Uncoupling protein 1 binds one nucleotide per monomer and is stabilized by tightly bound cardiolipin

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Uncoupling protein 1 (UCP1) catalyzes fatty acid-activated, purine nucleotide-sensitive proton leak across the mitochondrial inner membrane of brown adipose tissue to produce heat, and could help combat obesity and metabolic disease in humans. Studies over the last 30 years conclude that the protein is a dimer, binding one nucleotide molecule per two proteins, and unlike the related mitochondrial ADP/ATP carrier, does not bind cardiolipin. Here, we have developed novel methods to purify milligram amounts of UCP1 from native sources by using covalent chromatography that, unlike past methods, allows the protein to be prepared in defined conditions, free of excess detergent and lipid. Assessment of purified preparations by TLC reveal that UCP1 retains tightly bound cardiolipin, with a lipid phosphorus content equating to three molecules per protein, like the ADP/ATP carrier. Cardiolipin stabilizes UCP1, as demonstrated by reconstitution experiments and thermostability assays, indicating that the lipid has an integral role in the functioning of the protein, similar to other mitochondrial carriers. Furthermore, we find that UCP1 is not dimeric but monomeric, as indicated by size exclusion analysis, and has a ligand titration profile in isothermal calorimetric measurements that clearly shows that one nucleotide binds per monomer. These findings reveal the fundamental composition of UCP1, which is essential for understanding the mechanism of the protein. Our assessment of the properties of UCP1 indicate that it is not unique among mitochondrial carriers and so is likely to use a common exchange mechanism in its primary function in brown adipose tissue mitochondria.

membrane protein | cardiolipin binding | nucleotide binding | brown fat | oligomeric state

Brown adipose tissue oxidizes fatty acids to produce heat for thermoregulation in the cold and is present in adult humans, where it holds promise in combating obesity (1, 2). Human brown fat depots correlate with leanness (3, 4) and, in mice, thermogenesis by brown fat has been shown to clear blood triglycerides and dispose of blood glucose, reducing metabolic disease (5). Thermogenesis by brown adipose tissue depends on uncoupling protein 1 (UCP1), a 33-kDa mitochondrial carrier protein that, when activated, transports protons across the mitochondrial inner membrane, decoupling electron transfer from ATP synthesis to dissipate the protonmotive force as heat. During the adrenergic stimulation of brown adipocytes (e.g., due to cold exposure), long chain fatty acids released from lipid droplets activate UCP1, overcoming the inhibition of the protein by cytosolic purine nucleotides, to induce thermogenesis (6). Therapeutically activating UCP1 directly, without the need for physiological stimuli, could provide treatments for metabolic disease (6, 7).

The mechanism of proton conductance by UCP1 and the interplay that occurs between regulators is debated (refs. 8 and 9). Evidence largely from liposome studies has led to the proposals that fatty acid anions act as either cofactors, providing proton-binding sites in a transport channel within the protein (10), or transport substrates, which are exported by UCP1 and flip back across the inner membrane in a protonated state (11). Alternatively, patch-clamp studies with mitochondrial inner membranes suggest that either protonated or deprotonated fatty acid species can be transported by UCP1, which remain bound to the protein in the transport cycle to give a net proton transfer (9). During activation, fatty acids may compete with nucleotide binding directly, or indirectly through changes in the protein, as indicated by experiments with isolated mitochondria (12, 13). Claims of the involvement of other metabolites (e.g., ubiquinone-10 or 4-hydroxy-2-nonenal) in UCP1 activation have largely been refuted (14, 15).

For many years, there has been a general consensus on the fundamental composition of UCP1. Following the observation that one nucleotide binds to two proteins (16), UCP1 was believed to be a dimer. This notion was supported by analytical ultracentrifugation (17), nucleotide binding (18–21), and protein cross-linking studies (18, 22), as well as many reports indicating that the related mitochondrial ADP/ATP carrier is dimeric too (see ref. 23 and references therein). Distinct from other carriers, however, UCP1 was found to function independently of cardiolipin. This mitochondrial lipid enhances the activity of many carriers (24) and can be observed bound to the ADP/ATP carrier in the available atomic structures (25–27). Measurements of ^{31}P NMR have indicated that purified UCP1 preparations do not contain bound cardiolipin (28), in contrast to the three molecules reported to bind per ADP/ATP carrier (29), and reconstitution studies have indicated that the lipid is not required for UCP1 activity (e.g., refs. 30 and 31) but may alter the affinity of the protein for purine nucleotides (28).

