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

Etienne Rebuffet[a], Anna Frick[a], Michael Järvå[a], Susanna Törnroth-Horsefield[a,b,*]

[a] Department of Chemistry and Molecular Biology, University of Gothenburg, Box 462, SE-405 30 Gothenburg, Sweden
[b] Department of Biochemistry and Structural Biology, Centre for Molecular Protein Science, Lund University, Box 124, 221 00 Lund, Sweden

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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-

* Corresponding author at: Department of Biochemistry and Structural Biology, Centre for Molecular Protein Science, Lund University, Box 124, 221 00 Lund, Sweden.
E-mail address: susanna.horsefield@biochemistry.lu.se (S. Törnroth-Horsefield).

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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-

traniloyl guanidine di-phosphate (Mant-GDP) to hUCP1–3. Protein tryptophan residues (λ_{ex} = 280 nm, λ_{em} = 350 nm) were used as resonance energy transfer donor and Mant-GDP (λ_{ex} = 355 nm, λ_{em} = 448 nm) was the acceptor. A SPEX fluorolog 2 (HORIBA Jobin Yvon) was used to record emission spectra between 300 and 500 nm (λ_{ex} = 280 nm) of 1 mL freshly purified hUCP in a quartz cuvette containing a magnetic stirrer. The molar ratio hUCP/Mant-GDP was kept constant at 0.75 for all measurements. The normalised fluorescence resulting from energy transfer was calculated as: ((Protein + Mant-GDP) – Protein – Free Mant-GDP)/[Protein].

3. Results and discussion

3.1. Initial cell-free expression of hUCP1–3

As an initial test, 300 µL of cell-free expression reactions were set up for human UCP1–3 using previously published conditions [46]. During expression, Brij58 was added to a final concentration of 1% w/v to directly solubilise the proteins. The expression was evaluated using Western blot with an antibody directed against the His-tag. Clear bands could be seen at the correct mass, 33.4, 35.6 and 36.6 kDa for human UCP1–3 respectively (Fig. 1).

3.2. Optimisation of cell-free expression of hUCP1

To investigate whether the presence of compounds that naturally occurs in the inner mitochondrial membrane and have been suggested to interact with UCPs could have a positive effect on cell-free expression of UCP1, we added lauric acid and coenzyme Q_{10} to the expression media [49,50]. We also tested the effect of adding digitonin, a mild detergent that has been used previously to solubilise UCP [32,33,50]. UCP expression levels were estimated from Western dot blots using an antibody directed against the hexa-histidine tag (Fig. 2A).

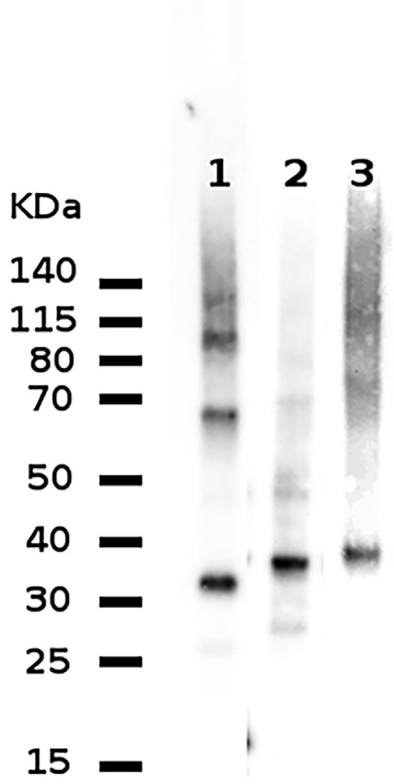

Fig. 1. Initial cell-free expression of human UCP1–3. Western blot showing initial expression of hUCP1–3 in 300 µL of cell-free reaction using previously published conditions [46]. For each reaction 5 µL were loaded. Lanes 1, 2 and 3 corresponds to hUCP1, 2 and 3 respectively.

