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Production of UCP1 a membrane protein from the inner mitochondrial membrane using the cell free expression system in the presence of a fluorinated surfactant

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ABSTRACT

Structural studies of membrane protein are still challenging due to several severe bottlenecks, the first being the overproduction of well-folded proteins. Several expression systems are often explored in parallel to fulfil this task, or alternately prokaryotic analogues are considered. Although, mitochondrial carriers play key roles in several metabolic pathways, only the structure of the ADP/ATP carrier purified from bovine heart mitochondria was determined so far. More generally, characterisations at the molecular level are restricted to ADP/ATP carrier or the uncoupling protein UCP1, another member of the mitochondrial carrier family, which is abundant in brown adipose tissues. Indeed, mitochondrial carriers have no prokaryotic homologues and very few efficient expression systems were described so far for these proteins. We succeeded in producing UCP1 using a cell free expression system based on *E. coli* extracts, in quantities that are compatible with structural approaches. The protein was synthesised in the presence of a fluorinated surfactant, which maintains the protein in a soluble form. Further biochemical and biophysical analysis such as size exclusion chromatography, circular dichroism and thermal stability, of the purified protein showed that the protein is non-aggregated, monodisperse and well-folded.

1. Introduction

UCP1 (uncoupling protein 1) belongs to the mitochondrial carrier family (MCF), which comprises more than 40 integral membrane proteins transporting metabolites across the inner mitochondrial membrane [1]. This transport is linked to major metabolic pathways occurring in mitochondria, which are essential to eukaryotic metabolism. Because of their functional importance, impairment of such carriers leads to severe diseases (for review see [2]). MCF members share several characteristics: (a) they are encoded by nuclear genes, (b) they have a molecular mass of 30–35 kDa, (c) six transmembrane helices are predicted from their sequence, (d) their sequence displays three repeated regions of about 100 amino acids each, and (e) strongly conserved amino acids forming a characteristic signature for these carriers, the MCF motif, are found in each repeated region. Despite of these common features each of them is highly specific to the transport of one metabolite [3]. The ADP/ATP carrier (AAC) is the most studied MCF carrier and is the first for which a high-resolution structure was solved [4]. From the location of MCF motifs in the structure, it is predicted that all MCF members have a similar folding. However, the specific recognition mode of the substrates as well as the conformation changes occurring during the transport, still are elusive. UCP1 is the second most studied MCF member (for review see [5]). By inducing a proton leak across the inner mitochondrial membrane, it is responsible for the dissipation of the proton gradient [6,7], and the uncoupling of oxidative phosphorylation from ATP synthesis. UCP1 is expressed in the brown adipose tissue (BAT) and because of the respiration uncoupling, fat stored into brown adipocytes converts into heat. UCP1 is a key component of non-shivering thermogenesis and several reports suggest its role in the protection against diet-induced obesity [8–10].

All the efforts to characterise biochemically and structurally MCF carriers are hindered by the difficulty in producing sufficient amounts of high-quality samples, known to be a major bottleneck for

Abbreviations: UCP1, uncoupling protein 1; MCF, mitochondrial carrier family; AAC, ADP/ATP carrier; BAT, brown adipose tissues; cmc, critical micellar concentration; CD, circular dichroism; RTS, Rapid Translation System

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membrane proteins. As predicted from the known AAC structure, MCF carriers consist in 6 transmembrane helices partially interacting with the lipid bilayer and partially accessible to the solvent, explaining their high lability. This topology was confirmed with the recent UCP2 structure [11]. Most of the published studies on purified MCF carriers have been made with protein purified from native tissues, which explains the more extensive studies on AAC extracted from heart or liver mitochondria (see *i.e.* [12–14] and for review [15]), and on UCP1 found in BAT (see *i.e.* [16–19], and for review [20]). Although AAC and UCP1 are quite abundant in heart or liver mitochondria and BAT, respectively, the purification of native proteins remains difficult to control and the production of mutants necessary for the study of structure–function relationships impossible. One of the major biochemical difficulties is that native MCF do not bind to chromatographic column and instead are generally isolated from the flow through of hydroxylapatite column when solubilised with Triton X-100 or LAPAO. A bacterial expression protocol including a large number of steps was recently published for UCP2 and led to the first structural insights of UCP2 by NMR [11]. Establishing efficient and robust protocols for the heterologous production and purification of functional mitochondrial carriers still remains an essential but challenging task for the study of the MCFs.

Two heterologous production systems of UCP1 have been described so far: (a) the production in *E. coli* as inclusion bodies [21–23] and (b) the expression in yeast [24–27]. Although, the oxoglutarate/malate carrier has been produced in large amounts from inclusion bodies [28], UCP1 could not be produced with similar yields. The final amounts of pure protein obtained from inclusion bodies or yeast are low and rather inadequate for structural studies. We present herein the production of UCP1 using the cell free system, which couples transcription and translation reactions into the same reaction medium [29]. This technique developed since a decade mainly for soluble proteins, emerged recently for membrane proteins. A considerable variety of prokaryotic or eukaryotic membrane proteins, belonging to different family, having up to twelve membrane helices and for some with molecular masses exceeding 100 kDa, have already been successfully expressed (for review see [30]). Among other advantages, cell free expression overcomes general limitations known for cellular expression such as targeting, translocation and insertion into the cell membrane, a limited volume of membranes into which proteins can be inserted, and toxic or inhibitory effects induced by the proteins on the host cell. In addition, the set-up of the cell free system offers a multitude of options to adapt the reaction conditions and to directly supply any compound favouring the expression or stability of proteins of interest. In order to produce UCP1 in the soluble fraction of the reaction medium, we added surfactants above their critical micellar concentrations (cmc) and explored various types of compounds from classical detergents to non commercial surfactants. Fluorinated surfactants [31] are of particular interest for stabilising membrane proteins [32] and were already shown to be compatible with the cell free machinery [33]. We were able to produce UCP1 in quantities that are compatible with structural studies in the presence of fluorinated surfactants. As shown from size exclusion chromatography and circular dichroism, including thermal stability, the protein is monodisperse, well-folded and sufficiently stable for structural studies.