Significance

Uncoupling protein 1 (UCP1) is a mitochondrial carrier protein responsible for thermogenesis in brown adipose tissue, and a putative therapeutic target for the treatment of obesity and related diseases. It is widely accepted that the protein is dimeric and regulated by binding one nucleotide per dimer, and unlike other mitochondrial carriers, functions independently of the lipid cardiolipin. Here, we have developed novel methods to purify UCP1 from native sources, and demonstrate that the protein is in fact monomeric, binding nucleotide in a 1:1 stoichiometry. Each protein also binds three cardiolipin molecules, which confer stability. These findings put in place the true composition of UCP1, overturning a paradigm, which is crucial to resolve the controversial mechanism of this important membrane protein.

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In recent years, several studies have demonstrated that the mitochondrial ADP/ATP carrier is monomeric, not dimeric (32–36), which raises questions on the oligomeric state of UCP1. Resolving the basic functional unit of UCP1 is paramount for understanding its mechanism. Here, we have developed novel methods to purify UCP1 from native sources, which have allowed us to reach new conclusions on the fundamental properties of the protein.

Results

Purification of UCP1 Using Covalent Chromatography. The recombinant expression of UCP1 in yeast or *Escherichia coli* can produce artifacts associated with misfolded protein (e.g., refs. 37 and 38), and so native material is essential to gain reliable information on the protein. Native UCP1 has typically been isolated from the brown adipose tissue of rodents, although we have found that newborn lambs are a rich and available source of the tissue. Established purification methods rely on the passage of Triton X-100 solubilized UCP1 through hydroxyapatite media (negative chromatography) (18), where excess detergent and endogenous lipid elute with the isolated protein and can interfere with downstream investigations. To avoid these issues, we have developed new procedures to purify native UCP1, exploiting the new maltose neopentyl glycol class of detergents (39) and a conserved cysteine residue at the C terminus of the protein, which exposes a reactive thiol group (22) (Fig. S1). In the method (summarized in Fig. 1*A*), UCP1 is first enriched through the removal of soluble and nonintegral membrane proteins by alkali treatment of mitochondria, and solubilized in decyl maltose neopentyl glycol (10MNG) detergent, before being desalted and passed through S and Q ion-exchange resins to remove the majority of protein contaminants. UCP1 is then covalently bound to a thiol propyl Sepharose (TPS) resin and washed, before being eluted off by reducing agent (DTT) in a defined buffer (see the gel profile of an example purification; Fig. 1*B*). We found that this strategy allowed UCP1 to be purified to homogeneity (Fig. 1*D*), free of ADP/ATP carrier (Fig. S2), with yields similar to those obtained with hydroxyapatite (~2 mg of UCP1 per 100 mg of starting mitochondria). The TPS-immobilization step could also be applied to hydroxyapatite preparations to facilitate buffer exchange and washing of the protein (Fig. 1 *C* and *D*).

When reconstituted into phosphatidylcholine liposomes, UCP1 prepared by our new methods exhibited high proton conductance rates in the presence of oleic acid upon induction of a membrane potential with valinomycin, comparable to UCP1 prepared by conventional hydroxyapatite methods (Fig. 2*A*). The activity was almost fully inhibited by 40 μM GDP in the assay buffer, where subsequent carbonyl cyanide 3-chlorophenylhydrazone (CCCP) additions confirmed that the total proton transfer capacity of the liposome system had not changed. The high proton conductance activity and nucleotide sensitivity demonstrate that the protein is intact and active with a predominantly externally oriented GDP binding site in liposomes. In the absence of interfering substances, such as Triton X-100, our UCP1 preparations were suitable for assessment by far UV circular dichroism, where a reliable signal down to ~185 nm could be obtained (Fig. 2*B* and Fig. S3). The profile revealed a strong alpha helical signature in the protein, in line with structural data for the related ADP/ATP carrier (25–27), with a positive band at 193 nm and negative bands at 209 nm and 220 nm. Various effectors did relatively little to the profile (Fig. S3*A*). However, SDS decreased the amplitude of the signal, particularly at ~193 nm (from 42,000 to 23,000 deg·cm^2·dmol^{-1}; Fig. 2*B*), consistent with the denaturation of UCP1 to an unfolded state, retaining significant secondary structural elements.

