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Over-expression in *E. coli* and purification of the human OCTN1 transport protein

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ABSTRACT

The hOCTN1 amplified from skin fibroblast RNA was cloned in pET-28a(+) or in pH6EX3 plasmid. The encoded recombinant hOCTN1 resulted in a 6-His tagged fusion protein with a 34 or 21 amino acid extra N-terminal sequence in the pET-28a(+)-hOCTN1 or in the pH6EX3-hOCTN1 constructs, respectively. Both constructs were used to express the hOCTN1 in *Escherichia coli* Rosetta(DE3)pLysS. The best over-expression was obtained with the pH6EX3-hOCTN1 after 6 h of induction with IPTG at 28 °C. The expressed protein with an apparent molecular mass of 54 kDa, was collected in the insoluble fraction of the cell lysate. Further improvement was obtained using the *E. coli* RosettaGami2(DE3)pLysS strain to express the protein encoded by pH6EX3-hOCTN1. After 6 h of induction with IPTG at 28 °C, hOCTN1 accounted for 30% of the total protein in the insoluble pellet. This protein fraction was washed with Triton X-100 and deoxycholate, solubilized with a buffer containing 0.8% Sarkosyl, 3 M urea and applied to a Ni^{2+}-chelating chromatography column. The homogeneously purified hOCTN1 was eluted with a buffer containing 50 mM imidazole, 0.1% Triton X-100 and 50 mM 2-mercaptoethanol. A yield of about 3 mg purified protein per liter of cell culture was obtained.

Introduction

Genes coding for membrane proteins represent about one fourth of the protein coding regions of sequenced genomes [1]. Membrane transporters are included in this class of proteins since all of them possess variable number of hydrophobic membrane spanning domains. Transporters are involved in nutrient uptake, elimination of metabolites and regulation of the homeostasis of several compounds which are essential for the life of cells and of whole organisms; thus, defects of the function of transporters are involved in human pathologies [2–7]. The molecular studies of transport systems are hampered by their insolubility in water and are particularly difficult in the case of higher eukaryote transporters which cannot be easily over-expressed in bacteria. These problems limit both the studies of the functional properties of transporters and the determination of the tertiary structures. Among the transporters which share importance in the cell metabolism and involvement in human pathology there is OCTN1 (SLC22A4). Defects of this transporter are associated with the Crohn disease [8]. This transporter belongs to the OCTN protein subfamily which includes three members, namely OCTN1, OCTN2 and OCTN3 [9]. These proteins share more than 66% identity with each other. The main functional properties of the OCTN1 transporter have been studied in intact oocytes or HEK293 cell systems, expressing the transporter in minute amount. In these systems it was found that OCTN1 catalyzed a pH dependent transport of tetraethylammonium (TEA)[1]. The transport was electroneutral and presumably driven by a counterflux of protons which balanced the positive charge of TEA [10]. The transport activity of OCTN1 was inhibited by several pharmacological compounds like cimetidine, quinine, verapamil and others [11]. More recently it was found that the main physiological substrate of OCTN1 is ergothioneine, which is transported in a sodium dependent mode [12]. In contrast, other authors showed that OCTN1 transported carnitine and was localized in the inner mitochondrial membrane [13]. Thus, some properties concerning the function and the biogenesis of this transporter are still controversial. So far, no examples of strategies of large scale over-expression and/or purification of the proteins belonging to the OCTN subfamily have been reported. In this paper the over-expression in *Escherichia coli* and the large scale purification to homogeneity of the human OCTN1 protein are described. This strategy represents the starting point for structural studies and for further functional characterization of the transporter.

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[1] *Abbreviation used:* TEA, tetraethylammonium.

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Materials and methods

Materials

All chemicals and the Anti-His antibody ("Monoclonal Anti-polyHistidine-Peroxidase antibody produced in mouse" A7058) were from Sigma–Aldrich, if not otherwise specified. pET-28a(+) plasmid, *E. coli* Rosetta(DE3) and RosettaGami2(DE3)pLysS strains were purchased from Novagen. Restriction endonucleases, other cloning reagents and prestained molecular mass standard proteins were purchased from Fermentas. pH6EX3 was kindly provided by Prof. M. Barile.

Cloning of cDNA coding for hOCTN1

Total RNA was isolated from primary human fibroblasts and reverse-transcribed. The 1656 bp cDNA corresponding to the encoding sequence for hOCTN1 (GenBank NM_003059) was amplified using the forward and reverse primers 5′-ATGCGGGACTACGACGAGGTGATCG-3′ and 5′-TCAGAATGCAGTTATTAGAACCTTG-3′, respectively. The amplified cDNA sequence was verified. Sequencing was performed three times by ABI 310 automated sequencer Applied Biosystems.

The full length cDNA coding for hOCTN1 was amplified using the forward and reverse primers 5′-GCGGATCCATGCGGGACTACGACGA-3′ and 5′-AAATAAGAATGCGGCCGCTCAGAATGCAGTTATT-3′, containing the BamHI and NotI sites, respectively and cloned in the BamHI/NotI sites of the pET-28a(+) expression vector. The resulting recombinant plasmid, defined as pET-28a(+)-hOCTN1, encoded a fusion protein corresponding to the hOCTN1 carrying the extra N-terminal sequence MGSSHHHHHHSSGLVPRGS HMASMTGGQQMGRGS. Alternatively, the full length cDNA coding for hOCTN1 was amplified using the forward and reverse primers 5′- CCGGAATTCCATGCGGGACTACGACGAGGT-3′ and 5′-CCGCTCGAGTCAGAATGCAGTTATTAGAAC-3′ containing the EcoRI and XhoI sites, respectively and cloned in the EcoRI/XhoI sites of the pH6EX3 expression vector. The resulting recombinant plasmid, defined as pH6EX3-hOCTN1, encodes a fusion protein corresponding to the hOCTN1 carrying the extra N-terminal sequence MSPIHH HHHHLVPRGSEASNS.

