Expression in *Escherichia coli*, purification and functional activity of recombinant human chaperonin 10

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Abstract We have recently reported the cloning of a cDNA coding for a stress inducible human chaperonin 10. The protein was shown to possess 100% identity with the bovine homologue and a single amino acid replacement (glycine to serine at position 52) compared to rat chaperonin 10. Here we report the heterologous expression of human chaperonin 10 in *Escherichia coli*, its purification and its functional characterization. The recombinant protein was purified to homogeneity as judged by different analytical techniques, and mass spectrometry analysis showed a MW of 10,801 Da in agreement with the predicted sequence. This molecular weight accounts for a protein which is not modified post-translationally. In fact, natural rat chaperonin 10 has been shown to be acetylated at the N-terminus, a feature suggested to be important for targeting and functional activity. Here we show that recombinant human chaperonin 10 is fully active in assisting the chaperonin 60 GroEL in the refolding of denatured yeast enolase, thereby showing that, at least in the present system, post-translational acetylation is not necessary for its activity.

Key words: Chaperonin 10; Human; GroES; GroEL; *E. coli*; Recombinant

1. Introduction

Molecular chaperons are proteins which facilitate correct folding and assembly of nascent polypeptide chains in bacteria, fungi, plants and animals [1,2] Members of this class of proteins are the *Escherichia coli* heat shock proteins GroEL (heat shock protein 60, HSP60) and GroES (HSP10), which have been termed also chaperonins 60 and 10. The GroEL/GroES complex, which assists protein folding in a catalytic process requiring K^+ and Mg-ATP [3], is the most studied chaperonin (cpn) system. In its native form, GroEL forms a double stacked ring, each ring comprising seven ≈60 kDa subunits, while GroES is made up of a single ring of seven ≈ 10 kDa subunits. Both share a high degree of homology with prokaryotic and eukaryotic equivalents, suggesting a general mechanism in promoting protein folding [4].

Thus, GroES homologues have been identified in many prokaryotes, plant chloroplasts and yeast, plant and mammalian mitochondria [5–9].

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Human cpn10 gene corresponds to HSPE1 according to the Guidelines for Human Gene Terminology [Shows et al. (1987) Cytogenet. Cell Genet. 44, 11].

Recently, we have identified and cloned a cDNA encoding the human cpn10 [10]. It showed a 100% homology with the bovine cpn10 [11] and possessed a single amino acid substitution, serine in place of glycine at position 52, compared to rat cpn10 [12]. A recent report has shown that human cpn10 is identical to the early pregnancy factor (EPF) [13]. EPF has been known for long time to be involved in a number of physiological processes which are apparently unrelated to the role of cpn10 in protein folding, but its characterisation has been elusive due to the difficulties encountered in its purification [13]. Here, we report the high-level expression of human cpn10 in *Escherichia coli*. A single-step purification procedure enabled us to purify the protein to homogeneity and to characterise it by mass spectroscopy and N-terminal sequencing. The recombinant human cpn10, which is not acetylated at the N-terminus residue, was fully functional in refolding experiments in vitro ere we employed GroEL as cpn60 and denatured yeast enolase as target protein.

2. Materials and methods

2.1. Materials

Guanidine hydrochloride (GdnHCl) was purchased from Fluka. Enolase, 2-phosphoglyceric acid, nucleotides were obtained from Sigma. GroES and GroEL and restriction enzymes were obtained from Boehringer. All other reagents, at the highest degree of purity, were purchased from Merck and Fluka.

2.2. Cloning of human chaperonin 10 into E. coli expression vector pET-11d

The total RNA extracted from heat shocked human hepatoma cell line HepG2 was amplified by reverse transcription-polimerase chain reaction (RT-PCR) using 21mer 5′ and 18mer 3′ oligonucleotides of rat cpn10 cDNA as primers [12].

