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Cell-free production and characterisation of human uncoupling protein 1–3

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ABSTRACT

The uncoupling proteins (UCPs) leak protons across the inner mitochondrial membrane, thus uncoupling the proton gradient from ATP synthesis. The main known physiological role for this is heat generation by UCP1 in brown adipose tissue. However, UCPs are also believed to be important for protection against reactive oxygen species, fine-tuning of metabolism and have been suggested to be involved in disease states such as obesity, diabetes and cancer.

Structural studies of UCPs have long been hampered by difficulties in sample preparation with neither expression in yeast nor refolding from inclusion bodies in *E. coli* yielding sufficient amounts of pure and stable protein. In this study, we have developed a protocol for cell-free expression of human UCP1, 2 and 3, resulting in 1 mg pure protein per 20 mL of expression media. Lauric acid, a natural UCP ligand, significantly improved protein thermal stability and was therefore added during purification. Secondary structure characterisation using circular dichroism spectroscopy revealed the proteins to consist of mostly α-helices, as expected. All three UCPs were able to bind GDP, a well-known physiological inhibitor, as shown by the Fluorescence Resonance Energy Transfer (FRET) technique, suggesting that the proteins are in a natively folded state.

1. Introduction

One of the major bottlenecks in structural studies of eukaryotic membrane proteins is obtaining sufficient amounts of protein material. Overexpression of membrane proteins often leads to cytotoxicity problems due to overloading of the translocon machinery or to excessive aggregation in the form of inclusion bodies [1–3]. Cell-free expression systems, in which a DNA template is added to a test tube containing reconstituted transcription and translation machinery, offer the opportunity to create a hydrophobic environment in the absence of cellular membranes. This allows for the soluble expression of membrane proteins while overcoming problems with translocon overload, membrane targeting and insertion into limited membrane volume, as well as toxic or inhibitory effects on the overproduction host cell [4]. Cell-free expression offers a fast way of screening for favourable expression conditions and to easily adapt these to suit the protein of interest. This is particularly useful for eukaryotic membrane proteins for which it is often necessary to optimise expression conditions in a protein-specific manner. During the last few years, a wide range of eukaryotic membrane proteins has been expressed, properly folded, using cell-free systems in sufficient amount for functional and structural studies [5–9]. It thus seems that cell-free expression is a plausible alternative for overproduction of eukaryotic membrane proteins.

Uncoupling proteins (UCPs) are integral membrane proteins found in the inner mitochondrial membrane, where they transport protons from the intermembrane space to the matrix. This proton leak activity dissipates the proton gradient formed by the electron transport chain, thus uncoupling the oxidation of fuels from ATP synthesis [10]. UCPs were first discovered in mitochondria from brown adipose tissue (BAT), where proton transport through UCP1 generates heat, enabling non-shivering thermogenesis [11]. Since then, four additional human homologues, UCP2-5, have been identified in mitochondria from various tissues [10,12–16]. In contrast to UCP1, the primary physiological function of these is not fully known, but they are believed to be involved in protection against reactive oxygen species and/or the regulation of ADP/ATP ratio [12,13,17]. As such, UCPs have been suggested to play a role in a number of human disease states, including obesity [18,19], diabetes [10,20] and cancer [21–23].

UCPs belong to the superfamily of mitochondrial solute carriers and share their common fold of approximately 300 amino acids forming six transmembrane helices [24]. Their proton transport activity is activated by long chain fatty acids, while purine nucleotides have an inhibitory effect [10]. The exact proton transport mechanism, and how this is regulated by fatty acids and nucleotides, is still under debate with several mechanisms having been proposed [11,25–30]. In order to allow for the controversies around UCP function to be resolved, high-

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resolution structural knowledge is crucial. However, heterologous overproduction of UCPs has proven difficult, with refolding from inclusion bodies in *Escherichia coli* [25,31–35] and expression in the yeast *Saccharomyces cerevisiae* [36–44] both failing to produce pure and stable in sufficient amounts for structural studies.

Recent research into the use of cell-free expression systems for UCP overproduction has identified it as a suitable alternative to conventional recombinant expression hosts. Rat UCP1 has been successfully expressed in a cell-free system in the presence of a fluorinated surfactant [45] and initial cell-free expression has also been demonstrated for several UCPs (human UCP1–3, mouse UCP1–2, rat UCP2) in a cell-free expression screen of 38 different membrane protein targets [46]. Here we present an optimised protocol for cell-free expression of human UCP1, 2 and 3 (hUCP1–3) at levels suitable for structural studies. We further show that addition of lauric acid, a physiological UCP-ligand, increases protein stability, wherefore this compound was added during purification. Characterisation using a Fluorescence Resonance Energy Transfer (FRET) based nucleotide binding assay together with secondary structure characterisation using CD spectroscopy indicate that all three proteins are properly folded, paving the way for further structural and functional characterisation.

2. Material and methods

2.1. Cloning and initial cell-free protein expression

Genes coding for complete hUCP1–3 were cloned into the pEXP5-NT plasmid (Invitrogen) [46]. All constructs contained an N-terminal hexahistidine tag followed by a TEV protease cleavage site. Proteins were expressed in cell-free batch mode using S12 *Escherichia coli* extract and 1% w/v final concentration of Brij58 to directly solubilise the expressed protein, as previously described [46,47]. Expression mixtures, with a maximum volume of 15 mL, were incubated for 4 h at 30 °C with 750 rpm shaking.