Importantly, during purification using TPS resin, we found that the wash buffers required supplementing with lipid (0.05 mg/mL cardiolipin or phosphatidylcholine), because lower yields were observed when UCP1 was washed in buffer alone, indicating that the protein becomes unstable. As an alternative to added lipid, we included GDP in the wash buffer to stabilize UCP1. The quantification of lipid phosphorus in various preparations confirmed that the total lipid was substantially lower where UCP1 had been bound to TPS and washed (Table 1), even with lipid supplementation. Where only GDP had been used in the absence of added lipid, however, we found that UCP1 still retained ~6 moles of endogenous lipid phosphorus per mole of UCP1, despite extensive washing (Table 1).

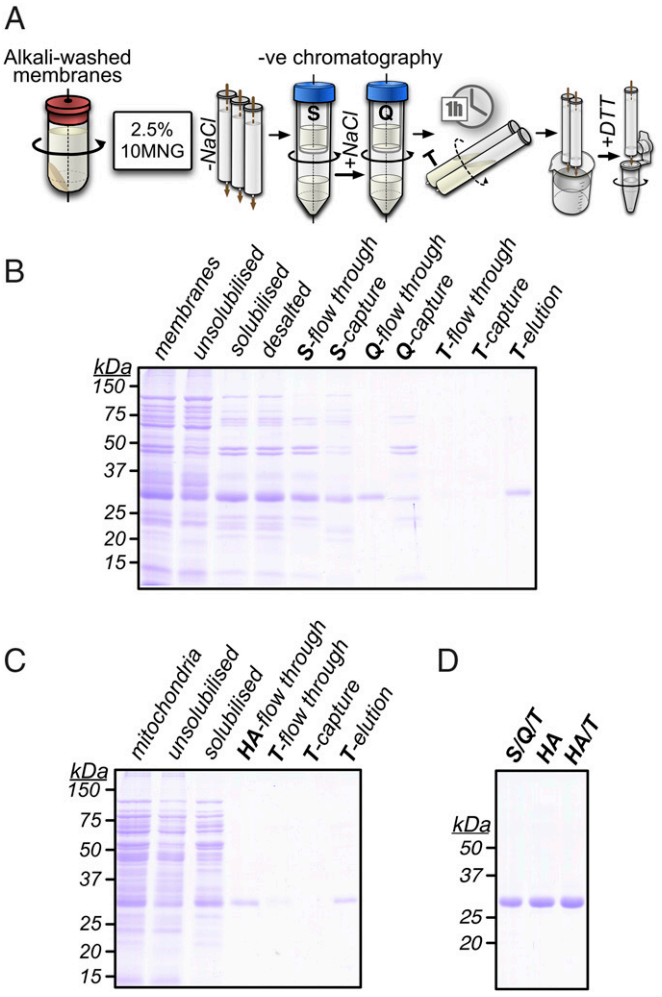

Fig. 1. Purification of native UCP1. Scheme of the procedure (*A*) and Coomassie-stained gel profile (*B*) of UCP1 purification by alkali wash treatment, passage through cation (S) and anion (Q) exchange media and binding to TPS resin (T). Ten micrograms of enriched membranes and equivalent fractions (by volume) of sample from subsequent steps in the procedure were loaded on to gels. (*C*) Equivalent profile of UCP1 purification using hydroxyapatite (HA) combined with TPS binding. (*D*) Coomassie-stained gel of final UCP1 samples (2 μg of protein per lane) purified as in *B* (lane 1) or *C* with hydroxyapatite alone (lane 2), or with hydroxyapatite and binding to TPS resin (lane 3). Note, buffers were supplemented with 18:1 cardiolipin during covalent chromatography (*Methods*).