In addition the primers included a *Nco*I and a *Bam*HI sites suitable for cloning the PCR product into pET-11d [14]. The complete sequences of the primers are 5′-GCGCGCGGATCCATGGCTGGACAAGCTTTTAGG-3′ and 5′-GCGCGCAAGCTTGG ATCCTCAGTCGACATACTTTCC-3′ for the human cpn10 5′ and 3′ ends, respectively. The 5′ end primer *Nco*I restriction site provided an ATG codon just upstream the GCA triplet that codes for the first residue of the native protein, an alanine. The T7 expression vector was obtained by cloning the *Nco*I and *Bam*HI restriction fragment from the human cpn10 cDNA into pET-11d after digestion with *Nco*I and *Bam*HI. The resulting plasmid coding for human cpn10 was named pET-hucpn10. The sequence analysis of the amplified product cloned into the expression vector was carried out with the Sequenase kit version 2.0 (United States Biochemical Co., Cleveland, OH, USA).

The accession number of human chaperonin 10 is EMBL Ac. No. X75821.

2.3. Expression of human cpn10 in E. coli

Plasmid pET-hucpn10 was transformed into BL21(DE3) pLysS. For expression, cells were grown at 37°C in ZBM9 medium supplemented

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with 50 μg/ml of ampicillin and 34 μg/ml of chloramphenicol to an OD_{600} of 0.6–1. Isopropyl β-D-thiogalactopyranoside was added to 0.4 mM, and cells were shaken for additional 3 h. The cells were harvested by centrifugation, resuspended in lysis buffer, 50 mM Tris-HCl, 2 mM EDTA, pH 8, at a concentration of 20 OD_{600}/ml and stored at −20°C until used.

2.4. Purification of recombinant human cpn10

Frozen cells were thawed at 4°C and sonicated over ice at an amplitude of 18 μm for 1 min intervals with 1 min cooling for a total of 10 min. Cell sonicate was spun in a Sorvall (DuPont) GSA rotor at 5000 rpm for 10 min at 4°C to remove debris and intact cells. The supernatant was centrifuged at 55,000 × *g* in a TST 2817 rotor with a Kontron Centrikon T-1065 for 1 h at 4°C. The resulting pellet was resuspended in 5 M urea, 0.045% trifluoroacetic acid (TFA), centrifuged in a Sorvall GSA rotor at 4000 rpm for 10 min at 4°C and then dialysed against 2% acetonitrile, 0.1% TFA (buffer A) overnight. The sample was centrifuged again as before and filtered through an 0.45 μm filter before chromatographic purification. The human cpn10 was then applied to a Vydac C18 reverse phase HPLC (RP-HPLC) column (10 × 300 mm) equilibrated in buffer A described above. Elution was carried out with a three-step gradient of acetonitrile containing 0.8% TFA: (1) from 0 to 32% in 91 min; (2) from 32 to 50% in 90 min; and (3) from 50 to 80% in 30 min, at flow rate of 2.5 ml/min. Column effluent was monitored at A_{220} nm. The purified recombinant product was analysed by RP-HPLC using an analytical Vydac C18 (4.6 × 150 mm) column using a linear gradient of buffer B from 0 to 55% of buffer B in 55 min.

2.5. Binding of recombinant human cpn10 to GroEL

Gel filtration experiments were performed using a Superose 6 FPLC column (Pharmacia) and the following eluent: 100 mM Tris-HCl, 10 mM KCl, 7 mM $MgSO_4$, pH 7.8 containing 0.25 mM ATP. Prior to injection, samples of GroEL and either GroES or human chaperonin 10 were prepared in the above buffer supplemented with 0.6 mM ATP. Controls were made without addition of ATP. After 15 min incubation at room temperature samples were applied onto the column and peaks corresponding to GroEL and GroEL–cpn complex were collected manually and lyophilised. Lyophilized material was resuspended in 200 μl of water and proteins were precipitated by the addition of 800 μl of cold acetone. After centrifugation precipitates were dissolved in SDS-buffer and analyzed by SDS-PAGE.

