have developed an expression construct for *Escherichia coli* expression of ^{WD40}Apaf-1.

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[1] *Abbreviations used:* Apaf-1, apoptotic protease activating factor; CARD, Caspase Recruitment Domain; HCCA, cyano-4-hydroxycinnamic acid; GST, glutathione S-transferase; CDNB, 1-chloro-2,4-dinitrobenzene.

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doi:10.1016/j.pep.2009.04.008

The gene construct excludes the N-terminal contiguous CARD and CED-4 homologous domains whose cloning and *E. coli* expression was described recently from this laboratory [19]. Here, we report on the cloning, *E. coli* expression, refolding, and cytochrome *c* binding properties of the WD40-repeat domain of the human Apaf-1XL isoform, called ^{WD40}Apaf-1 henceforth.

Materials and methods

Cloning and generation of expression construct for ^{WD40}Apaf-1

The gene sequence encoding the 2.5 kb ^{WD40}Apaf-1 (amino acid residues 424–1248) region was specifically amplified from pcDNA 3-Apaf-1XL, a generous gift from Gabriel Nunez's laboratory, by standard PCR reaction. The forward and reverse primers flanking the WD40 region of Apaf-1XL were:

*Bam*H1
WD40-F: 5′ CG**GGATCC**TTATTCTGTGATCGGAATGG 3′

WD40-R: 5′ CC**CTCGAG**TTATTCTAAAGTCTGTAAAATATA 3′
*Xho*1

Restriction sites were included at the 5′ end of each primer to facilitate cloning into pRSETa vector (Invitrogen), and the region of ~2.5 kb corresponding to ^{WD40}Apaf-1 was amplified using MBI-Fermentas long PCR enzyme mix. The 50 μL reaction mixture contained 5.0 μL 10× long PCR buffer, 10 pM each of forward and reverse primers, 100 ng of pcDNA 3-Apaf-1 XL as template, and 2.5 U/μL of long PCR enzyme mix. The PCR amplification involved initial denaturation at 94 °C for 2 min, followed by 30 cycles each consisting of a 30-s denaturation at 94 °C, a 30-s annealing at 55 °C, and a 2.5-min extension at 72 °C. To facilitate TA cloning, the final extension was allowed for 15 min at 72 °C. The amplified PCR product was analyzed by agarose gel electrophoresis. The 2.5 kb ^{WD40}Apaf-1 fragment was sliced from gel, eluted by using Qiagen gel extraction column, ligated into the TA vector pTZ57R/T (MBI-Fermentas), and transformed into DH5α *E. coli* cells. The cells were plated onto agar plates containing 100 μg/mL ampicillin.

Positive recombinant clones of ^{WD40}Apaf-1 were selected by colony PCR. Plasmids were isolated and digested with BamH1 and Xho1 restriction enzymes. The digested fragments were run on 1.0% agarose gel, and the digested 2.5 kb ^{WD40}Apaf-1 was sliced from gel and eluted by gel extraction columns. This was ligated between the BamH1 and Xho1 sites of pRSETa expression vector (Invitrogen). The recombinant pRSETa–^{WD40}Apaf-1 clones were selected by colony PCR, and confirmed by restriction digestion.

Protein expression

The recombinant pRSETa–^{WD40}Apaf-1 plasmids were sequenced before transforming into *E. coli* BL21 DE3 cells. Many singly isolated colonies were selected and screened for soluble expression of ^{WD40}Apaf-1. Unfortunately, we could not obtain any soluble expression of the protein. Use of different *E. coli* strains, and variable growth conditions, including temperature and IPTG concentration, did not ameliorate the situation. Facing this difficulty, we decided to proceed with inclusion bodies.

