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Human Ind1 expression causes over-expression of *E. coli* beta-lactamase ampicillin resistance protein

Vaishali Sharma Chakrabarti [a], Maciej Mikolajczyk [a,b], Francesca Boscaro [c], Vito Calderone [a,b,*]

[a] Magnetic Resonance Center – CERM, University of Florence, Via Luigi Sacconi 6, 50019 Sesto Fiorentino, Florence, Italy
[b] Department of Chemistry, University of Florence, Via della Lastruccia 3, 50019 Sesto Fiorentino, Florence, Italy
[c] Mass Spectrometry Centre – CISM, University of Florence, Via Ugo Schiff 6, 50019 Sesto Fiorentino, Florence, Italy

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ABSTRACT

Ind1, a mitochondrial P-loop NTPase is essential for assembly of respiratory complex-I. Respiratory complex-I (NADH: ubiquinone oxidoreductase), a large (mitochondrial inner membrane) enzyme, is made of 45 subunits and 8 iron–sulfur clusters. Ind1, an iron–sulfur cluster protein involved in the maturation of respiratory complex and binds an Fe/S cluster via a conserved CXXC motif in a labile way. Ind1 has been proposed as a specialized biogenesis factor involved in delivering the Fe/S clusters to the apo complex-I subunits. The IND1 gene is conserved in eukaryotes and is present in genomes of the species that retain functional respiratory complex-I. Depletion of human Ind1 causes ultra-structural changes in depleted mitochondria, including the loss of cristae membranes, massive remodeling of respiratory super complexes, and increased lactate production. Ind1 sequence bears known nucleotide binding domain motifs and was first classified as Nucleotide Binding Protein-Like (NUBPL). Despite the obvious importance of Ind1, very little is known about this protein; in particular its structure as well as its Fe/S cluster binding properties. In the present work we show that the expression of native huInd1 in *Escherichia coli* stimulates over-expression of the beta-lactamase TEM-1 from *E. coli*. The homology modeling of huInd1 shows hallmark of Rossmann fold, where a central beta sheet is covered by helices on either side. In the light of the modeled structure of huInd1, we hypothesize that huInd1 binds to the untranslated region (UTR) of the TEM-1 mRNA at 3′ site and thereby reducing the possibility of its endonucleolytic cleavage, resulting in over-expression of TEM-1.

Introduction

Human Ind1, an iron–sulfur cluster protein (huInd1 hereafter) is required for NADH Dehydrogenase activity. HuInd1 has also been known as human homologue of INDH (from plants) and was called NUBPL (Nucleotide Binding Protein-Like)[1] [1–3]. It has been recently identified as an iron–sulfur (Fe/S) cluster binding protein that is involved in the maturation of respiratory complex-I in mitochondria [3,4]. Complex-I is largest component of the respiratory chain and is comprised of forty-five protein subunits, out of which, seven proteins are encoded by the human mitochondrial genome [1]. In addition to this the complex-I contains stably bound cofactors, including flavin mononucleotide and eight iron–sulfur clusters. Assembly of this highly composite enzyme is not well understood so far but presumably involves several proteins [5,6]. Deficiencies in the complex-I activity are the most common cause of diseases of mitochondrial etiology [7–9]. It has been found that 50% of patients with complex-I deficiencies have no mutations in the individual subunits of complex-I. These facts are indicative of that, a number of assembly factors are relevant for disease [10]. Further, two assembly proteins, NDUFAF2/B17.2L [11] and NDUFAF1/CIA30 [10,12,13] have been studied and characterized. Mutations in the corresponding genes have been identified in the patients suffering from complex-I related diseases. A more recent study identified that patients harboring mutation/s in huInd1, lead to deficiencies in complex-I [14].

Besides fulfilling roles in the molecular chaperoning of complex-I protein subunits, assembly factors are also expected to be

* Corresponding author at: Magnetic Resonance Center – CERM, University of Florence, Via Luigi Sacconi 6, 50019 Sesto Fiorentino, Florence, Italy.
E-mail addresses: vaishalisharma_rs@yahoo.co.in (V.S. Chakrabarti), maciej.mikolajczyk@novartis.com (M. Mikolajczyk), francesca.boscaro@unifi.it (F. Boscaro), calderone@cerm.unifi.it (V. Calderone).

