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Assaying the proton transport and regulation of UCP1 using solid supported membranes

Iulia Blesneac · Stéphanie Ravaud · Paul Machillot · Manuela Zoonens · Sandrine Masscheylen · Bruno Miroux · Michel Vivaudou · Eva Pebay-Peyroula

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Abstract The uncoupling protein 1 (UCP1) is a mitochondrial protein that carries protons across the inner mitochondrial membrane. It has an important role in non-shivering thermogenesis, and recent evidence suggests its role in human adult metabolism. Using rapid solution exchange on solid supported membranes, we succeeded in measuring electrical currents generated by the transport activity of UCP1. The protein was purified from mouse brown adipose tissue, reconstituted in liposomes and absorbed on solid supported membranes. A fast pH jump activated the ion transport, and electrical signals could be recorded. The currents were characterized by a fast rise and a slow decay, were stable over time, inhibited by purine nucleotides and activated by fatty acids. This new assay permits direct observation of UCP1 activity in controlled cell-free conditions, and opens up new possibilities for UCP1 functional characterization and drug screening because of its robustness and its potential for automation.

Keywords Mitochondrial carriers · Uncoupling protein · Proteoliposomes · Solid supported membranes · Electrophysiology

I. Blesneac · S. Ravaud · P. Machillot · M. Vivaudou · E. Pebay-Peyroula
Institut de Biologie Structurale, Université Grenoble 1, 38027 Grenoble Cedex 1, France

I. Blesneac · S. Ravaud · P. Machillot · M. Vivaudou · E. Pebay-Peyroula (✉)
Institut de Biologie Structurale, CEA-CNRS, 41 Rue Jules Horowitz, 38027 Grenoble Cedex 1, France
e-mail: eva.pebay-peyroula@ibs.fr

Present Address:
I. Blesneac
Institut de Génomique Fonctionnelle, CNRS UMR5203, INSERM U661, Universités de Montpellier, 34094 Montpellier, France

Present Address:
P. Machillot
DSV/iBITec-S, CEA Saclay, 91191 Gif sur Yvette Cedex, France

M. Zoonens · S. Masscheylen · B. Miroux (✉)
Institut de Biologie Physico-Chimique, CNRS UMR 7099, LBPC-PM, 13 rue Pierre-et-Marie Curie, 75005 Paris, France
e-mail: bruno.miroux@ibpc.fr

M. Zoonens · S. Masscheylen · B. Miroux
Université Paris-Diderot, UMR 7099, Paris, France

Introduction

UCP1 (uncoupling protein 1), a member of the mitochondrial carrier family (MCF), is responsible for the dissipation of the proton gradient across the inner mitochondrial membrane. By carrying protons, it uncouples the respiratory chain from ATP synthesis [for review, see (Nicholls and Locke 1984), (Porter 2008)]. UCP1 is highly expressed in brown adipose tissue (BAT), whose main function is to convert fat into heat. UCP1 is essential in β-adrenergically controlled non-shivering thermogenesis, and evidence suggests a role in the protection against diet-induced obesity (Kopecky et al. 1996), (Feldmann et al. 2009). Recently, the presence of BAT with active UCP1 was demonstrated in adult humans, its activity being significantly diminished in overweight or obese humans (van Marken Lichtenbelt et al. 2009, Virtanen et al. 2009, Vijgen et al. 2011).

The transport mediated by UCP1 results in a net charge movement across the membrane, and is thus electrogenic. This transport is known to be activated by fatty acids and

inhibited by purine nucleotides (Lin and Klingenberg 1982, Rial et al. 1983). The functional properties of UCP1 have been assessed by indirect spectroscopic assays (Klingenberg and Winkler 1985), (Garlid et al. 1996), (Mozo et al. 2006) that are time and protein consuming, and do not allow real-time transport characterization. More sophisticated electrophysiological techniques, such as patch clamp (Huang and Klingenberg 1996) and planar lipid bilayer reconstitution (Urbankova et al. 2003), have also been employed, although these techniques remain challenging for routine use.

