Uncoupling protein 1 (UCP1) dissipates energy and generates heat by catalyzing back-flux of protons into the mitochondrial matrix, probably by a fatty acid cycling mechanism. If the newly discovered UCP2 and UCP3 function similarly, they will enhance peripheral energy expenditure and are potential molecular targets for the treatment of obesity. We expressed UCP2 and UCP3 in Escherichia coli and reconstituted the detergent-extracted proteins into liposomes. Ion flux studies show that purified UCP2 and UCP3 behave identically to UCP1. They catalyze electrochemical flux of protons and alkylsulfonates, and proton flux exhibits an obligatory requirement for fatty acids. Proton flux is inhibited by purine nucleotides but with much lower affinity than observed with UCP1. These findings are consistent with the hypothesis that UCP2 and UCP3 behave as uncoupling proteins in the cell.

Uncoupling protein 1 (UCP1)

of brown adipose tissue mitochondria occupies a special place in bioenergetics, because it is the exception that proves the rule of Mitchell’s elegant chemiosmotic theory (1), a protein designed to short circuit the redox proton pumps in order to generate heat and dissipate energy. UCP1 was identified from functional studies on brown adipose tissue mitochondria (2) and was one of the first membrane proteins to be sequenced (3).

For many years it was thought that UCP was expressed solely in mammalian brown adipose tissue; however, it now turns out that Nature has engineered at least five uncoupling proteins. In 1995, a plant uncoupling protein was discovered and later sequenced (4, 5), and 2 years later, UCP2 and UCP3 were identified (6–9). UCP4 was recently described as a brain-specific UCP (10). UCP2 maps to regions of human chromosome 11 and mouse chromosome 7 that have been linked to hyperinsulinemia and obesity, and it is hypothesized that UCP2 is the peripheral target for energy dissipation in the regulation of body weight. UCP2 is ubiquitously expressed in mammalian tissues, whereas UCP3 is expressed primarily in glycolytic skeletal muscle in humans and may account for the thermogenic effect of thyroid hormone (11). These aspects of this rapidly emerging area of research have been nicely reviewed by Boss et al. (12).

Virtual nothing is known about the transport functions of UCP2 and UCP3, and their putative physiological functions have been deduced primarily from their striking sequence identities with UCP1 (12). To address this problem, we expressed human UCP2 and UCP3 in Escherichia coli, where they accumulated in inclusion bodies. Following detergent extraction, we reconstituted the proteins into liposomes and measured H+ and K+ fluxes. Purified UCP2 and UCP3 both catalyzed electrochemical flux of protons and alkylsulfonates, and proton flux exhibited an obligatory requirement for fatty acids. We also found that FA-dependent proton transport by UCP2 and UCP3 was inhibited by purine nucleotides, albeit with lower apparent affinities for nucleotides than those observed with UCP1. From these results, we conclude that UCP2 and UCP3 are functional uncoupling proteins and that their biophysical properties are consistent with a physiological role in energy dissipation.

EXPERIMENTAL PROCEDURES

Expression of UCPs in Saccharomyces cerevisiae—UCPs were expressed in yeast as described previously (13). Briefly, the SacI/SphI fragments from M13mp19 plasmid containing wild-type rat UCP1 cDNA were subcloned into SacI/SphI-cut pCS110 E. coli/S. cerevisiae shuttle vector. The S. cerevisiae strain JB516 (MATa, ura3, ade1, leu2, his4, gal1) was transformed with the shuttle vector construct and plated on uracil-lacking selective plates. The resulting yeast transformants were grown at 30 °C in selective medium, and overexpression of UCP1 was induced by the addition of 0.2% galactose (13). Similar protocols were followed for UCP2 and UCP3.