2.2. Small scale expression of hUCP1 with additives

hUCP1 were expressed in 200 µL cell-free mixture as described above with the addition of 0.5 mM lauric acid, coenzyme Q_{10} or digitonin. At the end of the expression, media were centrifuged for 10 min at 7500*g* and 3 drops of 1 µL for each cell-free mixture were deposited on a nitrocellulose membrane. After incubation at 4 °C overnight in 100 mM phosphate buffer at pH 7.5 containing 0.2% v/v Tween-20% and 5% w/v dried milk, the membrane was incubated for 2 h at room temperature with a mouse anti-6xHis primary antibody followed by washing and a second incubation for 1 h with the secondary anti-mouse antibody coupled to horseradish peroxidase. Expression levels were determined by enhanced chemiluminescence detection using Luminol as the horseradish peroxidase substrate. The intensity of the signal was quantified using the Multi Gauge software (Fujifilm). The experiment was then repeated with the combination of lauric acid and coenzyme Q_{10}. Data were normalised against the expression of hUCP1 without additive, which was set to 100%. The data were explored for normality, where a one-sample Kolmogorov-Smirnov test was used to determine goodness of fit to a standard normal distribution. The test was found to be non-significant, thus the null hypothesis was accepted, i.e. that the population was normally distributed. Hence, parametric statistical tests were conducted to assess significant effects in the data. A one-way analysis of variance (ANOVA) test found a significant main effect ($F(4,14) = 12.06$, $p < 0.001$). Post-hoc tests were conducted to compare the expression level with the control. Post-hoc multiple-comparisons were computed (corrected using false discovery rate) and significant differences were found between the control and expression with lauric acid ($p = 0.037$), Coenzyme Q10 ($p = 0.009$), and lauric acid + Coenzyme Q10 ($p < 0.001$).

2.3. Purification of hUCP1 for thermostability assay

10 mL of cell free reaction mixture expressing hUCP1 was loaded on a 5 mL HisTrap column (GE healthcare) previously equilibrated with 20 mM Tris pH 8, 20 mM imidazole, 100 mM NaCl and 1% (w/v) Brij 58. The protein was eluted with a linear gradient of imidazole (20–500 mM). Fractions containing hUCP1 were concentrated using a Vivaspin concentrator with a MW cutoff of 50 kDa, after which the sample was loaded on a Superdex 200 10/300 (GE healthcare) previously equilibrated with 20 mM imidazole pH 8, 100 mM NaCl, 2 mM EDTA and 0.1% w/v C12E9. After SDS-PAGE analysis, fractions containing hUCP1 were pooled and concentrated to 1 mL using a Vivaspin concentrator (MW cutoff 50 kDa).

2.4. Thermostability assay for hUCP1

In order to investigate the protein stability, we adopted the fluorescent thermostability assay presented by Alexandrov et al. [48]. CPM dye (7-Diethylamino-3-(4-maleimidophenyl)-4-methylcoumarin) was purchased from Sigma-Aldrich, dissolved in DMSO to a final concentration of 4 mg mL^{-1} and stored at −80 °C. Prior to use, the CPM stock solution was diluted to 100 µM in gel filtration buffer and incubated at room temperature for 5 min while kept away from light. For the thermostability measurements, 10 µL of hUCP1 at 0.8 mg mL^{-1} was mixed with 35 µL buffer with or without 50 mM lauric acid and 5 µL CPM in a 96-well plate. The plate was read in a BioRad iCycler RT-PCR machine while being exposed to a ramp of temperature from 4 °C to 95 °C at 1 °C per minute. The excitation and emission wavelengths were set at 380 and 470 nm respectively. All samples were measured in triplicate. Data were processed using GraphPad (GraphPad Software Inc.). The melting temperature, T_m was determined by fitting the raw data to a Boltzmann sigmoidal function.

2.5. Optimised cell-free expression and purification of hUCP1–3

hUCP1–3 were expressed in 15 mL of cell-free expression media as above with the addition of 0.5 mM lauric acid, 0.5 mM Coenzyme Q_{10} and 7.5 mM cardiolipin. After incubation, the cell-free expression mixture was diluted two-fold with 100 mM Tris pH 8, 1 M NaCl, 80 mM imidazole, 10% v/v glycerol, 1.6% w/v C12E9 and 0.2 mM lauric acid. Following centrifugation (30 min at 117,000*g*) the sample was loaded on a 5 mL HisTrap column (GE healthcare) previously equilibrated with 0.5X dilution buffer. The protein was eluted with a 25 mL linear imidazole gradient (40–500 mM) at flow rate of 5 mL min^{-1}. UCP-containing fractions were pooled and concentrated to 1 mL using a Vivaspin 20 concentrator (MWCO 50 kDa) before being loaded on a Superdex 200 10/300 (GE healthcare) equilibrated with 10 mM Tris pH 8, 200 mM NaCl, 5% v/v glycerol, 0.4% w/v C12E9 and 0.1 mM lauric acid. Fractions were analysed using SDS-PAGE, pooled and concentrated as previously to a final concentration of 0.2 mg mL^{-1}.

2.6. CD spectroscopy

In order to minimise absorption of the buffer in the far UV-range, the protein buffer was exchanged by dialysis against 20 mM phosphate pH 8, 0.2% w/v C12E9 and 0.1 mM lauric acid. 20 CD spectra were recorded for each protein as well as buffer only on a ChiraScan circular dichroism spectrometer (Applied Photophysics) from 280 to 195 nm in 1 nm steps. The measurements were carried out in a 0.1 cm path-length quartz cuvette. The proteins were concentrated to 1.4 µM, 2.1 µM and 1.5 µM for hUCP1, hUCP2 and hUCP3 respectively. Reported spectra correspond to merged spectra after buffer subtraction.

2.7. GDP binding

The FRET technique was used to measure the binding of methylan-