Alternative techniques to record electrical currents associated with the activity of electrogenic transporters rely on solid supported membranes (SSM). The efficiency of these techniques to study transport mechanisms has been illustrated for various prokaryotic and eukaryotic carriers (Krause et al. 2009, Ganea et al. 2001, Zuber et al. 2005, Tadini Buoninsegni et al. 2004). Of special interest is the fast solution exchange on SSM. Proteoliposomes or native membranes are adsorbed to SSM and transporters activated using a rapid substrate concentration jump (Pintschovius and Fendler 1999). Transient currents generated by the transport are recorded via capacitive coupling and contain information about the amplitude and the rate of the transport process [for review see (Schulz et al. 2008)]. The power of the method was recently illustrated by the characterization of nucleotide transport by the ADP/ATP carrier, another MCF member (Watzke et al. 2010).

The present study reports the characterization of the transport activity of UCP1 using the SSM-based technology. The ion transport was induced by a fast pH jump from 7 to 6, and currents of a few nA were recorded. Based on the current measurements, the properties of the transport performed by UCP1, as well as the effects of GDP and lauric acid, were explored.

Materials and methods

UCP1 purification

UCP1 was purified from BAT mitochondria following a procedure adapted from Winkler and Klingenberg (1992). Briefly, BAT mitochondria isolated from 1-week cold-adapted mice were cleaned up with 3.2 % lubrol (30 min incubation) in buffer A (20 mM Na_2SO_4, 1 mM EDTA, 20 mM MOPS, pH 6.8). Protein solubilization was performed during 1 h in buffer A containing 2 % (w/v) $C_{10}E_5$ instead of 4.5 % as described in Winkler and Klingenberg (1992) to avoid contamination with porins and mitochondrial carriers other than UCP1 (Zoonens et al. in preparation). UCP1 was purified on a hydroxyapatite column previously equilibrated with buffer A containing 0.05 % (w/v) $C_{10}E_5$.

Preparation of proteoliposomes

A mixture of phosphatidylcholine, phosphatidylethanolamine and cardiolipin (Avanti Polar Lipids) was solubilized in chloroform in a glass tube at an 8/1/1 ratio (w/w/w). The chloroform was evaporated to dryness under a nitrogen stream. The lipidic film was resuspended in a buffer composed of 50 mM MOPS, pH 7, 140 mM potassium gluconate in order to have a final lipid concentration of 10 mg/mL. The solution was vortexed for several minutes and then sonicated in order to form small unilamelar vesicles (SUVs). The purified protein was then mixed with the SUVs at a lipid to protein ratio of 15/1 (w/w), giving a final protein concentration of 0.6–0.7 mg/mL. After 30 min equilibration at 4 °C, on a roller drum, 250 mg of SM2 Bio-Beads (Bio-Rad) was added to the solution and incubated overnight at 4 °C to remove the detergent. An additional incubation with 250 mg of Bio-Beads for 90 min allowed the removal of detergent traces. Bio-Beads were then removed, and the solution was recovered and centrifuged 25 min at 350,000*g* at 4 °C. The pellet was resuspended in 50 mM MOPS, pH 7, 140 mM potassium gluconate in order to have a final lipid concentration of 10 mg/mL. The liposomes were either used immediately for measurements or aliquoted, frozen in liquid nitrogen and stored at −80 °C.