Expression of UCP2 and UCP3 in E. coli—Human UCP2 and human UCP3 open reading frames were amplified by PCR and inserted into the NdeI and NotI sites of the pET21a vector (Novagen). From DNA sequencing, the constructs are predicted to encode proteins with an amino acid sequence identical to the wild-type UCP2 or UCP3 proteins (6–9). Plasmids were transformed into the bacterial strain BL21 (Novagen). Transformed cells were grown at 30 °C to an A600 of 0.6 and then induced with 1 mM isopropyl-β-D-thiogalactopyranoside at 30 °C for 6 h. Cells from a 700-ml culture were lysed in a French press in 20 ml of lysis buffer (10 mM Tris, pH 7, 1 mM EDTA, 1 mM dithiothreitol); the lysate was centrifuged at 27,000 × g for 15 min; and the pellet was resuspended in 20 ml of lysis buffer and centrifuged at 1000 × g for 3 min. 1-ml aliquots of the supernatant were centrifuged at 14,000 × g for 15 min in a microcentrifuge, and the resulting pellet inclusion bodies were stored frozen at −70 °C.
Inclusion bodies, and reconstituted into liposomes. Proteins were loaded onto each lane of the gel parallel to A port via UCP2. The proteins were expressed in E. coli, extracted from inclusion bodies, and reconstituted into liposomes. 10 mg of delipidated proteins were loaded onto each lane of the gel parallel to A port via UCP2. UCP2 and UCP3. The proteins were expressed in Blue-stained SDS-polyacrylamide gels from proteoliposomes containing FA and valinomycin were added sequentially. (Note that traces a and c are offset for clarity). B, traces follow changes in total intraliposomal K⁺ (\([\text{K}^+]_i\)), which were measured using potassium-binding benzofuran isophthalate fluorescence. Assay conditions and additions for each trace were identical with those described in Fig. 2 for UCP2, except that 40 \(\mu\)M laurate was used instead of palmitate. A, traces follow changes in intraliposomal acid (\([\text{H}^+]_i\)). B, traces follow changes in total intraliposomal K⁺ (\([\text{K}^+]_i\)). Except for trace c, liposomes contained UCP3. These data are representative of more than 20 experiments on 10 different UCP3 reconstitutions.

**Extraction of UCP2 and UCP3 from Inclusion Bodies**—We modified published protocols (14, 15) for solubilization of E. coli inclusion bodies. The pelleted inclusion bodies (about 2 mg of protein) were suspended and washed three times in wash buffer (tetraethylammonium (TEA⁺) salts of 0.15 M phosphate, 25 mM EDTA, 1 mM ATP, and 1 mM dithiothreitol, pH 7.8). The final pellet was solubilized in 0.4 ml of 50 mM TEA-TES, pH 7.2, containing 1.5% sodium laurylsarcosinate (SLS). The extract was supplemented with 10 mg/ml asolectin and 3% octylpentaoxyethylene (C8E5 detergent) and then dialyzed for 15 h against 3 × 400 ml of extraction buffer (TEA⁺ salts of 50 mM TES and 1 mM EDTA, pH 7.2) to remove SLS. In the first two dialysis periods (1 and 13 h), the extraction buffer was supplemented with 1 mM dithiothreitol and 0.03% sodium azide. These were removed from the final dialysis (1 h). Aliquots of the dialyzed extract, containing about 0.2 mg of protein, were stored at −20 °C.

**Reconstitution of Uncoupling Proteins into Liposomes**—Reconstitutions were carried out as described previously for UCP1 (16). Egg yolk phosphatidycholine (UCP1) or soybean phospholipids (UCP2 and UCP3) were supplemented with cardiolipin (2 mg/ml), dried, and stored under nitrogen. Internal medium (TEA⁺ salts of TES (30 mM), SO₄⁻ (80 mM), and EDTA (1 mM), pH 7.2) was added to give a final concentration of 40 mg of phospholipid/ml of proteoliposome stock. The mixture was vortexed and sonicated to clarity in a bath sonicator, and fluorescent probe was added. The final mixture (1.1 ml) was applied onto 2 ml of Bio-Bead SM-2 (Bio-Rad) column to remove the detergent. After 2 h of incubation, the column was centrifuged, and the resulting proteoliposomes were applied onto a new 2-ml Bio-Bead column, incubated for 30 min, and centrifuged. The formed vesicles (1 ml) were passed through a Sephadex G-25–300 column to remove external probe.

**Fluorescence Measurements of Ion Fluxes**—Ion flux in proteoliposomes was measured using ion-specific fluorescent probes and an SLM Amino 8000C spectrofluorometer. Measurements of H⁺ fluxes were obtained from changes in 6-methoxy-N-(3-sulfopropyl)quinolinium fluorescence due to quenching by the anion of TES buffer (17). Measurements of K⁺ fluxes, reflecting the movement of ionic charge across the membrane, were obtained from changes in potassium-binding benzofuran isophthalate fluorescence (16, 18). Internal and external media contained K⁺ or TEA⁺ salts of TES buffer (30 mM), SO₄⁻ (80 mM), and EDTA (1 mM), pH 7.2. TEA⁺ internal medium and K⁺ external medium were used for the experiments of Figs. 3 and 4 to measure nucleotide inhibition. Each proteoliposome preparation was individually calibrated for fluorescent probe response, and its internal volume was estimated from the volume of distribution of the fluorescent probe (16).

