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RESEARCH LETTER

Human mitochondrial uncoupling protein 3 functions as a metabolite transporter

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Since its discovery, a major debate about mitochondrial uncoupling protein 3 (UCP3) has been whether its metabolic actions result primarily from mitochondrial inner membrane proton transport, a process that decreases respiratory efficiency and ATP synthesis. However, UCP3 expression and activity are induced by conditions that would seem at odds with inefficient 'uncoupled' respiration, including fasting and exercise. Here, we demonstrate that the bacterially expressed human UCP3, reconstituted into liposomes, catalyses a strict exchange of aspartate, malate, sulphate and phosphate. The R282Q mutation abolishes the transport activity of the protein. Although the substrate specificity and inhibitor sensitivity of UCP3 display similarity with that of its close homolog UCP2, the two proteins significantly differ in their transport mode and kinetic constants.

Keywords: amino acid transport; anion transport; bioenergetics; mitochondrial metabolism; mitochondrial transport; uncoupling protein

Mitochondria are the principal energy regulators of eukaryotic cells. During oxidative phosphorylation, the oxidation of NADH and $FADH_2$, produced by the catabolic pathway, generates energy in the form of a proton gradient which is utilized by ATP synthase to synthesize ATP [1]. However, some energy may be lost as heat when protons re-enter into matrix independently of ATP synthesis [2,3]. This proton leakage across the mitochondrial inner membrane can be either basally active or inducible. Inducible proton leak relies on expression of specific mitochondrial proteins localized in the inner mitochondrial membrane, called uncoupling proteins (UCPs) [3,4]. UCP1, the first uncoupling protein to be discovered, is a member of mitochondrial carrier family (MCF or SLC25) and believed to play a key

Abbreviations
Asp, aspartate; BAT, brown adipose tissue; EDTA, ethylene diamine tetraacetic acid; IPTG, isopropyl β-d-1-thiogalactopyranoside; LOOH, lipid hydroxy peroxides; Mal, malate; MCF, mitochondrial carrier family; Pi, phosphate; PIPES, piperazine-N,N′-bis(2-ethanesulfonic acid); ROS, reactive oxygen species; SDS/PAGE, Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis; SLC25, solute carrier family 25; SO_4, sulphate; UCP, uncoupling protein.

role in nonshivering thermogenesis of brown adipose tissue (BAT) [5]. Based on homology to UCP1, five other MCF members have been classified as uncoupling proteins, UCP2-UCP6 [6]. These proteins share an amino acid sequence motif called 'uncoupling protein signatures' [7]. However, the uncoupling capability of these proteins remains controversial, particularly because of their wide tissue distribution challenges their presumable function as uncoupling or thermogenic proteins [8–10].

Recently, it has been reported that human UCP2 catalyses the transport of aspartate, malate and oxaloacetate from mitochondria against phosphate plus a proton, playing a role in the regulation of both glucose and glutamine metabolism [6,11–13]. Drosophila UCP4 catalyses a unidirectional transport of aspartate [6]. Similarly, it has been demonstrated that human UCP5 and UCP6 efficiently exchange metabolites, such as sulphate, thiosulphate, sulphite and phosphate and even, though weakly, dicarboxylates [14]. Uncoupling protein 3 (UCP3) shares 72% of protein sequence with UCP2, but unlike UCP2, it is mainly expressed in skeletal muscle, though has been found also in BAT and heart tissues [15]. Upregulation of UCP3 in skeletal muscle protects against diet-induced insulin resistance in mice and diabetic patients' UCP3 is reduced by 50% compared with health men [16]. Ablation of UCP3 in mice makes the heart more susceptible to oxidative stress insult by decreasing the oxygen consumption rate and antioxidant enzyme activity and by inducing mitochondrial structural changes thus increases the incidence of myocardial ischemia–reperfusion injury [17,18].