The addition of lauric acid or coenzyme Q_{10} individually resulted in an increase in hUCP1 expression by 10%. Addition of both lauric acid and coenzyme Q_{10} simultaneously had an additive effect, resulting in a 25% increase in expression yield compared to when no additive was added. In contrast to lauric acid and coenzyme Q_{10}, no effect was observed upon addition of digitonin. Based on these results, lauric acid and coenzyme Q_{10} (0.5 mM respectively) but not digitonin was included in the optimised cell-free expression conditions.

The effect of lauric acid is particularly interesting as fatty acids are known to bind UCP in the mitochondria resulting in activation of proton transport [10]. Coenzyme Q_{10} was suggested to bind and regulate UCPs [33,50] however this has since then been refuted [31,51], indicating that the positive effect of coenzyme Q_{10} on hUCP1 expression seen in our experiment may not necessarily result from direct hUCP interaction.

The final cell-free expression media also contains cardiolipin (7.5 mM), a lipid present in relatively high amounts in mitochondrial membrane. Cardiolipin has previously been shown to have a positive effect on UCP expression and stability and was shown to increase the stability of ovine UCP1 [52] as well the solubility of cell-free expressed rat UCP1 [45]. This effect was not further tested in this work.

3.3. Thermal stability of hUCP1

hUCP1 from 10 mL of cell-free expression reaction was purified using Ni-affinity chromatography and gel filtration and subjected to thermostability assay based on the thiol-specific fluorophore 7-Diethylamino-3-(4-maleimidophenyl)-4-methylcoumarin (CPM) [48]. In this assay, the protein is subjected to ramp in temperature whereby an increase in fluorescence is seen as the protein unfolds and CPM binds to cysteine residues that have been previously buried inside the protein, resulting in a melting curve. From this curve the melting temperature, T_m, can be determined, which gives an indication of protein stability.

Since lauric acid is well-known to bind directly to UCP and enhanced the cell-free expression of hUCP1 as described above, we tested if lauric acid has a positive effect hUCP1 stability in solution. As seen in Fig. 2B, addition of lauric acid dramatically increased hUCP1 stability increasing the T_m from 36.3 °C to 63.1 °C. It has been shown previously that fatty acids induce a conformational change of UCP1 in the brown adipose tissue mitochondrial membrane [26]. It may well be that this conformation of hUCP1 confers increased stability when the protein is in a detergent-solubilised state. Based on these positive results, lauric acid was added to all purification buffers.

3.4. Expression and purification of hUCP1–3

Assuming that conditions that favour hUCP1 stability would also be beneficial for cell-free expression and purification of its homologues hUCP2 and hUCP3, we set up 20 mL cell-free expression reactions of hUCP1–3 according to the optimised expression protocol described above. The reactions were left to incubate for 4 h. The proteins were purified in a two-step manner using Ni-affinity chromatography followed by gel filtration, resulting in a typical yield of 1 mg of pure protein per 20 mL cell-free reaction. Protein purity was analysed using SDS-PAGE (Fig. 3A). In addition to the expected band for the UCP monomer around 30–35 kDa, an additional band is observed around 65 kDa, possibly corresponding to UCP-dimers, a form that previously has been suggested to exist in the mitochondrial membrane [53]. A band at the same position is also seen in Western blot stained with antibodies against the His-tag (Fig. 1), further supporting this. In our experience, the appearance of oligomeric states on SDS-PAGE gels is common phenomenon for α-helical membrane proteins, a phenomenon that has also been described by others [54,55].

