Transport Kinetics of Uncoupling Proteins

ANALYSIS OF UCP1 RECONSTITUTED IN PLANAR LIPID BILAYERS*

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Members of mitochondrial protein subfamily, uncoupling proteins (UCP),1 uncouple substrate oxidation from mitochondrial ATP synthesis and thereby catalyze energy dissipation (for review see Refs. 1–4). First described as thermogenin or GDP-binding protein (5, 6), the 32-kDa UCP1 is so far the best investigated member of this protein subfamily. It is expressed exclusively in brown adipose tissue, an organ of non-shivering thermogenesis in newborn mammals, hibernators, and cold-adapted rodents. According to alternative hypotheses, UCP1 is either a proton channel or a fatty acid anion carrier. Transport across the proton channel along a chain of hydrogen bonds (Grotthus mechanism) (i) may include fatty acid carboxyl groups or occur in the absence of fatty acids. In this work, we demonstrate that planar bilayers reconstituted with UCP1 exhibit an increase in membrane conductivity exclusively in the presence of fatty acids. Hence, we can exclude the hypothesis considering a preexisting H+ channel in UCP1, which does not require fatty acid for function. The augmented conductivity is nearly completely blocked by ATP. Direct application of transmembrane voltage and precise current measurements allowed determination of ATP-sensitive conductances at 0 and 150 mV as 11.5 and 54.3 pS, respectively, by reconstituting nearly 3 × 105 copies of UCP1. The proton conductivity measurements carried out in presence of a pH gradient (0.4 units) allowed estimation of proton turnover numbers per UCP1 molecule. The observed transport rate of 14 s⁻¹ is compatible both with carrier and channel nature of UCP1.

According to alternative hypotheses, mitochondrial uncoupling protein 1 (UCP1) is either a proton channel (“buffering model”) or a fatty acid anion carrier (“fatty acid cycling”). Transport across the proton channel along a chain of hydrogen bonds (Grotthus mechanism) may include fatty acid carboxyl groups or occur in the absence of fatty acids. In this work, we demonstrate that planar bilayers reconstituted with UCP1 exhibit an increase in membrane conductivity exclusively in the presence of fatty acids. Hence, we can exclude the hypothesis considering a preexisting H⁺ channel in UCP1, which does not require fatty acid for function. The augmented conductivity is nearly completely blocked by ATP. Direct application of transmembrane voltage and precise current measurements allowed determination of ATP-sensitive conductances at 0 and 150 mV as 11.5 and 54.3 pS, respectively, by reconstituting nearly 3 × 10⁵ copies of UCP1. The proton conductivity measurements carried out in presence of a pH gradient (0.4 units) allowed estimation of proton turnover numbers per UCP1 molecule. The observed transport rate of 14 s⁻¹ is compatible both with carrier and channel nature of UCP1.

Members of mitochondrial protein subfamily, uncoupling proteins (UCP),¹ uncouple substrate oxidation from mitochondrial ATP synthesis and thereby catalyze energy dissipation (for review see Refs. 1–4). First described as thermogenin or GDP-binding protein (5, 6), the 32-kDa UCP1 is so far the best investigated member of this protein subfamily. It is expressed exclusively in brown adipose tissue, an organ of non-shivering thermogenesis in newborn mammals, hibernators, and cold-adapted rodents. According to alternative hypotheses, UCP1 is either a proton channel or a fatty acid anion carrier. Transport across the proton channel along a chain of hydrogen bonds (Grotthus mechanism) (i) may include fatty acid carboxyl groups (7, 8) or (ii) occurs in the absence of fatty acids (9). The second hypothesis of FA cycling presumes two transport steps: the flip-flop of protonated fatty acids along their interleaflet concentration gradient and the subsequent backward transport of the deprotonated (anionic) fatty acid by UCP (10, 11). FA anions are expelled from the matrix side by the negative membrane potential (ΔΨ) formed in respiring mitochondria in vivo.