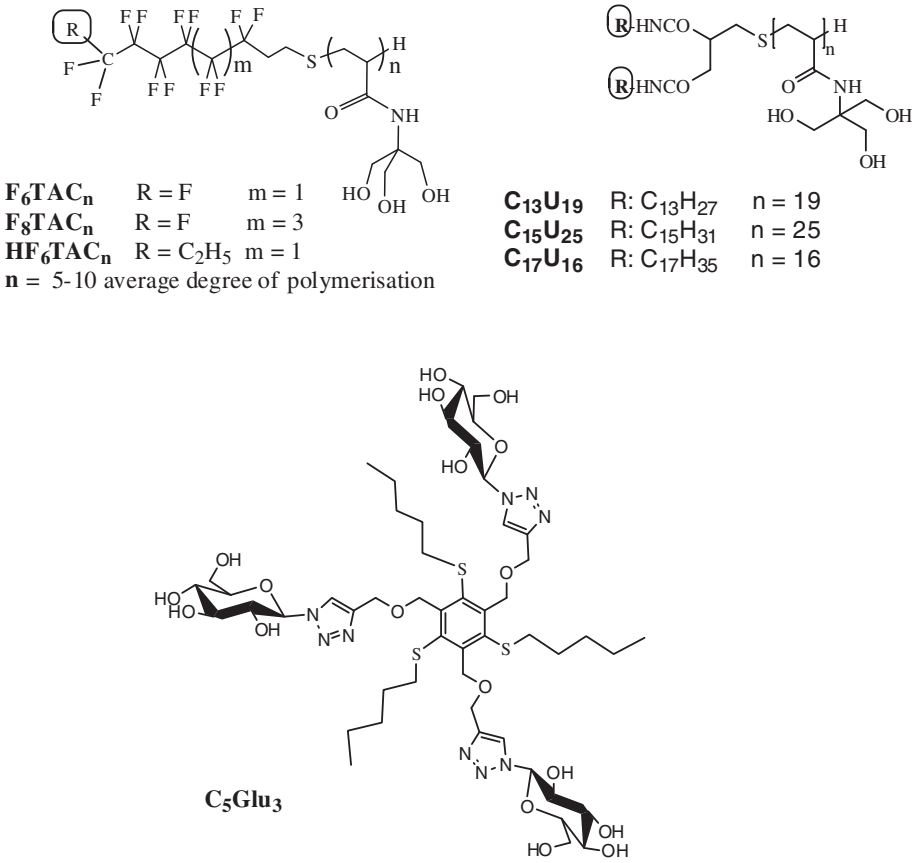

Fig. 1. Chemical structures of FTACs, C_nU_m and Tripod surfactants. FTACs are short oligomers of Tris (hydroxymethyl) acrylamidomethane (THAM) prepared in the presence of fluorinated mercaptans as transfer reagents, to introduce (hemi) fluorinated chain at the end of polymeric backbone [31]. C_nU_m are prepared from mercapto glycerol as starting material which is first endowed with two hydrophobic tails bearing C_n carbon atoms, then involved in telomerization reaction of THAM monomer to multiply the number of Tris moieties (m) as polar heads [34]. Three hydrocarbon chains and three glucosyl groups are grafted onto the central mesitylene forming the hydrophobic and hydrophilic faces of C_5Glu_3, respectively [35].

2. Material and methods

2.1. Surfactants and lipids

Detergents were purchased from Anatrace (except Triton X-100 from Sigma), and lipids from Avanti Polar Lipids. Hemifluorinated (HFTACs) or fluorinated surfactants (FTACs), phospholipid-like surfactants (C_nU_m) and facial amphiphiles C_5Glu_3 (Fig. 1), were synthesised by one coauthoring team (B. Pucci, A. Polidori and L.A. Barret). F_nTAC_m contain fluorinated hydrophobic tails consisting of n carbons (6 or 8 in this study), and m is the average degree of polymerization of the polar head [31]. Their physical–chemical properties (including cmc) are independent of the average degree of polymerization of the polar head. Three different FTACs were initially screened (Table 1). Only F_8TAC_5 was considered in additive screens and upscale experiments. In the corresponding sections, unless specified, FTAC refers to F_8TAC_5. Phospholipid-like surfactants C_nU_m have two hydrophobic tails bearing C_n carbon atoms, and m Tris moieties as polar heads [34]. The hydrophobic face of the facial amphiphile C_5Glu_3 consists of three short hydrocarbon chains (3 to 7 carbons each). Three glucosyl groups grafted onto a central mesitylene constitute the hydrophilic face [35]. The peptergents were a gift from S. Zhang (MIT, USA).