Although coenzyme Q_{10} showed a positive effect on hUCP1 expression, its direct binding to UCPs, initially suggested by Echtay et al. [33,50] is controversial and has since then been disproved by two

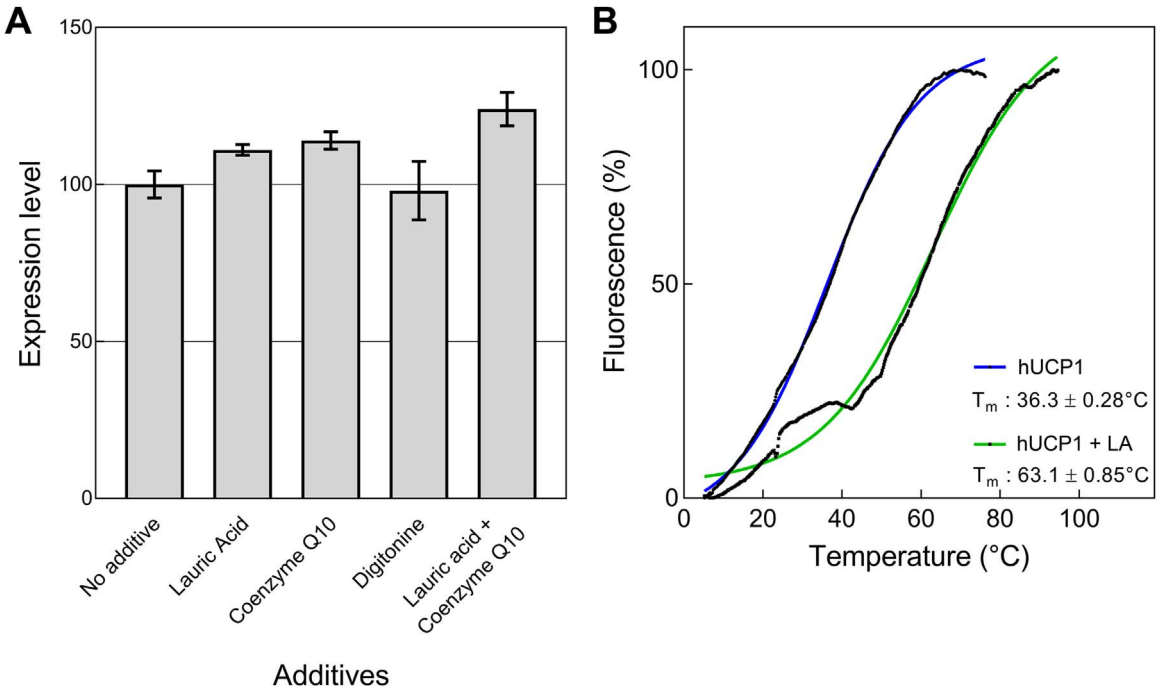

Fig. 2. Expression and stability of hUCP1 in the presence of different compounds. A) Bar chart displaying expression levels of hUCP1 without additives, in the presence of Coenzyme Q_{10}, lauric acid, digitonin and in the presence of lauric acid and Coenzyme Q_{10}. Expression levels were determined from Western dot blots using chemiluminescence detection. **B**) Thermostability of hUCP1 alone (control, blue) and in presence of lauric acid (green). Relative fluorescence where 100% equals the maximum fluorescence in each experiment is plotted against the temperature. T_m is calculated as the mid-point of the curve fitted to a Boltzmann sigmoidal equation.

independent studies [31,51]. In light of this, we decided to only add lauric acid, for which direct interaction is well established, and which had a dramatic effect on hUCP1 thermal stability, to the purification buffers. Moreover, since our optimised protocol yielded sufficient amounts of stable protein, we did not evaluate the effect of cardiolipin during purification. Nevertheless, this could potentially be interesting and we may look into it in future experiments.

3.5. Secondary structure characterisation of hUCP1–3

To further study the quality of purified hUCP1–3, we characterised their secondary structure using circular dichroism spectroscopy. CD spectra were recorded between 195 and 280 nm, revealing negative peaks at 208 and 222 nm respectively (Fig. 3B), typically corresponding to α-helices. The spectra correspond well to previously published CD-spectra for UCPs [32,35,45] and confirm that cell-free expressed hUCP1–3 contain mainly α-helices as expected.