Cardiolipin Copurifies with UCP1 and Is Required for Stability. To identify the lipids retained by UCP1, we separated solvent extracts of the purified protein by TLC. Because mitochondrial lipids from brown adipose tissue are predominantly unsaturated (40), a charring detection method was used that is insensitive to saturated lipids (such as those used for buffer supplementation; Fig. 3*A*) to identify the endogenous lipids in the UCP1 preparations.

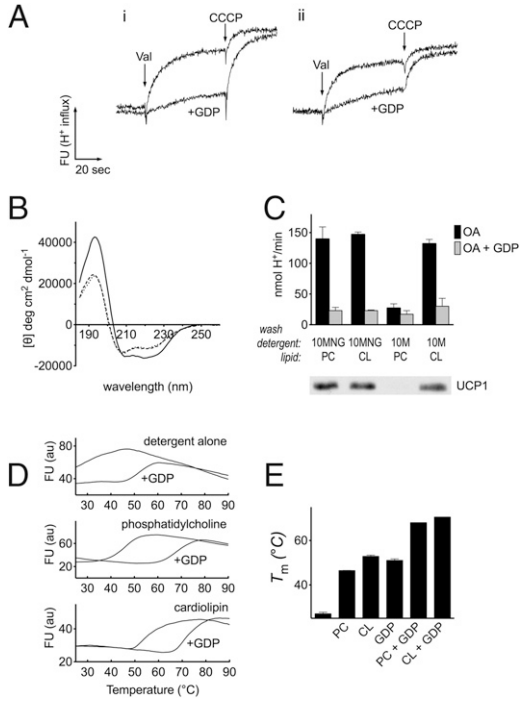

Fig. 2. Integrity of purified UCP1. (*A*) Proton uptake by liposomes (pure phosphatidylcholine) reconstituted with UCP1 purified by ion exchange/covalent chromatography (i) or conventional hydroxyapatite chromatography (ii). (*B*) CD spectra of native UCP1. Scans are of 0.6 mg/mL purified protein in buffer (10 mM Tris·HCl pH 7.4) containing 0.05% 10MNG in the absence (solid line) or presence of 0.5% or 1.0% SDS (dashed and dotted lines, respectively) at 10 °C. See Fig. S3*A* for other conditions and S3*B* for corresponding PMT dynode voltages. (*C*) Proton uptake rates (nmol/min) and relative UCP1 content (immunoblot) of liposomes reconstituted with UCP1 protein that had been washed in 10MNG or 10M detergent supplemented with 14:0 cardiolipin (CL) or 14:0 phosphatidylcholine (PC) during purification by ion exchange/covalent chromatography, as indicated (*Methods*). All proton uptake assays were in the presence of 200 μM oleic acid (1.6 mM methyl-β-cyclodextrin), ± 40 μM GDP, with rates driven by a membrane potential (positive outside) induced by addition of 2.5 μM valinomycin to liposomes with 100 mM internal and 1 mM external potassium present. Rates are averages (± SD) of three independent experiments. (*D* and *E*) The relative thermal stability of UCP1 purified by ion exchange/covalent chromatography in the absence or presence of lipid. Protein unfolding was monitored by the fluorescence of CPM-adduct formation at cysteine residues as they become solvent exposed due to thermal denaturation. Representative traces (*D*) and average (± SD; triplicate measurements) melt temperatures (*E*) of native UCP1 in assay buffer containing 10MNG, supplemented with lipid, as indicated, ± 2 mM GDP. Unless stated otherwise, UCP1 was purified by covalent chromatography in buffer supplemented with 18:1 cardiolipin.