2.2. Cloning

The rUCP1 cDNA was amplified by PCR using the primers UCP1-NOTI-FW (TATGAGCGGCCGCATGGTGAGTTCGACAACTTCCG) and UCP1-CFR9I-24A-REV (CGGATCCCGGGCTATGTGGTGCAGTCCACTGTCTGC) for insertion into the plasmid pIVEX 2.4a (Roche) or UCP1-CFR9I-MCS-REV (CGGATCCCGGGTGTGGTGCAGTCCACTGTCTGCC) for insertion into the plasmid pIVEX 2.3MCS (Roche). The PCR product was inserted between the NotI and Cfr9I restrictions sites of the vectors pIVEX 2.4a or pIVEX 2.3 MCS encoding for an N-terminal or C-terminal poly (His) 6 tag respectively. These vectors are optimised for expression in the Rapid Translation System (RTS) cell-free system used in this study. Constructs were checked by sequencing.

2.3. Cell free expression

Cell free expression experiments were performed using the Roche *E. coli* RTS system according to the manufacturer's instructions. For small scale (batch) production, 0.5 μg of plasmid DNA was incubated for 24 h, at temperatures ranging from 15 °C to 28 °C in the RTS 100 HY kit reaction mixture (final volume of 25 μL). The synthesis was followed by a centrifugation step at 18,000 g for 15 min (4 °C). The supernatant and the pellet resuspended in 50 mM Tris buffer, pH 7.5 were subsequently analysed by SDS-PAGE, Western blotting and immunodetection of the (His) 6-tag. The production of soluble protein was achieved by supplying into the reaction mixture different surfactants and additives listed in Tables 1 and 2.

For preparative experiments, 10 μg of plasmid DNA was incubated in the RTS 500 ProteoMaster HY lysate supplemented with 4 mM FTAC and 0.28 mM cardiolipin in a final volume of 1 mL. After 24 h incubation at 28 °C the reaction mixture was centrifuged 15 min at 18,000 g (4 °C) and the supernatant was used for the purification of the protein.

Table 2
Effect of additives on rUCP1 solubility. Solubility of the protein synthesised in the presence of (A) 4 mM FTAC and different additives or (B) different surfactants in the presence of 0.28 mM cardiolipin. The solubility was evaluated on the same scale as for Table 1. The surfactant and additives concentration used are given in the brackets. Some additives inhibited the synthesis. When scaled up to a 1 mL reaction mixture, in the presence of F_8TAC_5 and cardiolipins the quantity of protein is estimated to about 1 mg with 90% in the supernatant and 10% in the pellet.

A.

Additives	Solubility
No additive	6
E. coli lipids (0.4 g/L)	6
Cardiolipin (0.28 mM = 0.4 g/L)	9
Facial amphiphile C_5Glu_3 (0.25 mM)	6
Peptergent KA_6 (0.1 mM)	6
Lauric acid (0.1 mM)	No synthesis
GDP (0.1 mM)	No synthesis

B.

Surfactant	UCP1 solubility	
	No additive	Cardiolipin
F_8TAC_5 (4 mM)	6	9
$C_{13}U_{19}$ (0.8 mM)	6	4
DDM (1.7 mM)	3	1
Brij35 (3.3 mM)	5	9
Brij58 (0.9 mM)	5	0
Digitonin (32.2 mM)	5	5

Table 1
Effect of surfactants on rUCP1 solubility. For each surfactant the table indicates its charge, cmc, the concentration used (mM) and the result obtained. The solubility was evaluated on a scale from 0 to 10, 5 corresponding to half of the protein in the supernatant and 10 corresponding to the detection of the protein exclusively in the supernatant. N: non ionic, A: anionic, Z: zwitterionic.

Surfactant type	Surfactant	Charge	cmc (mM)	Concentration (mM)	UCP1 solubility
Glucosides	DDM	N	0.17	0.85	3
	Cymal 5	N	2.4	24	2
Polyethylene glycols	Triton X-100	N	0.23	2.3	0
Polyoxyethylenes	$C_{10}E_5$	N	0.81	8.1	0
	Brij35	N	0.091	3.3	5
	Brij58	N	0.004	0.9	5
Steroids	Digitonin	N	0.5	6.5	5
Amine oxides	LDAO	Z	1.00	10	No synthesis
	LAPAO	Z	1.56	15.6	No synthesis
Lipid like detergents	FC_{12}	Z	1.5	15	No synthesis
	FC_{14}	Z	0.12	1.2	No synthesis
	FC_{16}	Z	0.013	0.13	3
Phospholipid like surfactants	$C_{13}U_{19}$	N	0.008	0.08	6
	$C_{15}U_{25}$	N	0.0055	0.055	6
	$C_{17}U_{16}$	N	0.005	0.05	6
Fluorinated compounds	HFTAC	N	0.45	9	6
	F_6TAC_{10}	N	0.3	7.5	6
	F_6TAC_5	N	0.3	7.5	6
	F_8TAC_5	N	0.03	0.75	6
Facial amphiphiles	C_5Glu_3	N	0.12	1.23	0

2.4. Protein purification

rUCP1 was purified by affinity chromatography using a Ni-NTA resin. The supernatant recovered from the large scale synthesis was diluted ten times in 50 mM Hepes, pH 7.5, 200 mM NaCl containing 10 mM imidazole and incubated with 250 µL of preequilibrated resin (Ni-NTA Superflow, Qiagen). After 1 h incubation at 4 °C, under shaking, the suspension was poured into a column and extensively washed with buffer A (50 mM Hepes, pH 7, 200 mM NaCl, 0.014 mM cardiolipin, 0.15 mM FTAC) and increasing concentrations of imidazole (10, 20 and 40 mM). The protein was eluted at 200 mM imidazole.

