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Purification and characterisation of recombinant human eukaryotic elongation factor 1 gamma

Ikechukwu Achilonu *, Thendo P. Siganunu, Heini W. Dirr

Protein Structure-Function Research Unit, School of Molecular and Cell Biology, University of the Witwatersrand, Johannesburg 2050, South Africa

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ABSTRACT

The eukaryotic elongation factor 1 gamma (eEF1γ) is a multi-domain protein, which consist of a glutathione transferase (GST)-like N-terminus domain. In association with α, β and δ subunits, eEF1γ forms part of the eukaryotic elongation factor complex, which is mainly involved in protein biosynthesis. The N-terminus GST domain of eEF1γ interacts with the β subunit. eEF1γ subunit is over-expressed in human carcinoma. The role of human eEF1γ (heEF1γ) is poorly understood. A successful purification of recombinant heEF1γ is the first step towards determining unknown properties of the protein, including putative GST-like activities and the structure of the protein. This paper describes the over-expression, purification and characterisation of recombinant full-length, and the N- and C-terminus domains of heEF1γ. All three recombinant heEF1γ constructs over-expressed in the soluble *Escherichia coli* cell fraction and were purified to homogeneity. Secondary structure analysis indicates that the heEF1γ constructs have high α-helical structural character. The full-length and N-terminus domain are dimeric, while the C-terminus is monomeric. Both full-length and N-terminus domain interact with 8-anilino-1-naphthalene sulfonate (ANS) with K_D = 70.0 (±5.7) μM and with reduced glutathione (GSH). Glutathione sulfonate displaced ANS bound to hydrophobic binding sites in the recombinant N-terminus domain. Using the standard GSH-1-chloro-2,4-dinitrobenzene conjugation assay, the N-domain showed some enzyme activity (0.03 μmol min^{-1} mg^{-1} protein), while the full-length heEF1γ did not catalyse the GSH-CDNB conjugation. Consequently, we hypothesize the presence of a presumed GST-like active site structure in the heEF1γ, which comprises a glutathione binding site and a hydrophobic substrate binding site.

Introduction

Protein biosynthesis in eukaryotes involves three distinct steps, namely initiation, elongation and termination. The elongation step is catalysed by eukaryotic elongation factor 1 (eEF1γ) macromolecular complex, which consists of eEF1α, eEF1β, eEF1γ and eEF1δ subunits (renamed eEF1A, eEF1Bα, eEF1Bγ and eEF1Bδ) [1–3]. For simplicity sake, we will use the older terminologies in this publication. This energy-driven process catalyses the transfer of aminoacyl-tRNAs to ribosome, mediated by the hydrolysis of GTP [2]. During the elongation step, GDP-eEF1α complex is converted to GTP-eEF1α complex by eEF1βγδ complex-mediated GTP hydrolysis [2,4]. Studies have suggested that the main nucleotide exchange factor is the eEF1β, therefore the roles played by γ and δ subunits are not clearly understood [2,4–6]. Nevertheless, the four subunits have been shown to be highly expressed in most eukaryotic cells [1,7].

Studies have shown that eEF1γ is necessary for the nucleotide exchange between α and β subunits, but not directly involved in this process. This is because the absence of eEF1γ from the complex in protein synthesis does not affect the rate of translation [8]. Immunofluorescence studies have shown that eEF1γ forms part of the elongation factor complex with β and δ subunits in the endoplasmic reticulum of human fibroblast [9]. Studies have also shown that eEF1γ is remarkably over-expressed in tumour cells, when compared to the other three subunits [1,7]. In *Saccharomyces cerevisiae*, the depletion of eEF1γ results in resistance to oxidative stress [10]. This may be as a result of the presence of a GST-like domain on the N-terminus (NT) of heEF1γ[1] (Fig. 1) [3,11], which is also believed to be involved in the interaction of the β-subunit of the elongation factor complex [3]. GSTs are a very

* Corresponding author. Tel.: +27 11 717 6349; fax: +27 11 717 6351.
E-mail address: Ikechukwu.Achilonu@wits.ac.za (I. Achilonu).