SSM measurements

The liposomes were diluted ten times in 50 mM MOPS, pH 7, 140 mM potassium gluconate and sonicated 20 s (UP50H sonicator) at 30 % amplitude. Ten microliters of liposomes was deposited on a SSM sensor, previously prepared by following manufacturer's guidelines using the SensorPrepA and the SensorPrepB1 solutions (IonGate, Germany). The SSM sensors were then centrifuged for 30 min at 2,500*g* and kept at 4 °C for at least 2 h. For the detection of electrical currents, the sensor was inserted in the SURFE^{2}R One instrument (originally acquired from IonGate, Frankfurt, Germany; now available through Nanion, Munich, Germany) and the transport activated by a rapid exchange of a solution at pH 7 (50 mM MOPS, pH 7, 140 mM potassium gluconate) with a solution at pH 6 (50 mM MES, pH 6, 140 mM potassium gluconate). As a control, currents from protein-free liposomes prepared in the same conditions as the proteoliposomes were recorded. Currents were recorded at a sampling rate of 1 kHz after filtering at 300 Hz.

Results and discussion

Native UCP1 extracted from cold-adapted mice BATs and further purified was incorporated into liposomes formed by

a mixture of phosphatidylcholine, phosphatidylethanolamine and cardiolipin. This lipidic composition resembles that of the inner mitochondrial membrane, known to have a high cardiolipin content compared to other membranes (Zinser et al. 1991). The liposomes were deposited on sensors following the procedure described in Materials and methods, inserted in the SURFE^{2}R One instrument and rinsed with a non-activating pH 7 solution. After 2 s, this solution was rapidly exchanged with an activating pH 6 solution, thus generating a H^+ gradient across the membrane. For UCP1-containing liposomes the pH jump elicited a transient "on-signal" that decayed within 1 s. Switching back to the non-activating solution led to a transient current in the opposite direction, the "off-signal" (Fig. 1) corresponding to the proton backflow. As the conditions of this reverse reaction are less defined (behavior of valves and liposome charge), only the "on-signal" was used for the analysis (Balannik et al. 2010), (Schulz et al. 2010). A positive "on-signal" is indicative of a displacement of positive charges (H^+) from the bath to the membrane-supporting electrode (Schulz et al. 2008). The time course of the current was characterized by a fast rise and a slow decay similar to pH-jump-induced currents observed for other proton-translocating transporters (Balannik et al. 2010), (Schulz et al. 2010). The decay could be fitted with a single exponential decaying with a time constant in the range of 100–200 ms. Repeating measurements on the same sensor at different times (up to 140 min) showed that the characteristics of the transient current were stable over at least a couple of hours. The current amplitude varied from one sensor to another because of the quantity of adsorbed proteoliposomes onto the sensor, a parameter difficult to control.

GDP is a known inhibitor of UCP1 activity. The present model is that the nucleotide acts from the cytosolic side of UCP1 by entering a cavity and binding deep inside the protein (Arechaga et al. 2001). Its influence on the transient current induced on UCP1 proteoliposomes was therefore tested herein. A 38 ± 2 % decrease in the signal was observed in the presence of 100 µM GDP (Fig. 2a). The inhibitory effect was reversible by rinsing with a GDP-free solution. The partial inhibition may be explained by random orientation of the protein in the liposomes, with only a fraction of the proteins having the GDP binding site exposed to the outside. Alternately, part of the signal could be due to the protonation of the protein and therefore not be affected by GDP. However, GDP inhibition was diminished by 12.8 % in the presence of 5 mM Mg^{2+} ions and increased by 10.5 % in the presence of 1 mM of the divalent ion chelator EGTA (Fig. 2b). These data are consistent with previous observations showing that UCP1 preferentially binds Mg^{2+}-free nucleotides (Klingenberg 1988) and demonstrate that the transient GDP-sensitive current was indeed due to the transport activity of UCP1.