Patch clamp recordings of UCP1 that were undertaken after protein reconstitution into giant liposomes did not reveal proton but chloride channel properties (12). The artificial Cl⁻ conductance was by six orders of magnitude higher than the observed Cl⁻ unipor in mitochondria. It does not necessarily mean that proton channels are lacking but that proton channel activity may have been masked by the high Cl⁻ conductance or by the low proton concentration. In the absence of direct evidence for proton channels or carriers, the determination of proton transport rate may serve as a criterion to distinguish between channel and FA-cycling hypothesis, the latter being a special type of a carrier mechanism. The transport rate of 10⁵–10⁶ s⁻¹ would suggest a conductance by channels, because a carrier usually operates at rates of 10⁻³–10⁻⁴ s⁻¹. Slow rates, however, may be due to a channel with low conductance as well (13). Unfortunately, no clear picture has emerged so far. The turnover rates observed for UCP1 in reconstituted liposomes differ by several orders of magnitude. They range from 1000 s⁻¹ (7) via 20 s⁻¹ with native UCP1 (11) to 7 s⁻¹ (14) and 3 s⁻¹ with recombinant UCP1 (15). Differences in the experimental conditions, i.e. in ΔΨ, in concentration and species of fatty acids used may account for this divergence only marginally.

To solve the controversy, we have used a well defined system that allows current (I) measurements across planar bilayers with reconstituted UCP1. We have found that the presence of FA is absolutely essential for H⁺ conductance mediated by UCP1. Hence, we can exclude the hypothesis of Rial et al. (9) considering H⁺ channel activity in the absence of FA. We have also shown that ATP inhibits the UCP1-mediated H⁺ conductance. The transport rate observed is compatible both with carrier and channel nature of UCP1.

EXPERIMENTAL PROCEDURES

Preparation of Proteoliposomes Containing UCP1—Brown adipose tissue (BAT) mitochondria from Syrian hamsters were isolated by a standard procedure (16). The mitochondrial protein content was measured by RC-DC protein assay (Bio-Rad). For preparation of proteoliposomes with so-called lipid protection, the procedure described earlier was used (17, 18). Isolated mitochondria (usually 10–15 mg of protein) were washed by the cooled extraction medium (50 mM K₂SO₄, 20 mM K-MOPS (Fluka, Germany), 0.2 mM EGTA (Sigma), pH 6.7) and subsequently centrifuged (20,000 x g). Escherichia coli lipid extract or E. coli lipid extract/noyau extract (3:1, both from Avanti Polar Lipids) was dissolved by double amount of octylpentaaxyethylene detergent (Bachem Feinchemikalien, Büedendorf, Switzerland) in extraction medium. The mitochondrial pellet was extracted with the resulted lipid-detergent micelles. Extracted mitochondrial proteins were added to the
spin column containing 0.4 g of wet hydroxyapatite (Bio-Gel HTP, Bio-Rad). Centrifugation of the column yielded a flow-through fraction enriched with UCP1. This fraction was mixed with the rest of lipid-detergent micelles. This procedure resulted in partially purified UCP1 in lipid-detergent micelles (17). Proteoliposomes were prepared subsequently by the detergent-removal method while incubating the mixture overnight on a 3-ml column of wet Bio-Beads (Bio-Rad). After centrifugation of the Bio-Beads column, the flow-through fraction, already containing proteoliposomes, was recovered. To deplete it of detergent traces, a second Bio-Beads spin column was subsequently used. Proteoliposomes were kept on ice until used for the formation of planar membranes. The content of UCP1 in proteoliposomes was measured by the Amido Black method (19) and related to the lipid concentration (microgram of protein/milligram of lipids).

Formation of Planar Lipid Membranes—Planar lipid bilayers were made from proteoliposomes that spontaneously form monolayers on the top of a suspension. Two of such monolayers were combined to a bilayer membrane (20, 21) within an aperture (90–130 μm in diameter) of a thin polytetrafluoroethylene film (GoodFellow, Cambridge, United Kingdom). Prior to membrane formation, the polytetrafluoroethylene septum separating the two aqueous phases was pretreated by a hexadecane/hexane mixture (1:99). The solutions of UCP1-containing liposomes (proteoliposomes) or liposomes were used in final concentration of 1–2 mg of lipid/ml buffer. Fatty acids were added, if necessary, to dilute proteoliposomes in ethanol solution and incubated at least 1 h on ice before moving to the measurement chamber. The buffer contained, if not otherwise indicated, 50 mM K2SO4, 50 mM K-MOPS, and 0.6 mM EGTA, pH 7.7, at room temperature. Measurements were carried out by the conductance change (ΔG) of UCP1 containing bilayers after addition of stearic acid containing liposomes (40%) in buffer solution. The concentration of stearic acid is presented as its aqueous concentration in buffer solution.