[1] *Abbreviations used:* heEF1γ, human eukaryotic elongation factor 1 gamma; FL, full length; NT, amino terminus; CT, carboxylic terminus; CD, circular dichroism; H-site, hydrophobic binding site; G-site, glutathione binding site; GST, glutathione transferase; GSH, reduced glutathione; GSO_3^-, glutathione sulfonate; ANS, 8-anilino-1-napthalene sulfonate; CDNB, 1-chloro-2,4-dinitrobenzene; IMAC, immobilized metal ion affinity chromatography; FRET, Förster resonance energy transfer.

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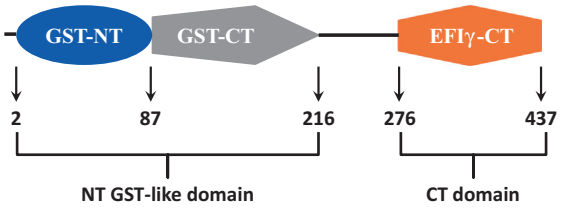

Fig. 1. A schematic illustration of the domain structure of heEF1γ as predicted by the ScanProsite server [19]. The numbers represent the amino acid corresponding to each domain structure.

important family of enzymes, involved in the detoxification of oxygen radical [12]. Therefore it is suggested that the presence of eEF1γ in many tumour cells alters the redox balance in these cells, thus enhancing the aggressiveness of the tumour [2,7]. The heEF1γ have been shown to interact with cytoskeleton [2,13], membrane-bound receptors such as dopamine D_3 receptor [14], RNA polymerase II [5] and tubulin [13,15].

Sheu and Traugh [8] showed that recombinant rabbit eEF1γ associates as a trimer of ~ 140 kDa. But works of Jeppesen et al. [16] show that the crystal structure indicates that the yeast NT GST-like domain of eEF1γ is monomeric, but that an extended form of the NT GST-like domain of yeast eEF1γ displays dimeric structure. Moreover the yeast NT eEF1γ does not exhibit a typical GST activity using GSH-CDNB conjugation assay. Vanwetswinkel and colleagues showed that recombinant full length heEF1γ subjected to limited proteolysis yielded a 25 kDa NT-domain and a highly protease-resistant CT-domain, and the CT-domain did not reveal any similarity with any existing protein family [17,18].

The critical role played by eEF1γ in protein biosynthesis and other suggested biological functions of the protein necessitates in-depth biochemical and structural studies on the heEF1γ, including its sub-domain structures. In this study, the ORF sequence of the heEF1γ was extracted from GenBank and recoded to enhance the over-expression of the protein in *Escherichia coli* host cell. The full length human eEF1γ (FL-heEF1γ), GST-like NT domain (NT-heEF1γ) and the C-terminus (CT) domain (CT-heEF1γ) were recombinantly over-expressed in *E. coli*. The tertiary and quaternary structure of purified full length and sub-domains of heEF1γ were analysed. The ligandin functions of heEF1γ, as well as enzyme activity towards conjugation of GSH to CDNB were also studied.

Materials and methods

Plasmid construction

The cDNA encoding heEF1γ (Gene ID NP_001395.1) was extracted from GenBank. The corresponding amino acid sequence was submitted to the ScanProsite server [19] for theoretical identification of protein domains on the heEF1γ polypeptide. The native mRNA sequence was re-coded (codon harmonization) [20,21] to match the natural codon usage frequency to what it will look like in *E. coli* [20]. The codon-harmonized gene was synthesized (by GenScript Inc, Piscataway, NJ) to incorporate a short *E. coli* thioredoxin peptide sequence, a 6 × His-tag and a thrombin cleavage site upstream the heEF1γ ORF sequence (Fig. S1). The synthesized gene was cloned into a pET-11a plasmid using incorporated *Nde*I and *Bam*HI to create pTFL-heEF1γ for expression in *E. coli* BL21Codon Plus strain (Stratagene). Using pTFL-heEF1γ as a template, site-directed mutagenesis was used to create a stop codon between the NT-heEF1γ and CT-heEF1γ domains (Fig. S1) to enable the over-expression of NT-heEF1γ in *E. coli*. The nucleotide sequence corresponding to the codon-harmonized CT-heEF1γ was synthesized to encode a short *E. coli* thioredoxin peptide, 6 × His-tag sequence and a thrombin cleavage site upstream the CT-heEF1γ sequence (Fig. S2). This synthetic gene was cloned into pET-11a for over-expression in *E. coli* using incorporated *Nde*I and *Bam*HI. All sequencing was done by GenScript.