The results show that UCP1 almost exclusively retained cardiolipin (with only a minor amount of phosphatidylcholine), even when washed in purification buffer with added 14:0 cardiolipin or 14:0 phosphatidylcholine (Fig. 3*B*). Similar washes in the harsher detergent decyl maltoside (10M) did not facilitate cardiolipin exchange even when the washes were extended (Fig. 3 *C* and *D*, respectively), indicating that the endogenous lipid is tightly bound to the protein (note, endogenous phosphatidylcholine could not be assessed because of interference by 10M). Even so, following reconstitution into pure phosphatidylcholine liposomes, UCP1 activity displayed a striking dependence on the cardiolipin that had been added to the wash buffer during purification, but only with the harsher 10M wash (Fig. 2*C*). GDP-sensitive rates of proton conductance were similar for protein that had been washed in 10MNG with either 14:0 cardiolipin or 14:0 phosphatidylcholine present, or 10M detergent with 14:0 cardiolipin present, but no activity was measured with protein that had been washed in 10M with phosphatidylcholine. The 10M wash may partially remove endogenous cardiolipin that is important for UCP1 activity, which can only be replaced by added 14:0 cardiolipin. Immunoblots of the final gel-filtered liposome fractions reveal that losses in activity relate to a complete loss of UCP1 incorporation into the liposomes rather than a decrease in specific activity of the protein (Fig. 2*C*), suggesting that cardiolipin is required for protein stability during reconstitution.

We tested the stability of purified UCP1 directly with a thermostability assay that monitors protein unfolding with the thiol-reactive dye *N*-[4-(7-diethylamino-4-methyl-3-coumarinyl) phenyl]maleimide (CPM). CPM reacts with protein-buried cysteine residues as they become solvent exposed due to thermal denaturation to give a fluorescent adduct (41, 42). In tests, ovine UCP1 exhibited a background fluorescence (Fig. 2*D*), indicating that at least one of the nine cysteine residues is solvent exposed in the native state. In each condition, the signal transitioned to a higher plateau with an increase in temperature as the protein unfolded, allowing an apparent denaturation or "melt" temperature (T_m) to be determined from the derivative of the profile. UCP1 proved to be highly unstable when purified and assessed in the absence of lipid or nucleotide, exhibiting an apparent T_m of 27.0 ± 0.7 °C (Fig. 2 *D* and *E*). When purified and assessed in the presence of phosphatidylcholine or cardiolipin, however, the apparent T_m was 46.4 ± 0.1 °C and 52.8 ± 0.6 °C, respectively (~20 and ~25 °C higher). GDP in the assay buffer increased the apparent T_m of UCP1 by ~24 °C and further improved the stability of UCP1 in the presence of lipids as well (Fig. 2*E*). Lipid supplementation, particularly cardiolipin, may reduce the dissociation of endogenous-bound cardiolipin during the assay, which, in turn, stabilizes the protein.

UCP1 Is Monomeric and Binds Nucleotide in a 1:1 Stoichiometry. To determine the oligomeric state of UCP1, we assessed the size of the purified protein by size exclusion chromatography. In 10MNG supplemented with 18:1 cardiolipin, UCP1 eluted with a major peak corresponding to a molecular mass of ~127 kDa when calibrated with molecular weight markers (Fig. 4*A*). Minor amounts of protein at higher molecular masses (see gel profile of the eluted fractions) were consistent with various oligomers associated with generic aggregation in detergent, as observed for the ADP/ATP carrier (35, 36). Quantification of the associated detergent and lipid present in the UCP1 peak fraction revealed that 2.3 g of detergent and 0.5 g of lipid per g of protein were bound. This amount equated to a detergent and lipid contribution of 77 kDa and 16 kDa, respectively, to the 127-kDa protein-detergent micelle, leaving 34 kDa of protein, which correlates well with the calculated mass of a single UCP1 polypeptide (33 kDa; Fig. 4*B*). In contrast to the prevailing view, therefore, UCP1 is monomeric, in line with recent findings for the ADP/ATP carrier (32–36).