2.6. Refolding assays

Yeast enolase refolding assay was performed as previously described [15]. Briefly, yeast enolase (Sigma, 80 units/mg) was denatured in 4 M GdnHCl at room temperature for at least 4 h and then diluted 60 fold into refolding buffer (50 mM Tris-HCl, pH 7.8, containing 10 mM $Mg(CH_3COO)_2$, 20 mM KCl, and 2 mM dithiothreitol, either with or without ADP 4 mM). GroEL, GroES and human chaperonin 10 where added to the refolding buffer where stated. Refolding reaction were carried out at 25°C.

The refolding mixture was diluted 25-fold into the assay buffer (50 mM Tris-HCl, pH 7.8, containing 1 mM 2-phosphoglyceric acid and 1 mM $MgCl_2$), and enolase activity was assayed at 240 nm monitoring the formation of phosphoenolpyruvate.

2.7. Additional analytical techniques

BCA protein assay reagent (Pierce) was utilised to determine protein content [16]. Sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) was performed with a 8–25 polyacrylamide gradient gel using a Phast System instrument (Pharmacia) according to the Laemmli method [17]. Protein sequencing was performed by automated Edman degradation using an Applied Biosystem 475A instrument. Electron Spray Ionization-Mass Spectrometry (ESI-MS) was performed on a Finnigan MAT model 700 instrument.

3. Results

RT-PCR amplification of total RNA from human hepatoma cell line HepG2, using the primers described in section 2, yielded a cDNA product of ≈300 bp (data not shown). The DNA was digested sequentially with *Nco*I and *Bam*HI and cloned directly into pET-11d plasmid *Nco*I and *Bam*HI restriction sites in order to generate expression plasmid pET-hucpn10. The recombinant plasmid was then inserted into *E. coli* strain BL21(DE3) pLysS for expression.

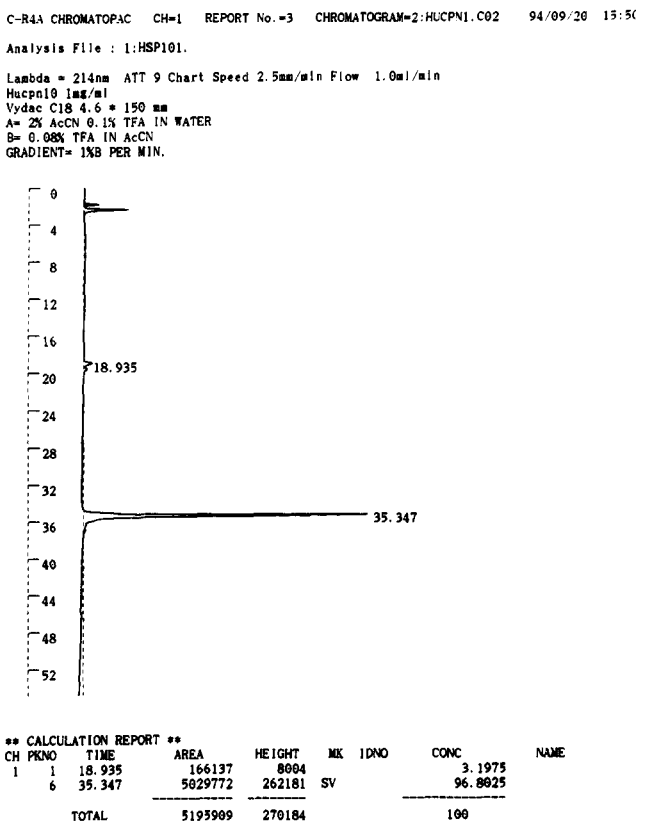

Fig. 1. RP-HPLC analysis of recombinant human cpn10 using a Vydac C18 column to assess purity of the protein (see section 2 for details).