Cell growth and harvest, cell lysis, and inclusion body washing

These steps were carried out using procedures already described for $^{\Delta WD40}$Apaf-1 [19] with minor modifications. Briefly, *E. coli* BL21 DE3 cells containing the pRSETa–^{WD40}Apaf-1 plasmid were grown in LB medium containing ampicillin (100 μg/mL). Typically, 10 mL of an overnight grown culture was added to a 1-L medium, and grown up OD_{600} = 0.5 at 37 °C. Protein expression was then induced by adding IPTG to a final concentration of 1 mM, and growth was continued for 5 h. Cells were harvested by centrifugation at 4000*g* for 10 min at 4 °C, washed with TE buffer (10 mM Tris–HCl, 1 mM EDTA pH 8.0), and frozen-stored at −80 °C. For lysis, 2 g of the cell pellet (wet weight) obtained from 1-L culture was suspended in 20 mL of the lysis buffer (20 mM Tris–HCl, 100 mM NaCl, 1 mM PMSF, pH 8.0, containing 10 μL of 1 mg/mL DNaseI), and sonicated at 4 °C with 10 cycles, each cycle consisting of 30 s on and 60 s off times. The lysate was treated with deoxycholic acid (4 mg per gram weight of *E. coli*), stirred for 30 min at room temperature, and centrifuged at 10,000 rpm for 15 min at 4 °C. The pellet was resuspended in ~9 volumes of a buffer consisting of 20 mM Tris, 100 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, pH 8.0, stirred at room temperature for 5 min and centrifuged at 10,000 rpm for 15 min at 4 °C. The step was repeated. Inclusion bodies were then washed with ~10 volumes of 20 mM Tris–HCl containing 100 mM NaCl at pH 8.0 three times to remove the Triton X-100. In the effort to remove the unwanted materials as far as possible, a final wash was given with ~10 volumes of 20 mM Tris–HCl containing 2.5 M GdnHCl at pH 8.0. Washed inclusion bodies are now ~95% pure.

Finding optimal pH for refolding

To measure the optimal pH for refolding, the inclusion bodies were first unfolded in buffer A (50 mM Tris–HCl, 0.15 M NaCl, 5 mM EDTA, 5 mM DTT, 6.0 M GdnHCl, pH 8.0), incubated for 2 h at room temperature, and spun at 20,000*g* for 15 min. The supernatant was then diluted 10-fold by adding buffer B (refolding buffer: 0.5 M NaCl, 0.25 M arginine, 0.5 M urea, 1 mM EDTA, and 1 mM DTT in 50 mM Tris–HCl for the 7–10.5 range of pH values, and in 50 mM sodium acetate for the 4.0–6.5 range of pH values). In all of these refolding trials, the final protein concentration was ~100 μg/mL. The refolded proteins samples were incubated for 2 h at 4 °C. The refolding efficacy was determined spectrophotometrically by turbidity measurement at 450 nm. By observing a dramatic decrease in turbidity at pH > 8.5, we decided to refold the protein at pH 8.8. It should be noted that attempts to refold the protein at lower pH values showed little success. However, in the steps followed for purification of the refolded protein, pH 8.0 was employed (see below). The multitude of refolding conditions checked by the turbidity measurement also suggested the inclusion of 400 mM arginine and 2 mM DTT in the refolding buffer. The buffer B mentioned above then would contain 0.4 M arginine, 2 mM DTT, and 0.1% glycerol.

Preparative refolding and purification of ^{WD40}Apaf-1

Inclusion bodies containing ~34 mg protein were solubilized in 2.5 mL of the unfolding buffer (buffer A as described above). Following 4 h of incubation at room temperature, the solution was centrifuged at 20,000*g* for 15 min at 4 °C. For refolding, the supernatant was diluted 10-fold by slow addition of the refolding buffer (buffer B: 50 mM Tris, 0.5 M NaCl, 0.5 M urea, 0.4 M arginine, 2 mM DTT, 0.1% glycerol, pH 8.8), and equilibrated overnight at 4 °C. The solution appeared a little turbid the following morning, but was clear when centrifuged at 20,000*g* for 15 min at 4 °C.

For purification of the refolded protein, two approaches were employed. In one, the supernatant obtained from the protein solution refolded at pH 8.8 was loaded onto a Ni–NTA column equilibrated in 20 mM Tris, 0.5 M NaCl, 0.5 M urea, 1 mM β-mercaptoethanol, pH 8.0, washed thoroughly with the same buffer, and eluted with 20 mM Tris containing 0.25 M imidazole at pH 8.0. Here, although the refolding was carried out at pH 8.8 outside the column, pH 8.0 was chosen to meet with the condition for Ni–NTA chromatography. Eluted fractions were pooled and dialyzed