Table 1. The content of phosphorus associated with phospholipids in UCP1 preparations

Purification method	Buffer supplement	Lipid phosphorus:UCP1, mol:mol	
HA	—	**115.2**	±9.3
HA/T	0.05 mg/mL CL	**24.2**	±0.6
S/Q/T	0.05 mg/mL CL	**21.8**	±2.2
S/Q/T	0.8 mM GDP	**6.2***	±2.0

*Corrected for GDP phosphorus.
See Fig. 1 for purification method abbreviations; CL, 14:0 cardiolipin.

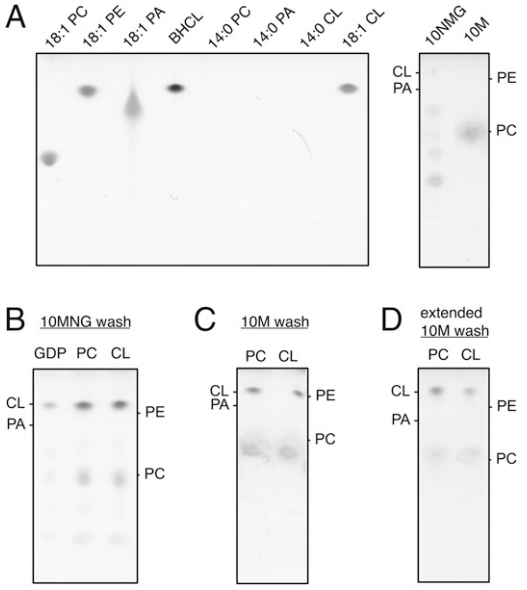

Fig. 3. Endogenous cardiolipin copurifies with UCP1. TLC of phospholipid/detergent standards (*A*) and lipid extracts from purified UCP1 after immobilizing and washing the protein on TPS resin (*B–D*). (*A*) The saturated and unsaturated phospholipid standards (2 μg of each) are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidic acid (PA), cardiolipin (CL), and beef heart cardiolipin (BHCL), and the detergent standards (80 μg of each) are decyl maltoside neopentyl glycol (10MNG) and decyl maltoside (10M). Note, saturated lipids, such as those used for supplementation during UCP1 purification, are not detected in the assay. (*B–D*) The lipids associated with UCP1 following ion exchange/covalent chromatography purification with 100-mL washes in 0.05% 10MNG detergent (*B*) or 0.2% 10M detergent (*C*), or extended 300 mL washes in 0.2% 10M detergent (*D*), supplemented with 2 mM GDP, 14:0 phosphatidylcholine or 14:0 cardiolipin to stabilize the protein, as indicated.

Original observations with isolated UCP1 indicated that one nucleotide is bound per two UCP1 (16). To obtain an accurate measure of the binding stoichiometry, we used isothermal titration calorimetry, which is well suited to assessing nucleotide–protein interactions. Purified UCP1, quantified by amino acid analysis, was titrated with known concentrations of GDP ($\varepsilon_{253} = 13{,}700\ M^{-1}{\cdot}cm^{-1}$), and the enthalpy changes associated with ligand binding were recorded (Fig. 4*C*). Importantly, the assay buffer used was kept free of the many anions that may compete with nucleotide binding to UCP1 (e.g., ref. 43). The resulting isotherms were fitted with an appropriate binding model (Fig. 4*C*), which revealed that 1.1 GDP molecules bind per UCP1, as indicated by the ligand/protein molar ratio at the center of the isotherm, with a K_D of 46 ± 3 nM in the absence of interfering salts (determined from 1/slope). This stoichiometry is in clear contrast to the original experimental work that led to the dimer premise (16).

Discussion

Despite intensive research, there are many inconsistencies and controversies on the nature of UCP1 activity. These problems may in part relate to the susceptibility of the protein to produce artifacts in recombinant systems, as well as the general difficulties associated with studying membrane proteins in isolation. Traditionally, native UCP1 has been purified in polyoxyethylene detergents (e.g., Triton X-100) by negative chromatography using hydroxyapatite media, and a consensus on the composition of the protein was established. The protein was understood to be dimeric and, unlike other carriers, operate without binding cardiolipin. Through the development of improved purification methods, however, we have found that these conclusions on the fundamental properties of the protein are incorrect. UCP1 is monomeric and tightly binds cardiolipin, which is required for its stability.