Expression of recombinant hOCTN1 protein in E. coli

To obtain the recombinant hOCTN1 protein, the expression host *E. coli* Rosetta(DE3) pLysS was transformed with the pET-28a(+)-hOCTN1 plasmid by calcium chloride treatment. Selection of transformed colonies was performed on LB-agar plates containing 30 μg/mL kanamycin and 34 μg/mL chloramphenicol. Cells carrying the recombinant plasmid were inoculated in 100 mL of LB medium (1% Bacto peptone, 0.5% Bacto yeast extract, 1% NaCl, pH 7.0) supplemented with 30 μg/mL kanamycin and 34 μg/mL chloramphenicol, and cultured overnight at 37 °C with rotary shaking (∼200 rpm). Fifty milliliters portion of the cell culture was transferred to 0.5 L of fresh LB medium supplemented with 30 μg/mL kanamycin and 34 μg/mL chloramphenicol and in the middle of the logarithmic phase (0.5–0.7 O.D. at 600 nm wavelength), 0.4 mM IPTG (except when differently specified) was added to induce the expression of the recombinant protein. Alternatively, the expression host *E. coli* Rosetta(DE3) pLysS or *E. coli* RosettaGami2(DE3) pLysS was transformed with the pH6EX3-hOCTN1 plasmid by calcium chloride treatment. Selection of transformed colonies was performed on LB-agar plates containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol and, only in the case of *E. coli* RosettaGami2(DE3) pLysS, 12.5 μg/mL tetracycline. Cells carrying the recombinant plasmid were inoculated in 100 mL of LB medium (1% Bacto peptone, 0.5% Bacto yeast extract, 1% NaCl, pH 7.0) supplemented with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol (and, in the case of *E. coli* RosettaGami2(DE3)pLysS, 12.5 μg/mL tetracycline), and cultured overnight at 37 °C with rotary shaking (∼200 rpm). Fifty milliliters portion of the cell culture was transferred to 0.5 L of fresh LB medium supplemented with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol (and, in the case of *E. coli* Rosettagami2(DE3) pLysS, 12.5 μg/mL tetracycline) and, in the middle of the logarithmic phase, 0.4 mM IPTG (except when differently specified) was added to induce the expression of the recombinant protein. In all the cases, after the induction two portions of 0.25 L each of cells were grown at 28 and 37 °C, respectively. Every 2 h, fractions of 50 mL were harvested by centrifugation at 3000*g* for 10 min at 4 °C and the pellet stored at −20 °C. The bacterial pellet (about 0.4 g wet weight) was thawed on ice for 15 min and resuspended in 5 mL 50 mM Hepes/Tris pH 7.5 supplemented with 20 μL of protease inhibitor cocktail (P8849, Sigma–Aldrich) and 0.5 mM PMSF. Cells were disrupted by mild sonication at 4 °C (10 min in pulses of 1 s sonication, 1 s intermission). The soluble and the insoluble cell fractions were separated by centrifugation of the cell lysate at 12,000*g* for 30 min at 4 °C. The protein patterns of the cell lysate fractions were analyzed by SDS–PAGE.

Purification of recombinant hOCTN1

The insoluble fraction obtained as described above, was washed twice with 50 mM Tris/HCl pH 7.5, 3% Triton X-100 and centrifuged at 12,000*g* for 10 min at 4 °C; the pellet was resuspended with 50 mM Tris/HCl pH 7.5, 3% deoxycholate and centrifuged again at 12,000*g* for 10 min at 4 °C. The resulting pellet was washed with 100 mM Tris/HCl pH 8.0 and centrifuged at 12,000*g* for 10 min at 4 °C. The resulting pellet was solubilized with a buffer containing 3 M urea, 0.8% Sarkosyl, 200 mM NaCl, 10 mM Tris/HCl pH 8.0 for 5 min at 25 °C and centrifuged at 12,000*g* for 10 min at 4 °C. One milliliters of the supernatant was applied onto a column filled with His-select Ni-Chelating affinity gel (0.5 cm diameter, 3 cm height) pre-conditioned with 10 mL of a buffer containing 0.1% Sarkosyl, 200 mM NaCl, 10 mM Tris/HCl pH 8.0. The elution was performed with 25 mL of a buffer containing 0.1% Triton X-100, 200 mM NaCl, 10 mM Tris/HCl pH 8.0, 50 mM 2-mercaptoethanol and then 6 mL of the same buffer containing 10 mM imidazole and then 6 mL of the same buffer containing 50 mM imidazole. Forty eight fractions of 1 mL were collected. The purified protein was eluted in the fractions 43–45.

Other methods

Protein concentration was measured by the method of Lowry, modified for the presence of detergents [14]. Proteins were separated by SDS–PAGE on 12% polyacrylamide gels performed according to Laemmli [15], using the Hoefer SE260 mini-vertical unit and stained by Coomassie-brilliant blue.

Quantitative evaluation of Coomassie-stained protein bands was carried out using the Chemidoc imaging system equipped with Quantity One software (Bio-Rad). The theoretical molecular mass of protein was determined by the pI/pMW tool at the web site http://www.expasy.org.

Results

The 1656 bp hOCTN1 cDNA was amplified from total human skin fibroblast RNA using forward and reverse primers constructed on the 5′ and 3′ ends of the hOCTN1 cDNA (GenBank NM_003059). The amplified cDNA showed an electrophoretic mobility on aga-