Transport Kinetics of Uncoupling Proteins

ANALYSIS OF UCP1 RECONSTITUTED IN PLANAR LIPID BILAYERS*

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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⁻ uniport 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 10²–10⁴ 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 × g). Escherichia coli lipid extract or E. coli lipid extract/soybean extract (3:1, both from Avanti Polar Lipids) was dissolved by double amount of octylpentaoxyethylene detergent (Bachem Feinchemikalien, Bubendorf, Switzerland) in extraction medium. The mitochondrial pellet was extracted with the resulted lipid-detergent micelles. Extracted mitochondrial proteins were added to the
Fatty Acid-induced Proton Transport by UCP1

spin of voltages on the interval between

Membrane conductivity

ator, Wavetek) operating at frequencies of 0.04

40 Hz and filtered with 1 Hz (Lampfit software, Axon Instruments). For

by an A/D converter (DigiData, Axon Instruments). It was sampled at

were measured by a patch clamp amplifier (GeneClamp 5000, Axon

depended neither on protein nor on fatty acid content.

K2SO4, 20 mM Tris, 20 mM HEPES, and 0.6 mM EGTA, pH 7.7, at room

temperature. Measurements the current-conductivity change (G(H)) of

UCP1 containing bilayers after addition of stearic acid containing liпо-

somes (40%) in buffer solution. The concentration of stearic acid is

presented as its aqueous concentration in buffer solution.

FIG. 1. The total conductivity of planar lipid membranes with-

out proteins (G(H), open diamonds) containing UCP1 (G, full cir-

cles) and containing 1.9 mM ATP (open circles). Protein content

was 0.5–4 μg of protein/mg of lipids. The buffer contained 50 mM

K2SO4, 20 mM Tris, 20 mM HEPES, and 0.6 mM EGTA, pH 7.7, at T =

22 °C. The buffer solution contained 50 mM K2SO4, 20 mM MOPS, and

0.6 mM EGTA, pH 6.7 or 7.0, or 50 mM K2SO4, 20 mM Tris (Sigma), 20

mM HEPES (Sigma), and 0.6 mM EGTA, pH 7.7. It was agitated by

magnetic bars. Membrane formation was monitored by capacitance

measurements (usually 0.89 ± 0.08 microfarad/cm²). The capacitance

depended neither on protein nor on fatty acid content.

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

conductivity measurements, a triangular voltage source (signal gener-

ator, Wavefun) 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(H)meas. The proton conductance of bilayers can be overshadowed by the

conductance of other ions. To determine the contribution of G(H)meas 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 concentra-

tions 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, Vr, because there are no gradients of buffer cations and

anions (22, 23). Vr was obtained from current-voltage characteristics

that were measured in the presence of a transmembrane pH gradient.

Therefore, current-voltage characteristics of membranes contain-

ing UCP1 and oleic acid (14.5%) were measured. In the range from

−50 to 50 mV, the current-voltage characteristics before (full trian-

gles) and after (open circles) the formation of the pH gradient were

collected. pHcis was adjusted to 7.5 using 7.6 mM HEPES and pHtrans

was adjusted to 7.9 using 7.6 mM Tris on the cis and trans sides of

the membrane, respectively (see “Experimental Procedures”).

where ΨN is the theoretical value of Nernst equilibrium potential (23.8

mV for a pH gradient of 0.4). The final proton conductivity G(H)meas

is calculated as shown in Equation 2,

\[ G_{\text{H}^+} = \frac{T_{\text{H}^+}}{\Psi_N} \]

where G is expressed in S/cm². Calculation of the number of UCP

molecules per cm² allows to estimate the turnover number in s⁻¹, r. If
one lipid molecule occupies an area of \(7.8 \times 10^{-12}\) m², the bilayer accommodates \(-2.6 \times 10^{14}\) lipids/cm². 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,

\[
r = \frac{G_{\text{HIGH}} \times p \times U}{6 \times 10^{10} e}\tag{Eq. 3}
\]

where \(e\) is the electron charge and \(U\) the applied voltage \(22, 23\). \(p = 2000\) corresponds to \(3 \times 10^9\) protein monomers/cm². 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/cm². It was comparable to \(G_0\) of a protein free membrane \((1.7 \times 10^{-8} \pm 0.8 \times 10^{-8}\) S/cm²). 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-surrounding buffer after membrane formation (ste-}

membrane-forming solution (oleic acid) or were added to the

membrane-forming solution. Quasi-exponential shape of this current-voltage characteristic 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 electrostrictive 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 \(11–39.3\) 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 for \(V_{\text{max}}\) for lauric acid and UCP1 reconstituted in liposomes \(11\). The apparent \(K_m\) for oleic acid was equal to
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 vary widely between 0.25 and 3 mM and FA can be rapidly transported across 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 property 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 ~ 1 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 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 × 10⁹ 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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REFERENCES