Whereas the CD spectra of hUCP2 and hUCP3 are very similar, the hUCP1 spectrum shows some dissimilarity at lower wavelengths. In particular, the intensity of the negative peak at 208 nm is lower than for hUCP2 and hUCP3, suggesting that hUCP1 could be structurally different. Indeed, hUCP2 and hUCP3 are very similar on the amino acid level, sharing 73% sequence identity. In contrast hUCP1 has a lower sequence identity, sharing only 60% of its sequence with hUCP2 and hUCP3, which could explain the structural differences suggested by the CD spectra measured in this study.

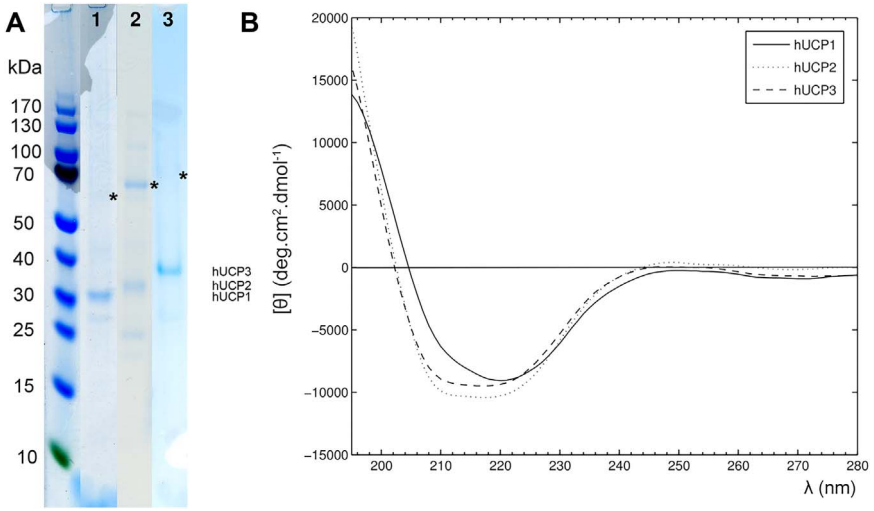

Fig. 3. Characterisation of purified hUCP1–3 A: SDS-PAGE showing typical fractions after gel filtration for hUCP1, hUCP2 and hUCP3 in lane 1–3 respectively. The expected monomeric size for each protein is highlighted with an arrow. A higher molecular weight band, possibly corresponding to a dimeric form is indicated with an asterisk (*). **B:** CD spectra of cell-free expressed hUCP1–3 in C12E9 micelles and 20 mM phosphate buffer at pH 8.