It has been suggested that UCP3 is involved in mammalian thermoregulation as a proton uncoupler; however, several lines of evidence have argued against a proposed uncoupling function. UCP3 upregulation does not increase mitochondrial uncoupling during fasting [19] or exercise [20,21]. UCP3 knockout (KO) mice are not obese and lack severe thermoregulatory issues. Importantly, UCP3 KO mitochondria have reduced proton leak when compared with their wild-type (WT) littermates [22]. Interestingly, UCP3 KO shows impaired sympathomimetic-mediated thermogenesis and completely absent lipopolysaccharide-induced thermogenesis [23]. Separately from an uncoupling function, several studies have reported that UCP3 may play a key role in fatty acid oxidation and mitochondrial ROS mitigation, via a transport mechanism that introduced fatty acids into the matrix [24]. In fact, mitochondria isolated from the skeletal muscle of UCP3 KO mice exhibited a reduced capability to oxidize fatty acid compared with their WT counterparts [25]. Yet another proposed function of UCP3 is that it may protect mitochondria by exporting lipid hydroxy peroxides (LOOH) out of mitochondria, preventing oxidative damage to mitochondrial DNA and proteins [26,27]. This mechanism was supported by the observation that, unlike the mitochondria of UCP3 KO mice, mitochondria from UCP3 WT mice translocate LOOH across the inner membrane, and this process was associated with uncoupling activity [28]. What all the above studies clearly indicate is that there remains much to be understood about the biochemical and functional role of UCP3.

In the present study, we hypothesized that UCP3 may act as a mitochondrial transporter of aspartate or other metabolic intermediates in a similar way to UCP2. By taking a systematic biochemical approach, we found that recombinant human UCP3, reconstituted into liposomes, transported aspartate, malate, oxaloacetate, sulphate and phosphate. Despite partial overlaps in substrate specificity, UCP3 and UCP2 differ in their transport mode and kinetic constants. The most unique and defining difference between human UCP2 and UCP3 is that UCP3 does not catalyse a unidirectional transport of any metabolites. These findings suggest that the two proteins may have distinct biochemical and physiological roles, possibly specific to different tissues.

Materials and methods

Plasmids construction and bacterial expression of UCP3

The coding sequence of UCP3 was amplified from a human skeletal muscle cDNA by PCR. The forward and reverse oligos carried a Nde*I* and Hind*III* restriction sites, respectively. The product was cloned into the expression vector pRUN [29]. Transformants of *Escherichia coli* NEB (New England Biolabs) were selected on ampicillin (100 $\mu g \cdot mL^{-1}$) and screened by colony PCR. We introduced the mutation Arg282Gln (R282Q) into the WT UCP3 cDNA by overlap-extension PCR [30,31] using the above-mentioned pRUN plasmid as template. All plasmids were sequence-verified. The WT and mutant proteins were overexpressed in *E. coli* C0214(DE3) [32]. Inclusion bodies were purified on a sucrose density gradient [33] and were washed at 4 °C, first with PE buffer (10 mM PIPES, 1 mM EDTA, pH 6.9), then twice with a buffer containing Triton X-114 (3%, w/v), 1 mM EDTA and 10 mM PIPES pH 6.9, and finally with PE buffer.

Reconstitution of recombinant UCP3 and $UCP3_{R282Q}$ into liposomes and transport measurements

UCP3 and $UCP3_{R282Q}$ from Inclusion bodies were solubilized in 1.75% sarkosyl and then diluted 5.6-fold with a buffer containing 3% Triton X-114, 1 mM EDTA and 10 mM Pipes·NaOH (pH 6.9). The solubilized recombinant proteins were centrifuged at 13 000 × ***g*** for 15 min at 4 °C to remove small insoluble residues. The composition of the initial mixture used for reconstitution was 55 µL solubilized proteins (about 2 µg), 70 µL 10% Triton X-114, 100 µL preformed liposomes [10% (wt/vol) L-a-phosphatidylcholine from egg yolk (Sigma-Aldrich, Milan, Italy) in 2 mM Pipes·NaOH, pH 6.9], 20 mM substrate to be tested, 1 $mg{\cdot}mL^{-1}$ cardiolipin, 20 mM Pipes·NaOH (pH 6.9) and water to a final volume of 700 µL. After vertexing, the mixture was recycled 15 times through a Bio-Beads SM-2 Resin (Amberlite) column (4.0 × 0.5 cm). All operations were performed at 4 °C, except the passages through Amberlite, which were carried out at room temperature. External substrate was removed from proteoliposomes on a Sephadex G-75 column pre-equilibrated with buffer A (100 mM sucrose, 10 mM Pipes·NaOH, pH 6.9). Recombinant human UCP2 (hUCP2) [12] was reconstituted under the same experimental conditions.