Conductivity Measurements—Current-voltage (I-V) characteristics were measured by a patch clamp amplifier (GeneClamp 5000, Axon Instruments). For the input into a computer, the signal was converted by an A/D converter (DigiData, Axon Instruments). It was sampled at 40 Hz and filtered with 1 Hz (Lampfit software, Axon Instruments). For conductance measurements, a triangular voltage source (signal generator, Wavetek) operating at frequencies of 0.04–0.07 Hz was used. Membrane conductivity G was determined at zero voltage from a linear fit of voltages on the interval between −50 and 50 mV.

The proton conductance was measured in the same setup. Because it cannot be distinguished from the OH− permeability, it is denoted as G\text{H+}. The proton conductance of bilayers can be overshadowed by the conductance of other ions. To determine the contribution of G\text{H+} to the total membrane conductance, G, the Nernst potential for protons was measured (22). For this purpose, a pair of well buffered solutions was chosen having similar osmolarities, ionic strength, and concentrations of all of the ions with the exception of H+ and OH−. The experimental H+ Nernst potential at a pH gradient is equal to the shift of the reversal potential due to the presence of a transmembrane pH gradient. Therefore, current-voltage characteristics of membranes containing UCP1 and oleic acid (14.5%) were measured. In the range from −50 to 50 mV, the current-voltage characteristics before (full triangles) and after (open circles) the formation of the pH gradient were collected.

\[ V_{\text{app}} = V_{\text{cis}} - V_{\text{trans}} \]

where \( V_{\text{cis}} \) and \( V_{\text{trans}} \) are the potentials across the cis and trans sides of the membrane, respectively (see “Experimental Procedures”).

H\textsuperscript{+} conductance in the presence of a transmembrane pH gradient was adjusted to 7.5 using 7.6 mM HEPES on the cis side and 7.6 mM Tris on the trans side. The transference number of H\textsuperscript{+}/OH\textsuperscript{−} was found as shown in Equation 1,

\[ T_{\text{H+}} = \frac{V_{\text{cis}}}{V_{\text{app}}} \]

where \( \Psi_{\text{N}} \) is the theoretical value of the Nernst equilibrium potential (23.8 mV for a pH gradient of 0.4). The final proton conductivity \( G_{\text{H+}} \) is calculated as shown in Equation 2,

\[ G_{\text{H+}} = G_{\text{H+}} \times G \]

where \( G \) is expressed in S/cm². Calculation of the number of UCP molecules per cm² allows to estimate the turnover number in s\textsuperscript{-1}, r. If
one lipid molecule occupies an area of $7.8 \times 10^{-12}$ m$^2$, the bilayer accommodates $\sim 2.6 \times 10^{14}$ lipids/cm$^2$. With respect to a lipid protein mass ratio, $p$, and molecular weights of 750 and 33,000 Da for the lipid and the protein, respectively, this transfers into Equation 3,

$$r = \frac{GH \times p \times U}{6 \times 10^7 \epsilon} \quad \text{(Eq. 3)}$$

where $\epsilon$ is the electron charge and $U$ the applied voltage (22, 23), $p = 2000$ corresponds to $3 \times 10^9$ protein monomers/cm$^2$. For comparison with literature data, $U$ is assumed to be equal to 180 mV. Because the $I-V$ relationships are non-linear and $G_{\text{HIGH}}$ is estimated from the conductivity at zero voltage, the turnover numbers represent the lowest estimations. Functioning of UCP1 as a dimer (4, 8, 24, 25) would tend to double all of the calculated turnovers.

RESULTS

Membrane Conductivity in the Presence of Uncoupling Protein UCP1—In the absence of fatty acids, the conductivity $G_0$ of planar lipid membranes reconstituted with UCP1 was $1.3 \times 10^{-8} \pm 0.5 \times 10^{-8}$ S/cm$^2$. It was comparable to $G_0$ of a protein-free membrane ($1.7 \times 10^{-8} \pm 0.8 \times 10^{-8}$ S/cm$^2$). This clearly indicates that UCP1 is incapable of increasing conductivity in sulfate medium in the absence of fatty acids.