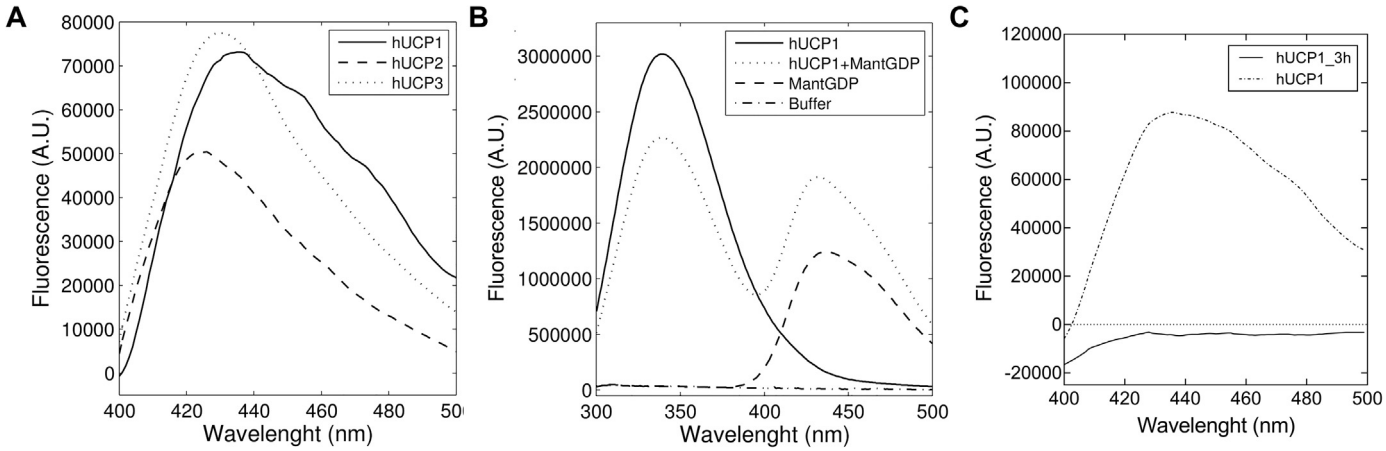

Fig. 4. FRET measurement of Mant-GDP binding to hUCP1–3. A: Normalised fluorescence resulting from energy transferred from hUCP1–3 to Mant-GDP. Fluorescence peaks can be observed at 435 nm, 426 nm, and 430 nm for hUCP1, hUCP2 and hUCP3 respectively. **B:** Emission spectra between 300 and 500 nm for hUCP1, Mant-GDP, buffer and hUCP1 + Mant-GDP. **C:** FRET signal for hUCP1/Mant-GDP at the beginning of the experiment and after 3 h, showing how the peak at 435 nm is lost.

3.6. GDP binding to hUCP1–3

Since purine nucleotides are known physiological inhibitors of UCPs [10], we investigated the ability of cell-free expressed hUCP1–3 to bind GDP using Fluorescence Energy Resonance Transfer (FRET). In this technique, the ligand becomes fluorescent only when energy is transferred from an excited state of the protein *via* protein-ligand bonds.

Human UCPs were mixed with the fluorescent GDP-analogue methylantraniloyl guanidine diphosphate (Mant-GDP) at a molar ratio of 0.75 and emission spectra were recorded between 300 and 500 nm. Protein tryptophans ($\lambda_{ex} = 280$ nm, $\lambda_{em} = 350$ nm) were used as resonance energy transfer donor and Mant-GDP ($\lambda_{ex} = 355$ nm, $\lambda_{em} = 448$ nm) was the acceptor. The experiment was carried out at pH 8 which is close to the pH in the mitochondrial matrix [56], where the purine nucleotide binding site is located *in vivo*. Fluorescence resulting from FRET could be measured for all three human UCPs with fluorescence peaks at 435 nm, 426 nm, and 430 nm for hUCP1, hUCP2 and hUCP3 respectively (Fig. 4A). The fluorescence from Mant-GDP in buffer without protein was significantly lower (peak at 438 nm) showing that resonance energy is only transferred when protein is present (Fig. 4B). It was further noted that when the FRET experiment was repeated multiple times with the same protein during several hours, the FRET signal was eventually lost (Fig. 4C). We interpret this as the protein becoming denatured over time due to heat build-up, supporting that the observed FRET signal is not due to unspecific binding.

The UCP nucleotide-binding site consists of three arginine residues well separated in sequence and residing on three different transmembrane helices [41]. It seems reasonable to assume that in order for this binding site to be formed, the entire protein needs to be correctly folded. Furthermore, the fluorescence of Mant-nucleotides is known to increase with the hydrophobicity of the environment, resulting in a blue-shift of the emission spectra maximum of approximately 10 nm [57]. This blue-shift is observed in our experiments (Fig. 4B), indicating that when the protein is present, the Mant-group is surrounded by a non-polar environment [57,58]. Thus, our results indicate that human UCP1–3 binds nucleotides at a site that is shielded from the polar environment rather than at the surface.

4. Conclusion

We present a fast, cheap and efficient way of producing human UCP1–3 using cell-free expression. Although cell free expression has been demonstrated for UCPs previously [45,46], this is the first time human UCPs has been produced at levels compatible with structural and functional studies. Our characterisation using CD spectroscopy and FRET suggest that the proteins are in a correctly folded state, although further structural and functional characterisation is needed to fully confirm this. This is an important step towards a deeper understanding of the structure-function relationships of these metabolically and medically important proteins.

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Appendix. Transparency document. Supporting information

Transparency data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2017.04.003.

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