Transport assays into liposomes

Transport at 25 °C was started by adding various labelled compounds to substrate-preloaded proteoliposomes (exchange) or to empty proteoliposomes (uniport). In both cases, transport was terminated by addition of a mixture of pyridoxal-5′-phosphate and bathophenanthroline 10 mM each. In controls, the inhibitors were added at the beginning together with the radioactive substrate according to the inhibitor stop method [33]. All transport measurements were carried out at the same internal and external pH value (pH 6.9). Finally, external radioactivity was removed from proteoliposomes using a Sephadex G-75 column. The proteoliposomes were eluted with a 50 mM NaCl solution, and their radioactivity was measured. The experimental values were adjusted by subtracting control values. The initial transport rate was determined based on the radioactivity taken up by proteoliposomes after 2 min, which falls within the initial linear range of substrate uptake. In the case of efflux measurements, proteoliposomes containing 2 mM unlabelled substrates were labelled with 5 µM of radioactive substrates through carrier-mediated exchange equilibration [34]. After a 30-min incubation period, the external radioactivity was removed by passing the proteoliposomes through Sephadex G-75. Efflux was initiated by adding unlabelled external substrate or NaCl to preloaded proteoliposomes and stopped by the addition of the aforementioned inhibitors at different time intervals. Finally, the external radioactivity was removed by a Sephadex G-75 column; the proteoliposomes were eluted with 50 mM NaCl, and their radioactivity was measured.

Other methods

The amounts of pure recombinant proteins were estimated by laser densitometry of stained samples, using carbonic anhydrase as protein standard [35]. The amount of protein incorporated into liposomes was measured as previously described [35] and varied between 12% and 20% of the protein added to the reconstitution mixture.

Results

Bacterial expression and functional reconstitution of recombinant UCP3 and $UCP3_{R282Q}$

Recombinant proteins, accumulated as inclusion bodies in *E. coli* C0214(DE3) strain, were purified by centrifugation on a sucrose gradient and washed. The isolated proteins gave an almost pure single band by SDS/PAGE with apparent molecular mass of 33 kDa (Fig. 1A). The 33 kDa band was not detected in bacteria collected immediately before the addition of the inducer, IPTG, nor in cells carrying the empty vector and harvested after induction (Fig. 1A). The WT recombinant UCP3 was reconstituted into liposomes and assayed in homo-exchange experiments (i.e. with the same substrate inside and outside the proteoliposomes). Using external and internal substrate concentrations of 1 and 10 mM, respectively, we initially tested the main substrates known to be transported by UCP2 [12].

The reconstituted UCP3 efficiently catalysed the homo-exchanges of aspartate, malate and to a lesser extent those of sulphate and phosphate, which were inhibited completely by pyridoxal-5′-phosphate and bathophenanthroline (Fig. 1B). No transport activity was found with ATP, ADP and AMP (not shown). To verify that the transport activity found was not due to any experimental artefact, the recombinant WT and $UCP3_{R282Q}$ were assayed in the same experimental conditions. The replacement of this conserved arginine in the sixth transmembrane a-helix with a glutamine abolishes the transport activity of other members of the MCF [11,36,37]. As expected, the recombinant $UCP3_{R282Q}$ failed to catalyse any of the homo-exchanges catalysed by the WT protein (Fig. 1B).