2.5. Size-exclusion chromatography

The apparent molecular mass and the Stokes radius of the rUCP1-surfactant complex (Rs) were analysed by size-exclusion chromatography using a Superdex 200 10/300 GL column (GE Healthcare). Standard proteins (ferritin, aldolase, ovalbumin, and ribonuclease; Bio-Rad) were used to calibrate the column with the same buffer. Prior to the size-exclusion analysis, imidazole was removed by dialysis and during concentration in Amicon Ultracel-30k concentrator (Millipore).

2.6. UCP1 purified from BAT

The purification procedure was described by Lin and Klingenberg [36]. Briefly, brown adipose tissue (BAT) mitochondria were isolated from one week cold-adapted mice. Mitochondria were cleaned up with 3.2% lubrol in buffer 20 mM MOPS pH 6.8, 20 mM Na_2SO_4, 1 mM EDTA. The proteins were then solubilised 30 min in 2% $C_{10}E_5$. UCP1 was purified on hydroxyapatite column previously washed with buffer containing 0.05% $C_{10}E_5$. The buffer was then exchanged for 10 mM KPi pH 6.5 by desalting on a HiTrap 5 mL column.

2.7. Circular dichroism spectroscopy

Prior to the circular dichroism experiments on cell-free expressed rUCP1, the buffer composition of the protein solution was changed to 25 mM KPi pH 7, 0.014 mM cardiolipin, 200 mM NaF (supplemented with 0.15 mM FTAC) by dialysis and during concentration in Amicon Ultracel-30k concentrator (Millipore). The measurements were performed using a Peltier temperature controlled spectropolarimeter (JASCO model J-810) equipped with a 0.1 cm path length cell. The CD spectrum of native UCP1, was recorded on a Jasco Model J-815 using a 0.1 cm path length cell. For both proteins, ten scans were collected at 20 °C from 260 to 200 nm at 0.5 nm intervals, then averaged and baseline-corrected by subtraction of blank buffer. After subtracting the blank signal, the CD signal (in millidegrees) was converted to mean molar residue ellipticity (in $deg.cm^2.dmol^{-1}$). Thermal denaturation of cell-free expressed rUCP1 was followed by continuously monitoring ellipticity changes at a fixed wavelength (220 nm) while the sample was heated up at a constant rate from 20 °C to 90 °C. Spectra were analysed with the Spectra Manager II software (Jasco) including the protein secondary structure determination.

3. Results

3.1. Small-scale cell free production of rUCP1

First, the expression of the two rUCP1 constructs (His-rUCP1 and rUCP1-His) was determined in detergent free batch reactions using RTS 100 kits. Interestingly, only His-rUCP1 was synthesised in detectable amounts. The influence of the position of the tag on the amount of proteins produced with the cell free system was already reported in a study of *Pseudomonas aeruginosa* proteins [37]. Although there is no general rule for the position of tag, the location of the His-tag in its N-terminus is important for an optimal synthesis of rUCP1.