Fatty acids are known activators of the UCP1 transport activity. As the UCP1 used in this study was purified from

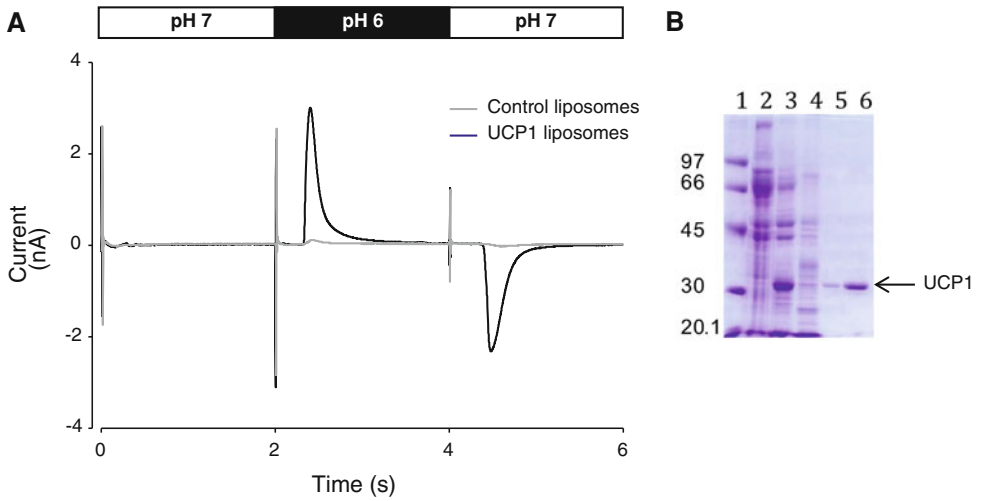

Fig. 1 Electrical currents associated with the presence of pure UCP1. **a** Sensors were prepared using liposomes devoid of UCP1 (Control liposomes) and liposomes enriched in UCP1 (UCP1 liposomes). pH-Dependent currents were evoked by a solution exchange protocol consisting of three successive 2-s flow steps, from pH 7 to pH 6 and back to pH 7. The non-activating pH 7 solution contained 50 mM MOPS and 140 mM potassium gluconate; the activating pH 6 solution contained 50 mM MES and 140 mM potassium gluconate. Traces are representative of measurements on at least 20 independent sensors. No significant difference was seen between sensors prepared from frozen or freshly prepared liposomes. **b** SDS-PAGE of UCP1 after purification from brown adipose tissue. Lanes from left to right: (1) Molecular weights of standards in kDa (Low Molecular Weight kit, GE Healthcare); (2) supernatant after washing mitochondria (30 min. incubation in 3.2 % lubrol); (3) supernatant after mitochondrial membrane solubilization step (1 h incubation in 2 % $C_{10}E_5$); (4) supernatant after a second step of solubilization of the non-solubilized pellet (overnight incubation in 2 % $C_{10}E_5$); (5 and 6) first and second fractions after hydroxyapatite column

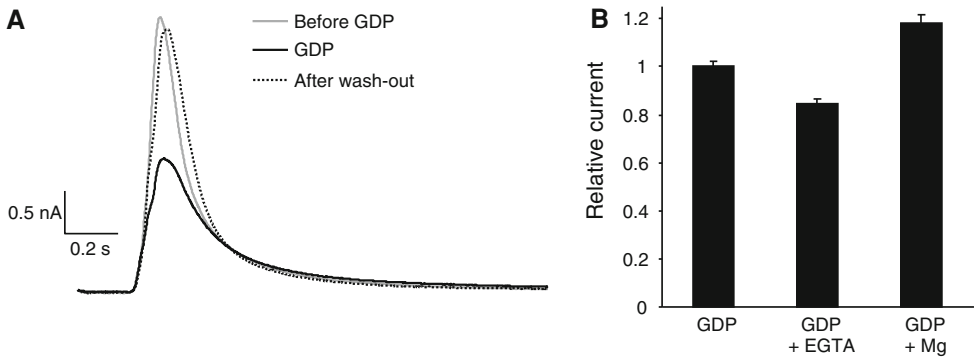

Fig. 2 Inhibition by GDP of pH-dependent transient currents. **a** Inhibitory effect of GDP. Signals generated by the pH jump in control (Before GDP), in the presence of 100 μM GDP (GDP) and after wash-out of GDP (After wash-out). The average inhibition of the peak amplitude of the transient current by GDP was 38 ± 2 % (mean ± SEM) of control, as measured in at least 100 different tests on at least 16 different sensors. **b** Histogram showing the effects of EGTA and Mg on inhibition by GDP. Peak currents were normalized to the control peak current obtained in the presence of 100 μM GDP. The data represent the mean ± SEM of at least ten different measurements on two different sensors. Mg^{2+} (5 mM) reduced GDP inhibition by 12.8 % and EGTA (1 mM) increased GDP inhibition by 10.5 %