Kinetic characteristics of the recombinant UCP3

The uptake of 1 mM [^{14}C]Aspartate into proteoliposomes was measured both as uniport (in the absence

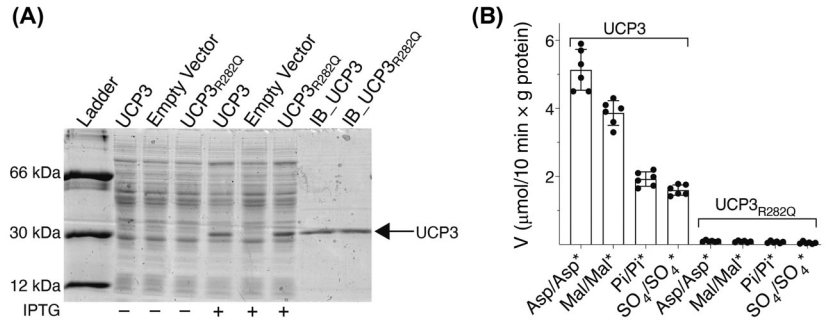

Fig. 1. Reconstituted recombinant uncoupling protein 3 (UCP3) catalyses an efficient homo-exchange of aspartate or malate. (A) Expression in *Escherichia coli* of UCP3 and $UCP3_{R282Q}$. Proteins were separated by SDS/PAGE and stained with Coomassie blue dye. The same number of bacteria were analysed in each sample (Lanes 1–6). Inclusion bodies purified from bacteria reported in the fourth and sixth lanes are shown. (B) Recombinant UCP3 and $UCP3_{R282Q}$ were reconstituted into liposomes and tested for their ability to catalyse various homo-exchanges [internal unlabelled substrate (10 mM) and external labelled substrate (*) (1 mM)]. Asp, aspartate; IB, Inclusion bodies; Mal, malate; Pi, phosphate; SO_4, sulphate. The error bars are displayed as mean ± SD values [data from two technical replicates of three independent experiments (B)].

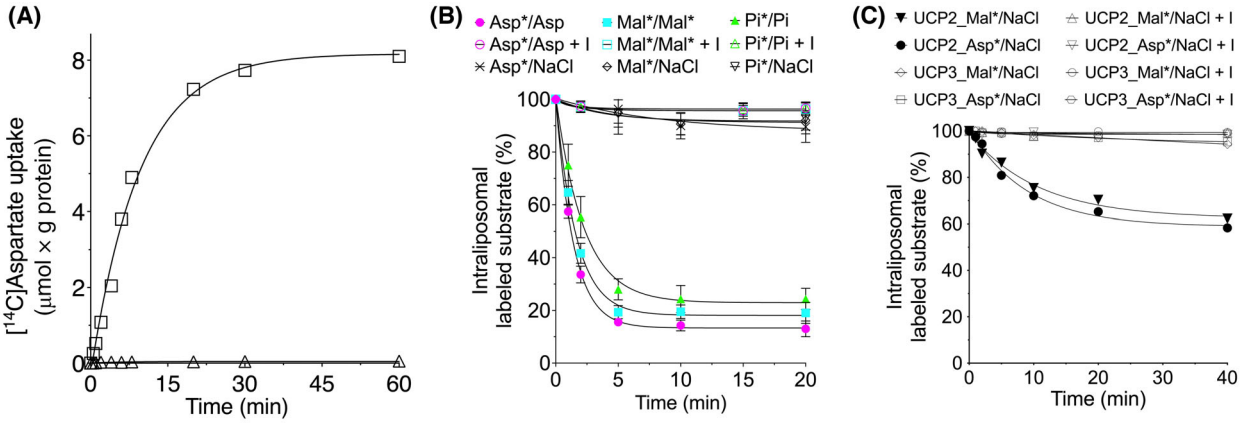

Fig. 2. Reconstituted recombinant uncoupling protein 3 (UCP3) functions as strict exchanger. (A) Time course of [^{14}C] aspartate uptake in proteoliposomes reconstituted with the recombinant UCP3. [^{14}C] aspartate (1 mM) was added to proteoliposomes containing 10 mM aspartate (□) (exchange reaction) or 10 mM NaCl (▵) (Unidirectional transport). A representative time course of three independent experiments is reported. (B) Efflux of various labelled substrates (*) (2 mM) from proteoliposomes reconstituted with UCP3. The internal substrate pool was labelled with 5 µM labelled substrate (*) by carrier-mediated exchange equilibration. Then, the proteoliposomes were passed through Sephadex G-75. Efflux was initiated by adding 10 mM of the indicated compounds. Asp, aspartate; I, inhibitors; Mal, malate; Pi, phosphate. Values are means ± SD from three independent experiments. (C) Unidirectional transport assay carried out on recombinant hUCP3 and hUCP2 reconstituted under the same experimental conditions established for UCP3. The transport assay was conducted as described in (B).