[1] *Abbreviations used:* NUBPL, Nucleotide Binding Protein-Like; Fe/S, iron–sulphur; DTT, Dithiothreitol; TFA, Trifluoroacetic acid; MW, molecular weight; AmpR, ampicillin resistance; cAMP, cyclic AMP; CAP, Catabolite Activator Protein; UTR, untranslated region; IRP1, iron regulatory protein 1; IREs, iron-responsive elements; TfR, transferrin receptor.

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necessary for the insertion of prosthetic groups into either monomeric complex-I subunits, complex-I sub-complexes, or the fully assembled apoenzyme. Fe/S clusters formation have been studied and it has been found that they require specialized and a dedicated molecular machinery for their synthesis and delivery to apoproteins [4]. The biogenesis factors/components and the molecular mechanisms for the Fe/S cluster protein assembly inside mitochondria have been largely investigated in yeast model system. Fifteen components of the mitochondrial assembly machinery are known to catalyze Fe/S cluster maturation. In short, sulfide is generated via desulfuration of cysteine, by cysteine desulfurase complex Nfs1-Isd11 and then it is combined with ferrous iron on a scaffold protein called Isu1. The Fe/S cluster assembly on Isu1 again depends on the electron transfer chain consisting of the ferredoxin reductase Arh1 and (two iron–two sulfur) [2Fe–2S] ferredoxin Yah1, which most likely receives its electrons from NADH. The Isu1-bound, labile Fe/S cluster is then transferred to apoproteins. This whole process is conducted by the dedicated chaperone proteins and monothiol glutaredoxin-5 [3,1].

HuInd1 is a 34.1 kDa protein expressed in cytosol and gets imported inside the mitochondria. A mitochondrial targeting sequence of 51 amino acids is cleaved before it enters mitochondria where it facilitates the correct assembly of the complex-I subunits containing Fe/S cofactors. Hence suggesting that huInd1 donates one or more Fe/S clusters to apo complex-I at the final stage of Fe/S cluster protein maturation [3]. According to this proposal, Ind1 is indeed able to bind a [4Fe–4S] cluster *in vitro* [3] and *in vivo*, in case of the yeast homologue [4]. HuInd1 contains a conserved nucleotide binding domain characteristic for the subclass of P-loop NTPases i.e. the Mrp/NBP35 subclass, and bears a sequence similarity to the cytosolic Nbp35/Cfd1 complex [15]. This complex acts as a scaffold for Fe/S cluster assembly in the cytosol of eukaryotic cells and binds up to three [4Fe–4S] clusters with the help of N- and C-terminal cysteine-rich motifs [16,17]. The C-terminal regions of Cfd1 and Nbp35 have a highly conserved $CX_{18}CPXCX_nC$ motif which binds metal or Fe/S cluster. HuInd1 does not completely conserve this motif, as it preserves a part of it which is the CPXC motif. This twin-cysteine-residue motif is, however, required for the function of huInd1 and is proposed to provide the ligands for a transiently bound [4Fe–4S] cluster probably by dimerization [3].

Despite the obvious importance of huInd1, very little is known about this protein; in particular, its structure as well as its Fe/S cluster binding properties are still unknown, and hence further investigations at the molecular level are required. In the attempt to express as a recombinant protein in *Escherichia coli*, we found that huInd1 expression causes over-expression of the *E. coli* beta-lactamase/ampicillin resistance TEM-1 protein. This result is discussed in the frame work of the Fe/S cluster binding properties of huInd1.