In the absence of lipid supplementation, we found that approximately six moles of lipid phosphorus are associated with UCP1, almost all in the form of cardiolipin. This stoichiometry equates to approximately three molecules of cardiolipin bound to each UCP1, the same ratio as observed for the mitochondrial ADP/ATP carrier (29). Crystal structures of the ADP/ATP carrier show that cardiolipin binds at three positions, consistent with the threefold pseudosymmetry of the structure, where each lipid head group links two domains toward the matrix side of the protein (25–27). A recent analysis of the molecular interactions involved indicate that the negatively charged phosphate groups in cardiolipin interact electrostatically with the positive pole of the helix dipoles of the even-numbered and matrix helices (27), and form hydrogen bonds with amide groups of the protein backbone. Residues from the [YF]xG and [YWF][KR]G motifs (present in each of the three repeat domains) play a key role in the architecture of the binding site. The glycine residues of the

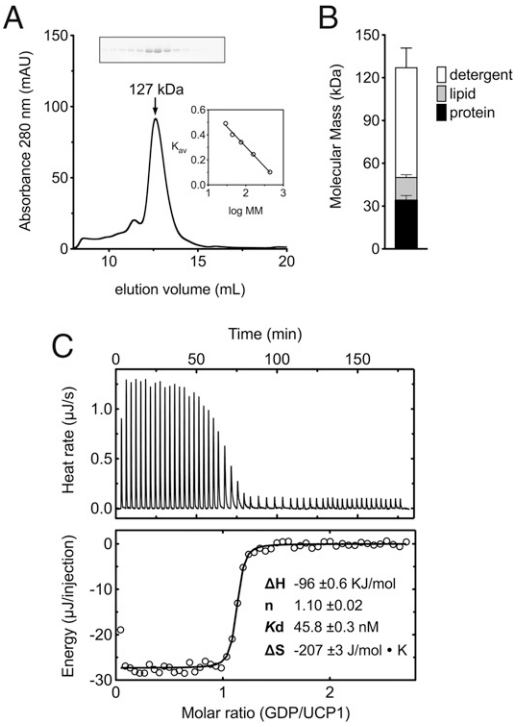

Fig. 4. Determining the oligomeric state and nucleotide binding stoichiometry of purified UCP1. (*A*) Assessment of the molecular mass of UCP1 in 0.05% 10MNG supplemented with 0.05 mg/mL 18:1 cardiolipin by exclusion chromatography. (*Inset Right*) Calibration of the size exclusion partition coefficient (K_{av}) to the molecular mass of a protein by using the markers carbonic anhydrase (29 kDa), ovalbumin (44 kDa), conalbumin (75 kDa), aldolase (158 kDa), and ferritin (440 kDa). (*Inset Top*) Coomassie-stained SDS gel of eluted fractions, aligned accordingly. (*B*) The mass composition of the major peak eluted during size exclusion chromatography. Protein, detergent, and phospholipid were quantified as described in *SI Materials and Methods*. (*C*) Isothermal titration calorimetry of purified UCP1 with GDP. The enthalpy changes associated with the titration of GDP into UCP1 at 4 °C (*Top*), and corresponding isotherms fitted to a one-site binding model with ΔH, K_d, and stoichiometry as fitting parameters (*Bottom*). (*Inset*) Average data (± SD) from three independent titration experiments (Fig. S4). UCP1 protein was purified by ion exchange, covalent and size exclusion chromatography, and experiments carried out in ITC buffer (50 mM cacodylate (TEA) pH 6.0, 0.01% 10MNG, 0.01% tetraoleoyl cardiolipin).

two motifs act as helix breakers, whereas the aromatic residues make hydrophobic contacts with the lipid acyl chains (25–27). Crucially, these motifs are present in UCP1 (although the glycine at position 248 is replaced by serine, also a helix breaker), indicating that UCP1 most likely binds cardiolipin in a similar manner as the mitochondrial ADP/ATP carrier. The observed stabilizing effect of cardiolipin and other lipids here is most likely achieved by increasing the size of the supporting detergent micelle (42), which, in turn, may impact on the behavior of the tightly bound species.