Recombinant protein expression

E. coli BL21 Codon Plus cells were transformed with plasmids encoding the full length, NT domain and the CT domain of heEF1γ. Single colonies were cultured overnight in 2 × YT (37 °C, 250 rpm) supplemented with 50 μg/ml of carbenicillin and 34 μg/ml chloramphenicol. The overnight cultures were diluted 1/50 with fresh 2 × YT supplemented with 50 μg/ml of carbenicillin and 34 μg/ml chloramphenicol and incubated (37 °C, 250 rpm) until the OD_{600} was approximately 0.5. The cultures were incubated on ice bath for 10 min and induced with IPTG to a final concentration of 1 mM and cultured for additional six hours (30 °C, 250 rpm). Cells were harvested by centrifugation (5000×*g*, 15 min, 10 °C). Approximately 11 g of wet cell pellet was obtained per liter culture, and the pellet was re-suspended in 35 ml of 50 mM Tris–HCl, pH 7.5, 500 mM NaCl, 15 mM imidazole and 0.02% (w/v) NaN_3 (buffer 1) and frozen (80 °C, 16 h). The frozen cell suspension was thawed at room temperature (22 °C) and disrupted by sonication on ice. Soluble cell fraction was obtained by centrifugation (25000×*g*, 25 min, 10 °C).

Protein purification

The soluble cell fraction was subjected to immobilized metal affinity chromatography (IMAC), using immobilized Co^{2+} (for FL- and NT-heEF1γ) or Ni^{2+} (for CT-heEF1γ) chelate resin pre-equilibrated with buffer 1. The IMAC resin was washed with buffer 1 to remove unbound protein, eluted with 50 mM Tris–HCl, pH 7.5, 500 mM NaCl, 250 mM imidazole and 0.02% (w/v) NaN_3. Fractions containing eluted proteins were pooled, dialysed (16 h, 2 changes, 4 °C) against 20 mM Tris–HCl, pH 8.3, 125 mM NaCl, 5 mM $CaCl_2$ and 0.02% (w/v) NaN_3 (buffer 2). The protein sample was treated with thrombin (0.5 U/mg of recombinant protein, 16 h, 22 °C). The thrombin-treated protein was subjected to a second affinity chromatography. For FL- and NT-heEF1γ, the protein sample was mixed with GSH-Agarose (Sigma) beads equilibrated with buffer 2 containing 5 mM DTT. Unbound proteins were eliminated from the beads by extensively washing with 50 mM Tris–HCl, pH 8.0, 1 M NaCl, 5 mM DTT, 1% (v/v) Triton X-100 and 0.02% (w/v) NaN_3, followed by 50 mM Tris–HCl, pH 8.0, 500 mM NaCl, 1 mM DTT, and 0.02% (w/v) NaN_3. Bound proteins were eluted with 10 mM glycine-NaOH, pH 10, 0.02% (w/v) NaN_3, and pH was immediately adjusted to ~8.0 by adding 1 M Tris–HCl, pH 7.5 at 20% (v/v) with the protein fraction. For CT-heEF1γ, a second immobilized Ni^{2+}-IMAC coupled to heparin-Agarose affinity chromatography was used to remove the His-tagged peptide, thrombin and other protein impurities. Fractions containing recombinant proteins were pooled and dialysed against (2 changes, 5 h, 4 °C) 5 mM Tris–HCl, pH 7.5, 1 mM DTT and 0.02% (w/v) NaN_3 (storage buffer). The purity was analysed by reducing SDS–PAGE [22] and the quantity of protein determined spectrophotometrically using theoretically estimated molar extinction coefficient; 86860 M^{-1} cm^{-1} (FL-heEF1γ), 37930 M^{-1} cm^{-1} (NT-heEF1γ) and is 48930 M^{-1} cm^{-1} (CT-heEF1γ).

Secondary structure characterization

Far-UV circular dichroism (Far-UV CD) measurements of FL-, NT- and CT-heEF1γ (re-suspended in 0.5 mM Tris–HCl buffer, pH 7.5, containing 0.001 mM EDTA, 0.005 mM DTT and 0.0002% NaN_3) was done at 22 °C in a Chirascan CD spectrometer. The software