Materials and methods

Cloning of huInd1

Hu*IND1* gene was commercially obtained from Imagenes (vector pCMV-SPORT6, Clone Sequence: BC024919.1). The DNA encoding mitochondrial version of huInd1 was amplified by PCR reaction using specific primers: Sense: CAC CAT GTC CCG AGG ACT TCC and Anti sense: TCA TTC TGA AGG TGA TGG CAA TC. The cloning was performed according to Gateway Technology (Invitrogen) using pENTR/SD/D-TOPO and pENTR/TEV/D-TOPO as donor vectors. Then, the gene was subcloned into the following destination vectors: pDEST14 (native), pDEST17 (His-tag fusion), pDEST MBP (MBP fusion), pDEST20A (Thioredoxin), pDEST30A (GST fusion) and pTH34 (GB1 domain fusion). All protein constructs were His-tagged at the N-terminus for purification purposes. All the pENTR plasmids were sequenced and the positive inserts were selected for further cloning into destination vector. In the case of native hu*IND1* gene, the pDEST14 was sequenced to verify the correct insertion and further used for protein expression.

Protein expression and purification

The expression and solubility tests of native huInd1 and fused with His-tag, MBP, GST, thioredoxin, GB1 domain were performed using *E. coli* strain BL21 (DE3) Gold, BL21 (DE3) pLysS, Rosetta-2 (DE3) and BL21 (DE3) C41. Bacterial cells were grown in absence and in presence of 100 μM $FeCl_3$ at 18 °C, 25 °C and 37 °C for 14–16 h. Cell culture of 2.5 L was induced with addition of 1 mM IPTG (final concentration). Cells were harvested by centrifugation at 6000×*g* and re-suspended in 50 ml of lysis buffer (Tris–HCl 50 mM, 10 mM NaCl, 10% glycerol, pH 7.6). Then cells were disrupted by sonication with a long pulse of 30 s with 50% amplitude (Ultronics) followed by few minutes of gap before another pulse and the procedure was repeated for 10 times. All the following purification steps were performed under anaerobic conditions. In the case of the native protein, the soluble extract, obtained from the cell lysate by ultracentrifugation at 40,000×*g*, was subjected to anion exchange chromatography using Q-column from GE Healthcare. The supernatant was loaded over Q-column, washed with 5 column volume, then 2 column with 20 mM NaCl buffer, followed by elution in 2.5 column volume with 60 mM NaCl added to wash buffer and these fractions were subjected to size exclusion chromatography. 1 L culture in enriched media yielded ~30 mg of protein. For the fused huInd1 constructs, the soluble extract was loaded on a Hi-Trap chelating HP column (GE Healthcare) previously charged with Ni^{+2} (for selective His-tag binding) and equilibrated with the lysis buffer. After washing the column with different imidazole concentrations (10 mM, 20 mM and 40 mM), the recombinant fused protein was eluted with the lysis buffer containing 500 mM imidazole. The tag cleavage was then performed using TEV protease (3 μl TEV/1 mg of protein) in 1× TEV Buffer (50 mM Tris–HCl, pH 8.0, 0.5 mM EDTA, and 1 mM DTT), incubated overnight at room temperature. The protein mixture was finally subjected to a reverse HiTrap chelating HP column to separate the cut protein and was further subjected to size exclusion chromatography.

Trypsin digestion for protein identification by mass spectroscopy In-gel trypsin digestion

The protein identification was accomplished after sequencing the protein by in-gel trypsin digestion [18] followed by mass spectroscopy. The gel bands of interest were cut into small pieces and first washed with distilled H_2O. Then gel pieces containing the protein were shrunk with acetonitrile and later rehydrated by 100 mM ammonium bicarbonate. Repeated cycles of shrinking and rehydration were carried out till the coomassie staining was completely removed. Reduction/alkylation of the protein sample was carried out by adding 40 μl of DTT (Dithiothreitol 0.01 mM) to the 20 μl of protein solution and incubated for 45 min at 56 °C. Then 55 mM iodoacetamide solution was added followed by 30 min incubation in dark, at room temperature. Protein digestion was done by adding 4 μl of trypsin [(12 ng/μl); type IX-S, from porcine pancreas, Sigma Aldrich] in 10 mM ammonium bicarbonate followed by overnight incubation at 37 °C. Digestion was stopped by 10% TFA (Trifluoroacetic acid) and the supernatant was extracted for the mass spectrometric analysis.

Mass spectroscopy

MALDI-MS experiments were performed on Bruker Daltonics Ultraflex III (Bruker Daltonics, Bremen, Germany). A MALDI-TOF/TOF