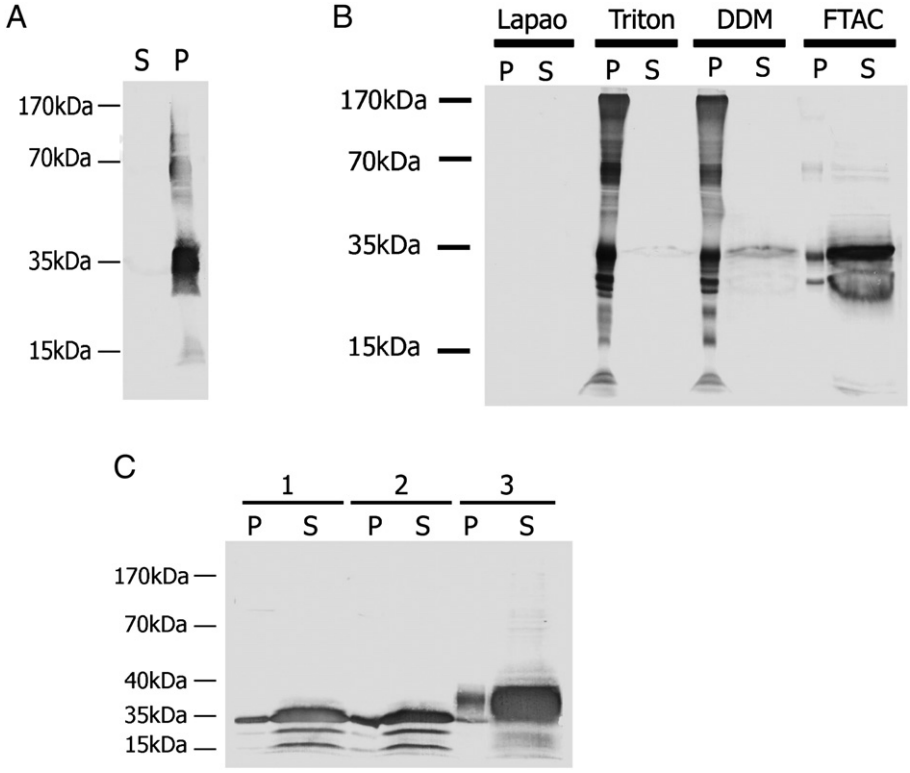

Fig. 2. Cell free production of rUCP1. (A) In the absence of surfactants. (B) In the presence of different surfactants. (C) In the presence of 4 mM FTAC and different additives: 1 — without any additives, 2 — with 1 mM A_6K-NH_2 (peptergent), and 3 — with 0.28 mM cardiolipin. The protein was synthesised as described in Material and methods section. The surfactants were used at the concentration described in Table 1. The notations used are as follows: Triton, Triton X-100; P, pellet; S, supernatant.

Therefore only the His-rUCP1 construct was used for further experiments. After the synthesis reaction of His-rUCP1, the supernatant and the pellet were separated by centrifugation and analysed by Western blots. As expected for a membrane protein, rUCP1 was detected exclusively in the pellet (Fig. 2A).

Three different strategies, commonly used for the cell free production of membrane proteins (for review see [30]) were then initially explored: (a) the production of the protein as a precipitate and the subsequent solubilisation of the pellet by detergents, (b) the synthesis of the protein in the presence of liposomes and (c) the synthesis of the protein in the presence of surfactants. While preliminary experiments following the first two strategies failed to produce soluble rUCP1, the cell free production of the protein in presence of surfactants proved to be efficient and was further explored.

3.2. rUCP1 cell free production in the presence of surfactants

To identify the ideal surfactant that results in the production of completely solubilised proteins while maintaining high expression levels, a panel of different types of surfactants was tested (see Table 1 and Fig. 2B). The panel not only contained classical detergents but also other amphiphilic molecules, which are able to surround membrane proteins and to maintain them in solution (Fig. 1). Some of them were already shown to be compatible with the cell free production of other membrane proteins, like DDM [38–40], Triton X-100 [39,41], Brij35 or Brij58 [39], digitonin [39,42] and fluorinated compounds [43]. LAPAO and $C_{10}E_5$ were never used in cell free protein production but are known to be efficient for the solubilisation of mitochondrial carriers [12,44,45]. The use of facial amphiphiles and phospholipid-like surfactants (C_nU_m) in a cell free expression system was also never reported.

In order to provide the appropriate environment necessary to maintain a membrane protein in solution, all the surfactants were tested at concentrations above their cmc, as specified in Table 1. In each case, the solubility of the protein was ranked according to the proportion of the protein present in the supernatant *versus* pellet, on a scale from 0 (all the protein in the pellet) to 10 (all the protein in the supernatant). Three categories of surfactants could be defined: (a) inhibitors for the synthesis reaction, (b) not capable to produce soluble rUCP1 and (c) capable to produce at least partially soluble rUCP1.

Most of the zwitterionic surfactants (LDAO, LAPAO, FC_{12}, FC_{14}) were inhibitory for the synthesis reaction at the used concentrations, except FC_{16}, which even increased the level of soluble protein. All the fos-cholines were used at ten times their cmc. Given the low cmc of FC_{16}, the concentration used for FC_{16} was significantly lower than for FC_{12} and FC_{14}, which probably explain its lack of toxicity. Indeed, increasing ten times the FC_{16} concentration proved to be also inhibitory for the cell free reaction (data not shown). The capacity of fos-cholines to inhibit the cell free synthesis of protein when used at high concentration was already described by Kaiser et al. [42].

Triton X-100, a non ionic surfactant widely used for the purification of MCFs from native tissues, was compatible with the *in vitro* synthesis but surprisingly not efficient for the production of soluble protein (Fig. 2B). Same result was obtained with the facial amphiphile C_5Glu_3. Maltosides (DDM and Cymal5) only partially maintained the protein in solution. Among polyoxyethylenes, $C_{10}E_5$ was not able to produce soluble proteins, but about 50% of the proteins was obtained in the soluble fraction in the presence of dodecyl or hexadecyl polyoxyethylenes analogues like Brij35 and Brij58. Even surfactants with similar chemical nature displayed different effects and similar deviations were already reported for the cell free synthesis of GPCRs [46]. From all these data, it is not obvious whether a longer hydrophobic tail or polydispersity of the surfactant favours the production of soluble protein. Good results were also obtained with the steroidian detergent, digitonin (about half of the protein was soluble). The best yields for the production of soluble rUCP1 were obtained with less commonly used surfactants like phospholipid-like surfactants (C_nU_m) [34] and fluorinated surfactants (FTACs). About 60% of the protein was detected in the supernatant in the presence of the six fluorinated surfactants and the three C_nU_m tested independently of the length of the fluorinated or hydrocarbon chains.

However, among all the surfactants tested, none of them was able to produce 100% of the protein in the soluble fraction. Increasing the DDM, C_nU_m and FTAC concentrations did not enhance the fraction of soluble protein and even reduced it for DDM (results not shown). Varying the temperature of the synthesis reaction to 15 °C, 20 °C or 28 °C did not improve the proportion of soluble protein and even significantly reduced the total amount of protein at 15 °C (results not shown). We therefore investigated the effect of additives.