**Chemicals and Reagents**—Potassium-binding benzofuran isophthalate and 6-methoxy-N-(3-sulfopropyl)quinolinium were purchased from Molecular Probes, Inc. (Eugene, OR). Undecanesulfonate was purchased in Research Plus, Inc. Asolectin (45% L-α- phosphatidylcholine) was purchased from Avanti Polar Lipids, Inc. Sulfuric acid was purchased from Fisher. Materials for UCP1 expression in yeast were from sources listed previously (17). All other chemicals were from Sigma. Purine nucleotides were adjusted to pH 7.2 with Tris base.

**RESULTS**

**Isolation and Reconstitution of UCPs Expressed in E. coli**—The development of a functional yeast expression system al-
allowed us to investigate structure-function relationships of UCP1 using site-directed mutagenesis (19, 20). Similarly, we attempted to use yeast expression for human UCP2 and UCP3 in order to study their function. As opposed to UCP1, however, both UCP2 and UCP3 expressed in lower quantities in yeast and were difficult to purify. Expression in *E. coli* yielded high amounts of UCP2 and UCP3, which accumulated in inclusion bodies. However, when the proteins were extracted using SLS detergent and reconstituted into liposomes, the proteins were found to be inactive (not shown). We obtained functionally active protein by supplementing the extract with asolectin and octylpentaoxyethylene and subjecting the extract to prolonged dialysis against SLS-free buffer. The dialysis may have reduced the SLS concentration, but this was not assayed. The proteoliposomes typically had an internal volume of 1.2 \( \text{m}^3/\text{mg} \) of lipid and contained 3–5 \( \mu \text{g} \) protein/\( \mu \text{g} \) of lipid. To estimate the purity of reconstituted UCP2 and UCP3, the proteoliposomes were delipidated and subjected to SDS-polyacrylamide gel electrophoresis, with the results shown in Fig. 1.

**Undecanesulfonate and Fatty Acids Induce Electrophoretic Fluxes in Liposomes Reconstituted with UCP2 and UCP3**—The representative ion flux traces in Fig. 2 show that UCP2 catalyzes FA-dependent, electrophoretic proton flux. The traces in Fig. 2A follow \( \text{H}^+ \) movement across the membrane. It can be seen that FA induce a strong \( \text{H}^+ \) flux (Fig. 2A, trace a) that is absent in the absence of FA (trace b) and does not occur in liposomes without protein (trace c). Undecanesulfonate, an analogue of laurate, does not support \( \text{H}^+ \) transport (trace d). The traces in Fig. 2B follow \( \text{K}^+ \) movement. It can be seen that charge movement across the membrane exactly matches FA-induced \( \text{H}^+ \) flux (trace a), confirming that the \( \text{H}^+ \) flux is electrophoretic. Undecanesulfonate induced a strong \( \text{K}^+ \) flux (trace d), demonstrating that the sulfonate anion is transported by UCP2.

A rapid intraliposomal acidification ensues upon the addition of FA (Fig. 2A, traces a and c). This is due to flip-flop of the protonated FA and acid-base equilibration (21). It is an electroneutral process, as evidenced by the lack of a corresponding \( \text{K}^+ \) jump upon the addition of FA (Fig. 2B).

The representative ion flux traces in Fig. 3 were obtained with UCP3, and they are qualitatively identical in every detail with those of UCP2. These results are highly reproducible and are representative of more than 20 assays from 10 or more preparations of each UCP.

**Preliminary Results from a Kinetic Study of UCP2 and UCP3**—The \( \text{K}_m \) values for FA are similar among all three UCPs (10–20 \( \mu \text{mol} \) of FA/mg of lipid), and the \( \text{V}_{\text{max}} \) values for palmitate are also similar (10–30 \( \mu \text{mol} \)/mg/min). However, the \( \text{V}_{\text{max}} \) for laurate is much lower in UCP2 than in UCP1 or UCP3, indicating a preference for long-chain FA by UCP2.