of internal substrate) or as exchange (in the presence of internal 10 mM aspartate; Fig. 2A). The uptake of [^{14}C]Aspartate followed a first-order kinetics (rate constant 0.105 min^{-1}; initial rate 0.85 µmol·min^{-1} × g protein) with isotopic equilibrium being approached exponentially (Fig. 2A). Conversely, no [^{14}C]aspartate uptake was found into proteoliposomes loaded with NaCl (no internal substrate), indicating that the recombinant UCP3 was unable to catalyse the transport of substrate unidirectionally. Since UCP2 was able to catalyse also a unidirectional transport of substrate [12], we aimed to further confirm that UCP3 was a strict exchanger. To achieve this, we measured the efflux of the main labelled transported substrates from prelabelled active proteoliposomes, as it provides a more convenient assay for detecting a unidirectional transport [38]. In the absence of the external substrates (10 mM of NaCl), no efflux was observed after a 20-min incubation period (Fig. 2B), whereas extensive effluxes occurred upon the addition of 10 mM of external aspartate or malate or phosphate (Fig. 2B). These results confirmed the obligatory exchange reaction of substrates catalysed by the reconstituted UCP3.

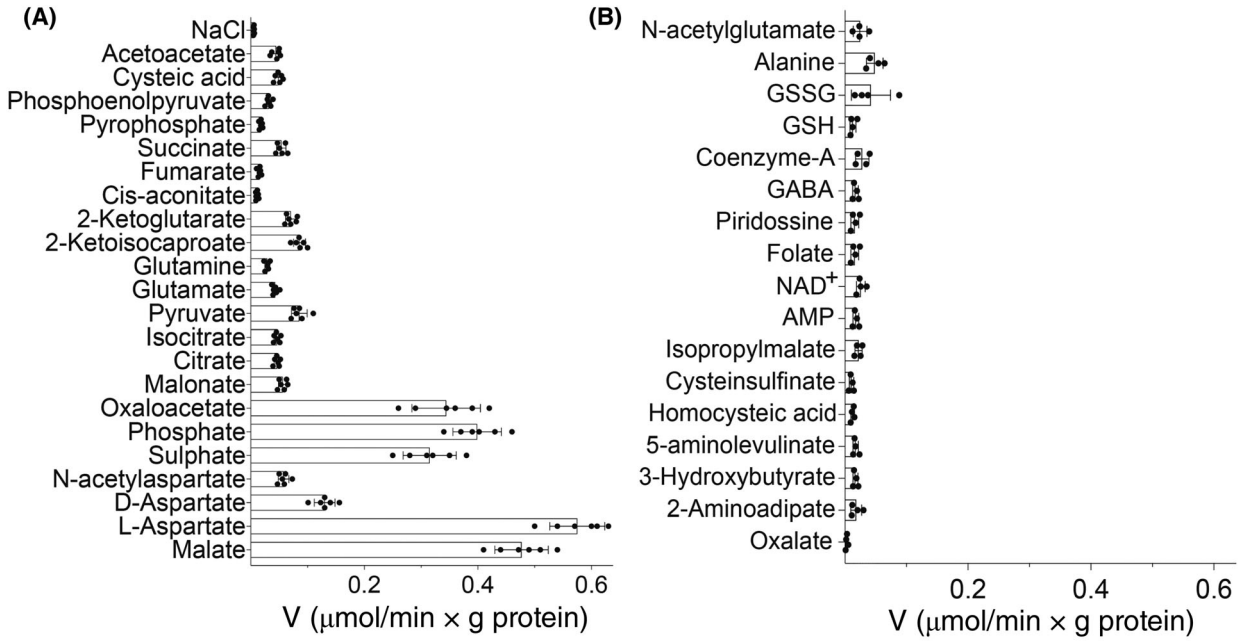

Fig. 3. Aspartate uptake into liposomes reconstituted with the recombinant uncoupling protein 3 (UCP3) and preloaded with various substates. (A, B) Proteoliposomes were preloaded internally with various substrates (concentration 10 mM). Transport was started by adding 1 mM [^{14}C] aspartate to proteoliposomes reconstituted with UCP3 and terminated after 2 min. The error bars are displayed as mean ± SD values [data from two technical replicates of three (A) or two (B) independent experiments].