3.3. Effect of additives on rUCP1 solubility

A wide range of additives was systematically tested to enhance the solubility of protein produced in presence of FTAC (Table 2A and Fig. 2C). They were chosen based on their stabilising effect reported on other membrane proteins (peptergents [47] or lipids) or on their functional effect on UCP1 (lauric acid or GDP known to activate or inhibit UCP1, respectively [48,49]). Peptergents or *E. coli* lipids did not increase the soluble fraction of the protein. Activators, like lauric acid, or inhibitors, like GDP, could favour a single conformation and thus prevent flexibility due to conformational disorder. However, adding lauric acid or GDP inhibited the synthesis reaction. Most interestingly, in the presence of cardiolipins almost all the protein was detected in the supernatant. Cardiolipins were then added in combination with surfactants other than FTAC like $C_{13}U_{19}$, Brij35, Brij58, digitonin and DDM (Table 2B). Surprisingly, the cardiolipin effect was very different depending on the surfactant. The soluble fraction of the protein was improved in the presence of Brij35 while no effect was observed with digitonin. The solubility of the protein was even decreased when cardiolipin was added to Brij58, DDM or $C_{13}U_{19}$.

3.4. Preparative scale expression of rUCP1 and purification

The production was scaled up using the RTS 500 system and the best conditions determined with the batch experiments were first tested. The synthesis was carried out (a) in the presence of 4 mM FTAC and 0.28 mM cardiolipin or (b) in the presence of 3.3 mM Brij35 and 0.28 mM cardiolipin. Then, the reaction mixture was centrifuged and rUCP1 was mainly detected in the supernatant for both of these conditions. The protein was purified in a one step affinity purification on Ni-NTA resin. Preliminary tests revealed that the yield could be easily improved when working with FTAC. Therefore, further

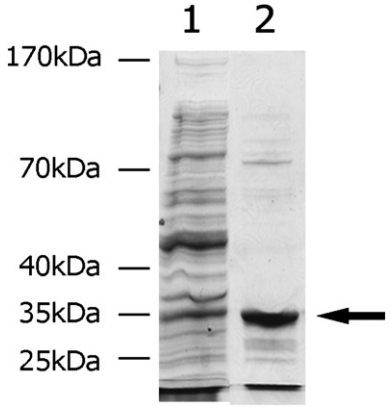

Fig. 3. Purification of rUCP1 as analysed by SDS-PAGE. rUCP1 was purified as described in the Material and methods section; Lane 1, supernatant of the reaction mixture before purification; Lane 2, protein eluted from the Ni-NTA resin. Protein standards in kDa are indicated on the left side.

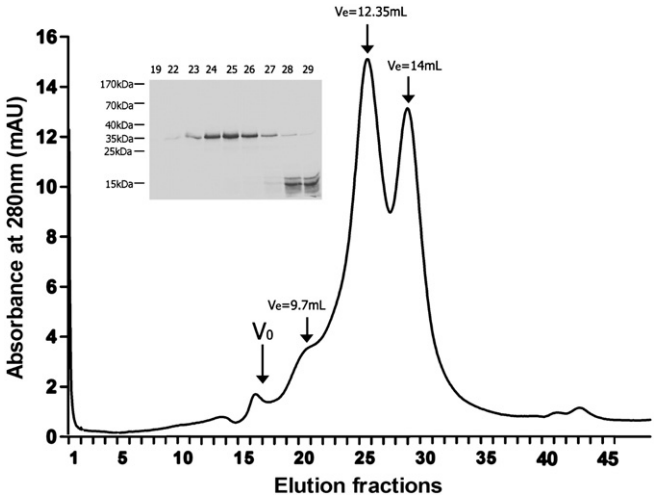

Fig. 4. Size exclusion chromatography analysis of purified rUCP1. Purified rUCP1 in the presence of FTAC was applied to a Superdex 200 HR 10/30 column. The column was equilibrated with 25 mM Hepes, pH 7, 200 mM NaCl supplemented with 0.15 mM FTAC and 0.014 mM cardiolipin. Fractions of 0.5 mL were collected starting from 0 mL. Western blots with anti His-tag immunodetection of the protein present in various elution fractions are shown in the insets. V_0 is the void volume.

optimisations were mainly restricted to the protein produced in FTAC and cardiolipin. Most impurities were washed off with sequential washing steps at 10, 20 and 40 mM imidazole and the bound protein eluted with 200 mM imidazole. The purity of the eluted protein was analysed on a Coomassie blue stained SDS-PAGE gel (Fig. 3). Except the band corresponding to the expected mass of rUCP1, one band at about 75 kDa and several bands at lower molecular masses could be noticed. Lower molecular mass bands could correspond to not fully synthesised proteins, a common problem encountered in cell free expression. Indeed, all the fragments that contain the N-terminal His-tag are recovered after Ni-NTA purification. The yield of purified protein ranged from 0.6 to 0.8 mg per mL of reaction mixture. This amount is quite high and compatible with structural studies. Furthermore, the method from expression to purification is very simple and fast as compared to the production from inclusion bodies.