brown adipose tissue, a tissue very rich in fatty acids, it is possible that fatty acids are still present in the proteoliposome preparation. In order to test this hypothesis, bovine serum albumin (BSA, a protein known to bind fatty acids) was first added in the recording buffers. Indeed, the current measured on UCP1-containing liposomes was inhibited in the presence of 15 μM BSA (fatty acid free). After washing out BSA, the current could be activated again by adding lauric acid to the activating solution (Fig. 3).

Our experimental results show the relevance of the method used herein to measure proton transport by UCP1.

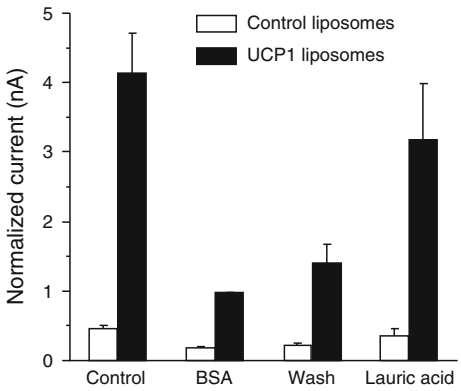

Fig. 3 Modulation of UCP1 currents by fatty acids. Currents measured in response to jumps from pH 7 to pH 6 were recorded in the following sequential conditions: (1) in the absence of effectors (Control); (2) in the presence of 15 μM BSA for 20–30 min (BSA); (3) after wash-out of BSA (Wash); (4) in the presence of 7.5 μM lauric acid (Lauric acid). Experiments were conducted using sensors prepared with UCP1-containing proteoliposomes (*black bars*) or with empty liposomes as a control (*white bars*). In order to allow comparison between control and UCP1 liposomes, currents were normalized to the current measured in BSA, then rescaled to current units by multiplying by the average current measured in BSA. The data represent the mean ± SEM of at least 30 different measurements on three different sensors

We believe that the present SSM method has several advantages over other previously reported methods of studying UCP1, not the least of them being that it requires little technical expertise. The patch-clamp technique has so far only been used to characterize the chloride transport of UCP1 (Huang and Klingenberg 1996). Because it is designed to record the activity of single channel proteins, it is very sensitive to undetectable amounts of protein contaminants such as ion channels or porins, and is not suited to measure the small fluxes of proton transporters. Spectroscopic measurements or the use of pH electrodes on proteoliposomes are more indirect as they measure the consequences of a pH or membrane potential modification (Klingenberg and Winkler 1985, Garlid et al. 1996, Mozo et al. 2006). In addition, the SSM method allows multiple trials on the same sensor chip, which is unique to proteoliposome-based approaches. The method described herein necessitates at least two orders of magnitude less protein quantity than most liposome methods as seen from the comparison of experimental details in the various publications. Such a decrease in protein quantity opens the possibility to perform protein transport measurements on low amount of recombinant proteins and therefore also mutants. This would constitute a major step forward in deciphering structure–function properties.

Conclusion

Deciphering the molecular mechanism of action of UCP1 is of great interest. Here we present a new assay for assessing the activity of this membrane protein that is based on the measurement of electrical currents associated with its transport activity. The currents recorded were stable for at least a couple of hours, modulated by UCP1

activators (fatty acids) and inhibitors (nucleotides). The development of this simple and robust functional assay paves the way for the detailed characterization of UCP1 and the large-scale screening of activatory and inhibitory compounds.

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