SDS-PAGE analysis of induced total cell lysate indicated the presence of a band of ≈10 kDa present in the recombinant strain, but absent in a non-recombinant one (data not shown). Most of the recombinant protein was found in the pellet obtained after ultra centrifugation indicating that the product was insoluble. Therefore, the pellet was resuspended in 5 M urea containing 0.045% TFA. The resuspended pellet from 1 litre culture was dialysed overnight against 2% acetonitrile, 0.1% TFA and then applied onto a semi-preparative C18 Vydac RP-HPLC column where the recombinant human cpn10 was recovered as a single peak. Fig. 1 shows an analytical RP-HPLC run with an aliquot of purified material, indicating a purity of the recombinant protein of >95%. Moreover, this preparation is not contaminated with detectable levels of GroES which shows a different mobility both in SDS-PAGE and RP-HPLC (data not shown). The estimated yields were about 10 mg of purified protein per litre of culture. Although the recombinant human cpn10 was originally found to be insoluble, after purification the lyophilised protein showed to be fully soluble in water. ESI-MS of the recombinant human cpn10 showed a molecular weight of the recombinant protein of 10801 Da as predicted from its deduced amino acid sequence lacking starting methionine (Fig. 2). N-terminal sequencing of the first 10 residues of the purified protein confirmed the expected sequence for the human cpn10 [10] indicating again that

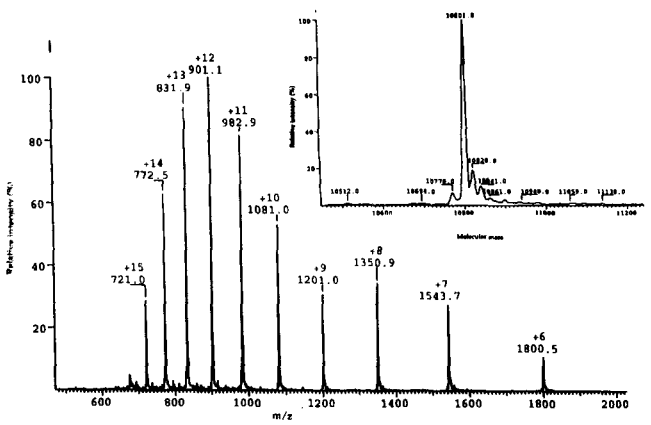

Fig. 2. ESI-MS of recombinant human cpn10, displayed as multiply protonated molecular ions. The inset shows computer reconstruction of the molecular mass.

the initial methionine introduced during the amplification of the cDNA was removed by *E. coli* [18].

The recombinant human cpn10 was then tested for its ability to bind the *E. coli* cpn60 GroEL. Fig. 3 shows a silver-stained SDS-PAGE analysis of peaks obtained after gel filtration experiments. Upon addition of ATP to the incubation buffer the presence of a band corresponding to human cpn10 was detectable analysing the GroEL peak. The band was undetectable omitting the nucleotide in the incubation buffer. We then tested recombinant human cpn10 in the refolding assay of yeast enolase. When yeast enolase is denatured with GdnHCl, its subsequent spontaneous refolding can be arrested by the presence of GroEL. GroES is required for enolase to be released from GroEL in the presence of ADP [15]. In a similar manner, recombinant human cpn10 was capable to effectively release partially folded molecules of yeast enolase from GroEL (Fig. 4). The above results also clearly indicate that the initial denaturation of the recombinant protein and the subsequent RP-HPLC purification did not affect its functionality.