3.5. Size exclusion chromatography

The protein was subjected to size exclusion chromatography in order to investigate the dispersity and homogeneity of the sample. We analysed the Stokes' radii and the apparent molecular mass of the rUCP1-surfactant-lipid complex in the presence of FTAC (Fig. 4). The size-exclusion elution profile of rUCP1 contained two main peaks with an elution volume of 12.35 mL and 14 mL, respectively (Fig. 4). A small shoulder at higher molecular mass (elution volume 9.7 mL) was also observed. The Western blot analysis showed that the first peak was essentially composed of full-length rUCP1 while the second contained only fragments of not fully synthesised protein. Of note, fragments of rUCP1 were efficiently separated from the full-length protein by this additional SEC purification step, therefore useful to improve the purity of the sample. After removal of the second peak, rUCP1 was monodisperse and eluted with an apparent molecular mass of 179 kDa. Using a calibration curve, a Stokes radius of 4.6 nm was determined for the protein-surfactant-lipid complex. No significant peak corresponding to the void volume was evidenced in the size exclusion profile, showing that the protein did not aggregate.

3.6. Circular dichroism

To further investigate the *in vitro* synthesised protein after purification, its secondary structure was analysed by CD spectroscopy. As shown in Fig. 5A, the far-UV CD spectra of rUCP1 purified in FTAC in the presence of cardiolipin exhibited two negative maxima near 222 nm and 208 nm indicating a significant α-helix content in the secondary structure. Comparison of the spectra obtained in the absence or in the presence of 100 μM GTP did not indicate any significant differences at the level of the secondary-structure (data not shown). Thermal denaturation of rUCP1 was also investigated by CD by continuous recording of ellipticity at 220 nm while heating up the sample from 20 °C to 90 °C (Fig. 5C). The Tm value measured for the protein purified in FTAC was 64 °C.

4. Discussion

The cell free system has become over the last few years an interesting alternative for the production of membrane proteins (for review, see [30]). Cell free synthesised human VDAC could even be crystallised, thus demonstrating the structural homogeneity of the protein [40]. Fluorinated surfactants were successfully used to stabilise membrane proteins (see *i.e.* [50]) and proved to be efficient for the cell free synthesis of bacterial proteins [33]. As mitochondrial carriers are particularly labile eukaryotic proteins, cell free synthesis in the presence of fluorinated compounds or other amphiphiles was therefore explored herein. The best results for producing a large soluble fraction of rUCP1 were obtained with phospholipid-like surfactants (C_nU_m)

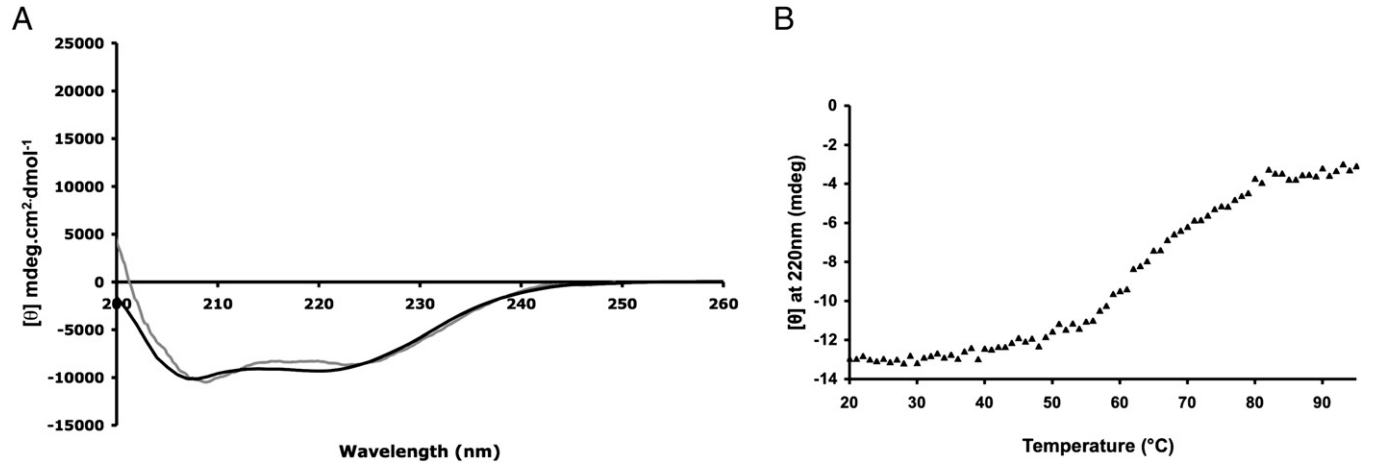

Fig. 5. Circular dichroism spectroscopy of purified UCP1. (A) CD spectra of the cell-free expressed rUCP1 purified in the presence of FTAC (black line) and of native UCP1 (grey line). The data were recorded at 20 °C in a 0.1 cm path length cell. Ten scans were collected at 0.5 nm intervals from 260 to 200 nm, then averaged and baseline-corrected by subtraction of blank buffer. (B) Melting curve of cell-free produced rUCP1 purified in the presence of FTAC.