Although UCP3 was expressed and reconstituted under nearly the same conditions as those previously employed for the functional characterization of UCP2 [12], we tested the unidirectional transport of both proteins side by side using exactly the experimental setup used for UCP3. As expected UCP2 was able to catalyse a unidirectional transport of aspartate or malate whereas no detectable efflux of substrates was observed from proteoliposomes reconstituted with UCP3 (Fig. 2C) The kinetic constants of the recombinant UCP3 were determined by measuring the initial transport rate at various external labelled substrates concentrations in the presence of a constant saturating internal concentration of unlabelled substrates (10 mM). The transport affinities (K_m) for aspartate, malate, sulphate and phosphate were 0.92 ± 0.08, 1.58 ± 0.16, 0.91 ± 0.11 and 0.86 ± 0.09 mM respectively; the specific activities (V_{max}) for aspartate and malate was 1.52 ± 0.2 μmol × min × g of protein, whereas that of sulphate and phosphate was 0.36 ± 0.06 μmol × min × g of protein. For all substrates, at least four independent experiments were done. Interestingly, UCP3 exhibited a Km for aspartate approximately seven times lower than that previously determined for UCP2 [12]. This difference was further confirmed by reconstituting the two proteins side by side. In two experiments, UCP3 and UCP2 showed Km values for aspartate of 0.87 ± 0.11 and 6.92 ± 0.85 mM, respectively.

Substrate specificity of the recombinant UCP3

The substrate specificity of recombinant UCP3 was determined by measuring the uptake of [^{14}C] aspartate into proteoliposomes preloaded with a variety of potential substrates (Fig. 3A,B). The highest [^{14}C] aspartate uptake into proteoliposomes was measured with internal aspartate and malate, to a lesser extent were also exchanged sulphate, phosphate and oxaloacetate. A low transport activity was found in proteoliposomes internally preloaded with D-aspartate, N-acetylaspartate, malonate, citrate, isocitrate, pyruvate, 2-Ketoisocaproate, 2-Ketoglutarate, succinate, cysteic acid and acetoacetate. All the remaining substrates tested were poorly exchanged against aspartate (Fig. 3A,B). A comparable substrate specificity was recently reported for the recombinant murine UCP3 at the Annual Meeting of the Biophysical Society [39].

Inhibitor sensitivity of the recombinant UCP3

The effect of inhibitors on the [^{14}C] aspartate/aspartate exchange reaction catalysed by the recombinant UCP3 were also determined. The transporter was strongly

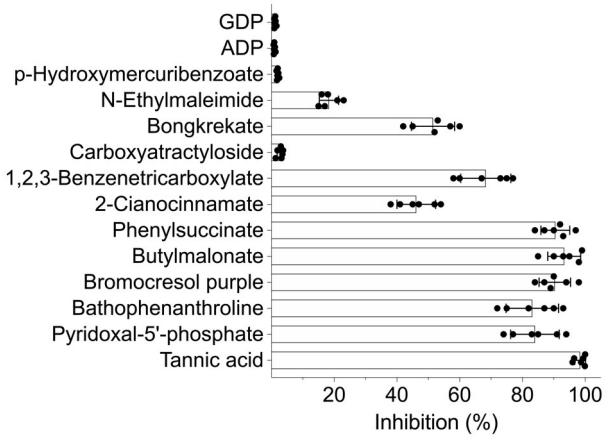

Fig. 4. Effect of inhibitors on the [^{14}C] aspartate/aspartate exchange catalysed by uncoupling protein 3 (UCP3). Proteoliposomes were preloaded internally with 10 mM aspartate. Transport was initiated by adding 1 mM [^{14}C] aspartate to proteoliposomes reconstituted with UCP3 and was stopped after 2 min. The sulfhydryl reagents were added 2 min before the labelled substrate. The remaining inhibitors were added together with the labelled substrate. The final inhibitor concentrations were 1 mM N-ethylmaleimide, 10 μM p-hydroxymercuribenzoate, bongkrekate and carboxyatractyloside, 10 mM for the remaining. The extent of inhibition (%) is reported. The error bars are displayed as mean ± SD values (two technical replicates of three independent experiments).

inhibited by well-known inhibitors of other MCF members as tannic acid, pyridoxal-5′-phosphate, bathophenanthroline and bromocresol purple [29,40–43] (Fig. 4).