Fatty acids were either immediately introduced into the membrane-forming solution (oleic acid) or were added to the membrane-surrounding buffer after membrane formation (stearic acid). Incorporation of oleic acid into a protein-free planar membrane (up to 20 weight %) led to only a small conductivity increase proportionally to the FA/lipid ratio up to $5 \times 10^{-8}$ S/cm$^2$ (Fig. 1, open diamonds). The same concentrations of oleic acid resulted in a pronounced (10–30-fold) conductivity increase of bilayers reconstituted with UCP1 (Fig. 1, full circles). Note that UCP1 alone in planar membranes free of FA did not increase the measured conductivity. The FA-mediated conductivity was inhibited by 1.9 mM ATP (Fig. 1, open circles). Reproducible results were achieved only if the nucleotide was allowed to equilibrate with the proteoliposome suspension before the membrane was formed. ATP addition subsequent to UCP1 stimulation by FA revealed a large variability of the inhibitory effect. Most probably, it was due to the circumstances that (i) two lipid phases containing UCP1 (proteoliposomes and planar bilayer) were present at the same time in the chamber; (ii) a large bulk volume (1.5 ml on both sides of the membrane) surrounded the bilayer; and (iii) in sulfate media used to exclude chloride, ATP has a low affinity to UCP1 (8). To circumvent these difficulties, ATP was added directly to the membrane-forming solution.

Substitution of oleic for stearic acid also resulted in a concentration-dependent conductivity increase. Because of its addition into the aqueous solution, its final membrane concentration was smaller and, hence, the conductivity increase was less pronounced (Fig. 1, inset).

Current-Voltage Characteristics of Membranes Reconstituted with UCP1—The current-voltage characteristics of reconstituted planar membranes and protein-free membranes did not differ from each other (Fig. 2). These findings also support the conclusion that UCP1 requires fatty acids to uncouple. A significant increase in conductivity was observed with 16% (w/w) oleic acid corresponding to $\sim 45$ mol % in the bilayer-forming solution. Quasi-exponential shape of this current-voltage characteristics yielded apparent conductances of 16.2 pS at 0 mV and 59 pS at 150 mV. The conductivity was inhibited by 1.9 mM ATP added to the membrane-forming solution. The current-voltage characteristics measured after inhibition by ATP were linear in the interval from $-100$ to $+100$ mV, yielding a conductance of 4.7 pS. Non-linearities observed at voltages of around $-150$ mV may be due to electrostriction effects. Correcting for the base-line conductivity, the ATP-sensitive portion of conductance accounts for 11.5 pS at 0 mV and 54.3 pS at 150 mV. Because $3 \times 10^5$ UCP1 molecules were reconstituted, the single-molecule conductance corresponds to $2 \times 10^{-17}$ or $10 \times 10^{-17}$ S, respectively.

Oleic Acid-mediated Proton Conductivity of Planar Membranes Reconstituted with UCP1—To measure proton conductance avoiding background effects produced by other ions, ionically balanced buffer mixtures were used (26). A transmembrane pH gradient across planar bilayers (0.4 units pH) was produced by adding of 7.6 mM HEPES and 7.6 mM Tris on the cis and trans sides of the membrane, respectively. Current-voltage characteristics of bilayers containing UCP1 and oleic acid (Fig. 3, open circles) and bilayers containing only UCP1 (Fig. 3, full triangles) were measured in the range from $-50$ to 50 mV. $G_{\text{HIGH}}$ was derived from the shift of the reversal potential in the presence of the pH gradient (for details see “Experimental Procedures”). It increased significantly only if both UCP1 and oleic acid were present (Fig. 3).

UCP1 Turnover Numbers—Based on current measurements with five independent proteoliposome preparations, the final proton conductivity, $G_{\text{HIGH}}$, and substrate turnover numbers per protein molecule, $r$, were calculated according to Equations 2 and 3 (Fig. 4). The concentration of oleic acid in lipid was varied from 4.2 to 14.8 weight % (corresponding to $\sim 11–39.3$ mol %). At saturating oleic acid concentration, a turnover number of $14 \pm 5$ s$^{-1}$ was estimated (Fig. 4), which is very close to values found as $V_{\text{max}}$ for lauric acid and UCP1 reconstituted in liposomes (11). The apparent $K_m$ for oleic acid was equal to
−8 ± 5% (w/w oleic acid/lipid). 1.9 mM ATP decreased the turnover rates by at least one order of magnitude (Fig. 2).