[34] and fluorinated surfactants (FTACs). Combined with cardiolipins as additives, the solubility of rUCP1 synthesised in FTACs and also Brij35 was highly enhanced. The use of Brij35 in a cell free system was already described [30], while the use of C_nU_m is novel. C_nU_m are water-soluble non-ionic surfactants. They display interesting low denaturing properties and have stabilising capacities. Endowed with two hydrophobic tails, they mimic natural phospholipids and are suggested to associate with membrane proteins and maintain them soluble in aqueous solution without prohibitive unfolding [34]. The use of FTACs was earlier explored for the stabilisation of membrane proteins. Bacteriorhodopsin and cytochrome b_6f have been shown to be more stable once transferred to FTACs than in the presence of detergents [32]. A similar effect was shown on Smoothened, a human G-protein coupled receptor [51]. Fluorinated surfactants are highly hydrophobic but also lipophobic. Using dye-labelled FTACs, it could be demonstrated by fluorescence correlation spectroscopy that these molecules do not interact with lipids [52]. In contrast to detergents, FTACs do not compete with protein–lipid, but most probably also with protein–protein or intra-protein interactions. In combination to the relative stiffness of their chains compared to detergents, these properties are favourable to the maintenance of membrane protein integrity and function. For example, specific ATPase activity of the yeast F1–F0 ATP-synthase was reported to be higher in FTAC than in DDM, and in addition, the sensitivity towards inhibitors remained strong even after 6 weeks [50]. Fluorinated surfactants were also described as possible chemical chaperones as inferred from their efficiency in forming the membrane-competent form of diphtheria toxin T-domain by acidification [52]. When synthesising rUCP1 in the cell free system, this chaperone property could explain the higher yield of soluble protein observed in the presence of FTAC. FTACs were already used with success for the cell free production of a bacterial mechanosensitive ion channel MscL [43]. Here, we demonstrate that FTAC is also a valuable surfactant for expressing a labile mammalian membrane protein.

Cardiolipin is highly abundant in the inner mitochondrial membrane. Being a dimer of phosphatidylglycerol, it contains two polar heads and four aliphatic chains instead of two as for most of the phospholipids. Several studies showed that cardiolipins play an important role for the structural integrity of mitochondrial carriers [53,54]. The high resolution structures of AAC revealed three cardiolipins that surround the carrier and that are tightly bound through several interactions [53,4]. The phosphate groups of the three lipid molecules mainly interact with main chain nitrogens of AAC, while their acyl chains stack to aromatic residues located in MCF motifs. The first type of interaction is related to the overall fold of MCF carriers, and the second to residues conserved in MCF motifs. Therefore both types of interactions are not specific to AAC and possibly exist in other MCF carriers. In particular in UCPs, the increase in the solubility of the cell free produced rUCP1 could be explained by a stabilising effect by cardiolipins. Because FTACS are poorly miscible with lipids, they do not perturb putative interactions between rUCP1 and cardiolipins in contrast to detergents as for example DDM. A similar effect was observed for the yeast F1–F0 ATP-synthase. Relipidation of this complex in DDM did not rescue its specific ATPase activity nor its sensitivity to inhibitors. In contrast, in FTAC both properties were maintained for more than 40 days after extraction [50].

Although no functional assays have been developed so far on rUCP1 produced in the cell free system, the protein was characterised by several means. The Stock radius of 4.6 nm and the apparent molecular mass of 179 kDa measured from size exclusion chromatography, were consistent with data reported for the ADP/ATP carrier, predicted to have a similar overall shape as UCP1. Indeed, in the presence of various detergents having hydrophobic chains of similar length to FTAC (such as $C_{12}M$, $C_{13}M$ or LAPAO), Rs values for AAC-detergent complex were determined in the range of 4 to 4.5 nm by two groups [55,56]. In both studies, AAC was shown to be monomeric. The Rs value of 4.6 nm would be therefore consistent with a monomeric rUCP1 in FTAC. Our CD spectra are comparable with those previously reported for UCP1 purified from inclusion bodies [23] and from yeast [57] but also with the CD spectrum recorded for native UCP1 purified from BAT (Fig. 5A), suggesting that the secondary structures are similar. Indeed, similarly to [57], the shape between 200 and 240 nm is characterised by two negative peaks located at 208 and 222 nm. In addition, the addition of GDP does not modify significantly the secondary structure of the protein as reported in [57]. Overall, CD analyses showed that rUCP1 produced in the cell free system in the presence of FTAC is folded mainly as α-helices as expected. We estimate that 60% of cell-free expressed UCP1 is α-helical. From their CD data, Douette et al. evaluated the α-helical structure to 68% while Jelokhani-Niaraki et al. reported 40 to 50%. In addition, the known structures of members of the same MCF family, bAAC1 [4] and mUCP2 [11] highlight 67% (55% in the TM helices and 12% within the structured part of the matrix loops) for both bAAC1 and mUCP2. Our data are consistent with all these values, considering structural variability of matrix loops (they vary depending on the conformational state of the carriers) and also lack of accuracy of the value calculated herein due to the missing part below 200 nm. Finally, thermal denaturation over 60 °C is a good indication of a well-defined tertiary structure below Tm. In comparison with other membrane proteins that were successfully purified in the presence of FTAC and functional, we anticipate that rUCP1 produced in the cell free system in the presence of FTAC is adequate for structure–function studies.

5. Conclusion

We demonstrate herein that rUCP1 can be produced in a cell free system in the presence of a fluorinated surfactant FTAC. This is the first example of the use of fluorinated surfactants for producing large quantities of eukaryotic membrane proteins in the cell free system. This method is fast and simple compared to the production from inclusion bodies. It is also flexible and constitutes a valuable alternative to the expression of MCF carriers. The protein forms monodisperse protein-surfactant complexes whose size is compatible with a monomeric state. The protein is mainly structured in α-helices and the amounts obtained after purification are compatible with structural studies.

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