The substrate specificity of UCP3 towards malate was further confirmed by the strong inhibitory effect exerted by butylmalonate and phenylsuccinate, two well-known inhibitors of the dicarboxylate carrier [32] and the oxoglutarate/malate carrier [44], as well as by 1,2,3-benzentricarboxylate a specific inhibitor of the citrate carrier which exchanges citrate against malate [45,46]. Interestingly, UCP3 showed a different sensitivity against two well-known inhibitors of the mitochondrial adenine nucleotide carrier, bongkrekate and carboxyatractyloside, being significantly inhibited by the former and not affected at all by the latter. The sulfhydryl reagents p-chloromercuribenzoate and N-ethylmaleimide had little or no effect on the [^{14}C] aspartate/aspartate exchange reaction.

Discussion

For many years, the biochemical function of UCP3 has been a subject of debate, with the fatty acid-dependent protonophoric function hypothesis being the most widely supported. However, recent patch-clamping experiments conducted on the inner mitochondrial membrane have definitively ruled out this hypothesis [10]. The data presented in this study, for the first time, demonstrate that UCP3 functions as a metabolite transporter, similar to most members of the mitochondrial carrier family [47]. This confirms its structural characteristics, which are typical of the small carboxylic or keto acids class of mitochondrial carriers [48]. Although this preliminary study demonstrates that UCP3 shares discrete similarities in substrate specificity and inhibitor sensitivity with UCP2 [12], the two reconstituted proteins differ in several aspects: (a) UCP3 does not transport malonate, a three-carbon dicarboxylate transported by UCP2 and the dicarboxylate carrier [32]; (b) Unlike UCP2, UCP3 is unable to catalyse the unidirectional transport of substrates (Fig. 2); (c) UCP3 transports aspartate and malate more efficiently than sulphate, phosphate and oxaloacetate (Figs 1B and 3A), with a V_{max} value for the aspartate/aspartate or malate/malate exchanges about fivefold higher than that found for phosphate/phosphate or sulphate/sulphate exchanges. In contrast, UCP2 primarily transports phosphate and aspartate, with the remaining substrates transported with similar efficiency [11,12]; and (d) most importantly, UCP3 exhibits a much higher affinity for aspartate, approximately sevenfold higher than that of UCP2 [12]. Although the degree of protein identity between UCP3 and UCP2 (72%) is similar to or even higher than that found in other MCF isoforms, such as the glutamate carrier or the aspartate/glutamate carrier [40,49], the aforementioned differences between the two reconstituted proteins suggest that they may not be necessary isoforms. While differences in the kinetic parameters between MCF isoforms are quite common [40,49,50], none of them has ever shown any difference in the transport mode (uniporter or exchanger) or variations in substrate specificity. While the functional characterization of most recombinant mitochondrial transporters into liposomes has been confirmed at the physiological level, it is important to note that this study has been conducted using *in vitro* refolded protein and requires further confirmation through alternative experimental approaches. Therefore, additional research in cell or mouse models is necessary to gain a deeper understanding of the precise biochemical and physiological role of UCP3 *in vivo*. This study marks the beginning of illuminating why UCP2 and UCP3 exhibit differential regulation within the same cell types and counteractively modulate the same metabolic pathway [24] or play opposing roles in cancer [51–53].

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Author contributions

FD, AA, AV, SNB and DD performed experiments; FD and LC analysed data. JD, ST and GF project administration. ST, JD, VD, LP, CLR and GF wrote the manuscript. VD, LP and GF made manuscript revisions.

Peer review

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Data accessibility

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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