**Inhibition of UCPs by Purine Nucleotides**—A second essential property of UCP1 is inhibition of fatty acid-induced proton fluxes by nucleotide (9). Similarly, all three UCPs are inhibited by nucleotides. The results shown in Figs. 4 and 5 were obtained with 50 \( \mu \text{M} \) laurate for UCP1 and UCP3, and 50 \( \mu \text{M} \) palmitate for UCP2. 100% inhibition was estimated in a manner similar to that shown in Fig. 4. The representative ion flux traces in Fig. 4 (A and B) show that FA-induced \( \text{H}^+ \) flux (trace a) is inhibited by 50 \( \mu \text{M} \) ATP in the presence of 50 \( \mu \text{M} \) laurate (Fig. 4A) and 50 \( \mu \text{M} \) palmitate (Fig. 4B). The traces in Fig. 4A follow \( \text{H}^+ \) movement across the membrane. It can be seen that FA induce a strong \( \text{H}^+ \) flux (trace a) that is absent in the absence of FA (trace b) and does not occur in liposomes without protein (trace c). Undecanesulfonate, an analogue of laurate, does not support \( \text{H}^+ \) transport (trace d). The traces in Fig. 2B follow \( \text{K}^+ \) movement. It can be seen that charge movement across the membrane exactly matches FA-induced \( \text{H}^+ \) flux (trace a), confirming that the \( \text{H}^+ \) flux is electrophoretic. Undecanesulfonate induced a strong \( \text{K}^+ \) flux (trace d), demonstrating that the sulfonate anion is transported by UCP2.
This location is shielded from the aqueous, which causes the partitions in the lipid bilayer with its head group at the level of the acyl uncoupling cycle consists of the following five steps. (i) FA anion partition in membranes to be 3–4 units higher than their values in solution (34). There is no significant flux of FA anion, because the bilayer energy barrier is too high (35). (ii) The FA anion diffuses laterally in the bilayer to reach a subsurface binding site on UCP that is shielded from the bulk aqueous phase (36). (iii) The energy barrier to FA anion transport is lowered by a weak binding site located about halfway through the UCP transport pathway (37). The electric field created by redox-linked proton ejection drives the anionic head group to the energy well. (iv) The FA carboxylate group is transported to the other side of the membrane transport pathway (37). The electric field created by redox-linked proton ejection drives the anionic head group to the energy well. (v) The FA is protonated, and the protonated FA rapidly flip-flops again, delivering protons electroneutrally to the mitochondrial matrix and completing the cycle.

Inhibition by purine nucleotides is also an essential property of UCP1. Since FA have no effect on the Ki for nucleotide inhibition (22), it is generally agreed that transport and inhibition take place on different domains. The nucleotide binding domain in UCP1 is extensive and reasonably well characterized. The sugar-base moiety reacts with three residues located on the matrix segment that connects helices 5 and 6, an interaction that may confer selectivity among nucleotides (27–29). A glutamate, Glu190, in the fourth transmembrane helix is the pH sensor for nucleotide binding (30). Three arginines, located in the transmembrane helices 2, 4, and 6, are required for nucleotide inhibition and have been shown to bind the nucleoside phosphates (20). Site-directed mutagenesis studies have led to a three-stage binding-conformational change model for nucleotide binding and inhibition in UCP1 (20).

It is noteworthy that the seven residues involved in nucleotide inhibition are largely conserved in UCP2, UCP3, and plant uncoupling protein, suggesting not only that these proteins would be regulated by nucleotides but also that regulation would be similar among the UCPs. Surprisingly, there are striking differences in nucleotide sensitivity among the UCPs, with UCP2 and UCP3 being only weakly sensitive to GDP, for example (Fig. 4, Table I). Similarly, plant uncoupling protein was also only weakly sensitive to purine nucleotides (31).

The physiological significance of variations in nucleotide inhibition is unclear, because it is not known how any of the UCPs are opened in vivo. In the case of UCP1, a common view is that uncoupling is initiated by dissociation of ATP (32). In our view, nucleotide debinding is an unlikely opening mechanism; to regulate important physiological processes, Nature normally relies on specific signaling pathways and not on the law of mass action. Regulation may involve post-translational modification of the proteins; however, no such signaling pathway has yet been demonstrated in the opening of any of the UCPs.

A major value of studies such as these on isolated, reconsti-
tuted UCPs is that they permit direct comparison with similar studies obtained using UCP1. In this regard, our most noteworthy finding is that the three mammalian UCPs are qualitatively identical in mediating FA-dependent proton transport. Studies on whole cells and isolated mitochondria containing native UCP2 and UCP3 are urgently needed to advance the field. It is hoped that the biophysical approach described here will prove useful as a guide to studies on the native system.

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