DISCUSSION

Since the discovery of UCP1, it has been debated whether fatty acids are essential for protein uncoupling activity and whether UCP1 is a channel or a carrier. To elucidate these issues, we have investigated proton transport in planar bilayers reconstituted with purified UCP1. The first functional reconstitution of UCP1 in planar membranes demonstrates that fatty acids are necessary and sufficient to enable UCP-mediated proton transport. Thus, the hypothesis of Rial et al. (9), concerning preexisting H⁺ channel that does not require FA for protein function, can be excluded. The increase of membrane conductivity depends on FA concentration and localization. 80 µM oleic acid in the aqueous solution augmented the conductivity "only" 2-fold (Fig. 1, insert), whereas 15% (w/w) oleic acid introduced directly into the lipid membrane resulted in a 30-fold conductivity increase (Fig. 1). This observation is in line with the hypothesis that UCP1 recruits fatty acids from the lipid phase (7, 27). Similar high FA-to-lipid molar ratios were required to stimulate proton transport by UCP1 reconstituted into liposomes (7, 27). A comparison with experiments carried out on native mitochondria can be done by considering that BAT mitochondria that were isolated without bovine serum albumin contained ~1.3 mol % FA in lipids (28). Moreover, maximum stimulation by FA has been reported at 600 nmol FA/mg rat BAT mitochondrial protein, which corresponds up to ~45 mol % (see Fig. 15 in Ref. 29). A physiological relevance of high FA levels is supported by findings that FA concentrations up to 20 mm (1) occur in BAT on activation of lipolysis (8). Generally, venous blood FA concentrations vary widely between 0.25 and 3 mM and FA can be rapidly transported across the plasma membrane (30). Due to the high partition coefficient (>10⁶), >90% of total FA amount is distributed in the membranes.

UCP1 and other UCPs are structural homologues of other mitochondrial anion carriers (altogether ~46 human genes), such as the ADP/ATP carrier or the phosphate carrier. A carrier-type activity of UCP1 protonophoric activity seems to be in agreement with its genetic origin. UCP1 has also long been known to conduct halide anions and a wide variety of monovalent unipolar anions (4). Hence, it shares the anion transport activity with other carriers. To explain the protonophoric activity of UCP1, the FA cycling mechanism was suggested by Skulachev (10). According to this hypothesis, UCP1 does not accomplish forward proton transport but rather the backward transport of fatty acid anions. Forward proton transport is realized by the spontaneous flip-flop of protonated fatty acids (31, 32). The flip-flop rate of stearic and oleic acids is ~5 s⁻¹ in large unilamellar vesicles (33) and ~45 s⁻¹ in small vesicles (33). Within reasonable limits of accuracy, the rates of fatty acid flip-flop and the substrate turnover rates of UCP1 are similar. Their striking coincidence suggests that fatty acid flip-flop may be the rate-limiting step in the transport cycle. The view is supported by the observation that both the substrate turnover rate of UCP1 (8) and the flip-flop rate of fatty acids (34) rise with increasing unsaturation and decreasing fatty acid chain length (8). A counterargument is that fatty acid molecules outnumber the protein molecules by a factor of 10,000 under our experimental conditions. Consequently, rate limitations due to fatty acid flip-flop can only apply if the UCP transporter cycles one and the same fatty acid multiple times. This possibility cannot be ruled out easily because fatty acid desorption from the membrane surface is extremely slow. For example, oleic acid desorbs at a rate of 2 s⁻¹ (35). Thus, a molecule that once has been transported by UCP may stay long enough in the vicinity of the binding center to be transported a second or a third time.

We had to insert 3 × 10³ copies of the protein into the bilayer to augment its conductivity by an order of magnitude. This extreme number suggests that UCP1 does not operate as a typical transmembrane channel. However, the distinction of carriers from channels based on the lower turnover rate of carriers does not work in the case of proton channels. The low proton transport rate of ~14 s⁻¹ found in our experiments does not preclude UCP1 being a channel. The substrate turnover of UCP1 is comparable to the one of the Na-K-ATPase (36) or the Ca-ATPase (37). Glucose transporters, in contrast, have a substrate turnover that is from 10 to 100-fold faster (38). A big variety of turnover rates were found for different proton channels (for review see Ref. 13). It ranges from 7–26 H⁺/s for the M₂ viral H⁺ channel to 2.2 × 10⁶ for gramicidin. Thus, the transport rate alone does not allow to consider the UCP1 to be a carrier or a channel.

In summary, the first functional reconstitution of UCP1 in planar membranes demonstrates that fatty acids are necessary and sufficient for UCP-mediated proton transport. Direct application of transmembrane voltage and precise current measurements allowed us to determine that the maximal substrate turnover per UCP1 molecule is 14 s⁻¹. It also showed that voltage dependence is not linear as suggested previously (14).

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