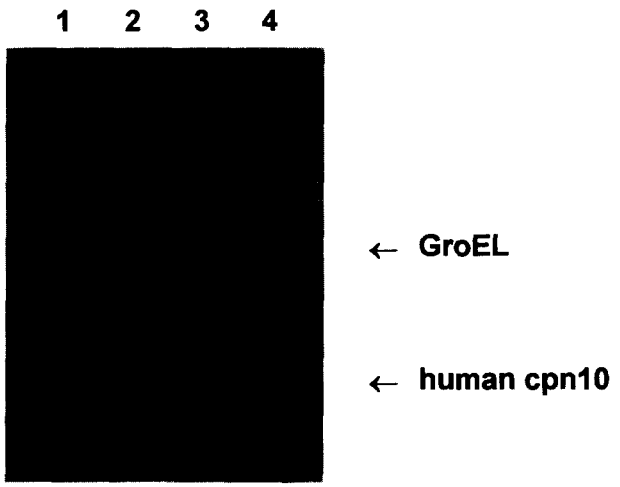

Fig. 3. Silver stained 8–25% SDS-PAGE of GroEL peaks recovered by gel filtration. GroEL was pre incubated with recombinant human cpn10 in the presence or absence of ATP (0.6 mM). Lane 1 shows the GroEL peak obtained without the addition of ATP; lane 2 shows the GroEL peak in the presence of ATP. Lane 3 and 4 show pure recombinant human cpn10 (Hu-cpn10) and GroEL, respectively.

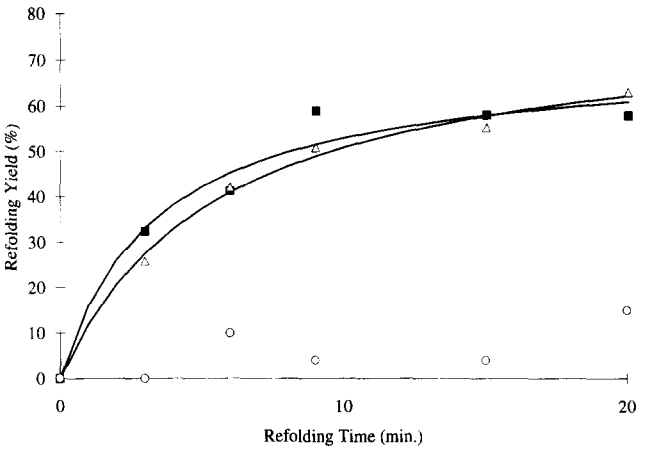

Fig. 4. Refolding profile of enolase in the presence of GroEL, GroES and ADP (open triangles) and in the presence of GroEL, recombinant human cpn10 and ADP (closed squares). The negative control (open circles) is performed in the presence of GroEL and ADP. The refolding was performed at 25°C, as described in section 2.

4. Discussion

Several eukaryotic cpn10 have now been identified and characterised at the molecular level [6–9,19–20]. Common features of these molecules seem to be: (1) lack of any N-terminal presequence necessary for their targeting to the mitochondrial compartment; (2) removal of the starting methionine, followed by N-terminal acetylation of the first amino acid of the mature polypeptide; (3) capacity to bind to GroEL, in the presence of K^+ ions and Mg-ATP; and, consequently (4) the ability to replace GroES in in vitro assisted refolding experiments, using GroEL as cpn60. Conversely, however, GroES was not able to replace eukaryotic cpn10 using mammalian cpn60 [21].

The first three features have been recently confirmed for pure natural human cpn10 [13]. In addition, the complete primary structure of rat cpn10 has been established [22], indicating again modification at the N-terminal amino acid. The authors of this report hypothesize that N-terminal acetylation could be important for the interaction with cpn60.

In the present study we have been able to demonstrate that the recombinant form of human cpn10, lacking N-terminal acetylation could still bind to a cpn60 and assist it in the folding of a denatured protein. Although it must be stressed that we employed an heterologous refolding system using *E. coli* GroEL as cpn60 and yeast enolase as the target for refolding, the present results suggest that N-terminal acetylation is dispensable for the functionality of eukaryotic cpn10, at least as its role in protein refolding is concerned.

These results extend those published in recent reports obtained using a C-terminal modified recombinant mouse cpn10 [23] and a synthetic rat cpn10 [24]. In light of recent findings suggesting that cpn10 are multifunctional molecules one may also speculate that N-terminal acetylation could be relevant in functions other than assisting cpn60 in protein folding. Comparative studies of natural and recombinant cpn10 in those biological systems that have been studied so far only with the natural form [13] may help clarify this issue. The availability of recombinant human cpn10 will facilitate to address these studies.

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