UCP1, which has a molecular mass of 33 kDa, migrated as an ∼127-kDa species in size exclusion chromatography, yet the carrier is monomeric after accounting for the contributions of the detergent and lipid. Similar observations were made with the mitochondrial ADP/ATP carrier where the apparent size of the monomer varied dramatically with different detergents because of the size of the associated micelle (34). The apparent size of the ADP/ATP carrier was also heavily influenced by lipids, which increased the Stokes radius of the associated detergent micelle, giving a considerable increase in overall mass (44). Previous estimates of the mass of UCP1 in Triton X-100 detergent (∼180 kDa; ref. 17) may not have been suitably adjusted for the high amount of detergent and lipid present following purification using hydroxyapatite. It is likely, therefore, that the protein was monomeric in past work too.

Nucleotides were reported to bind to UCP1 in a 1:2 stoichiometry, supporting the idea that the protein forms dimers. However, accurate isothermal calorimetry titrations unambiguously demonstrate that GDP binds in a 1:1 stoichiometry. Past studies typically relied on a modified Lowry method to quantify protein (16, 18–21), which is likely to have overestimated protein amounts in the presence of high concentrations of Triton X-100 (45). Our data support a simple model where each UCP1 monomer is capable of binding a single nucleotide. In line with this model, past observations with UCP1 provide clues on the way that nucleotides interact with the protein. Binding occurs in a similar manner to carboxy-atractyloside inhibition of the ADP/ATP carrier, where the nucleotide binds to a central substrate-binding site in UCP1 from the cytosolic side. Note that nucleotides increase the stability (e.g., Fig. 2*D*) and resistance to trypsinolysis (13) of UCP1, similar to the effects of carboxy-atractyloside on the ADP/ATP carrier (46), indicating that inhibition may occur in the cytoplasmic state of the protein. The mutagenesis of three highly conserved arginine residues in UCP1 (related by threefold pseudosymmetry in the monomer) abolishes nucleotide binding (47, 48). These residues are the contact points of the substrate-binding site in other mitochondrial carrier proteins where they are well placed to engage in electrostatic interactions with the phosphate moiety of nucleotides (49, 50). An interaction in this region of the protein would be consistent with the loss of nucleotide binding associated with the covalent modification of Glu-190 in UCP1 by *N*-ethyl-5-phenylisoxazolium 3′-sulfonate (Woodward's Reagent K) (51). This conserved residue is involved in the pH regulation of nucleotide binding and is predicted to face the central cavity toward the cytosolic side of the UCP1 monomer, where adduct formation would obstruct access to the central binding site. Our results infer that nucleotide regulation of UCP1 occurs through a relatively simple binding process, without the complication of dimer cross-talk (e.g., ref. 10), and provide a core structural basis for future investigations. The development of compounds that compete with nucleotides at the binding site without inhibiting proton flux may provide therapeutic means to switch thermogenesis on in brown fat artificially.

Our findings have implications on the mechanism of UCP1, which is the subject of ongoing debate. UCP1 is monomeric and dependent on cardiolipin, and so is more similar to other mitochondrial carriers than previously thought. Amino acid sequence analysis indicates that UCP1 has retained all of the key residues for carrier function, such as those involved in the formation of two salt bridge networks that gate access to a central substrate-binding site in the carrier transport cycle (27, 50), as well as those involved in the binding of cardiolipin. These observations put considerable constraint on how the protein is likely to operate and implies that the primary function of UCP1 relies on a conventional carrier transport mechanism. Based on symmetry analysis across carriers, uncoupling proteins were predicted to transport small carboxylic or keto acids via a strict exchange process (49, 50). In line with this notion, recombinant UCP2 was shown to exchange C4 metabolites, such as aspartate, malate, malonate, and oxaloacetate (52). However, recombinant UCP1 did not transport these substrates in parallel tests. The properties of the protein described here would favor the only mechanistic model of those currently debated that can be described by a conventional carrier exchange mechanism. In the "shuttling model" (9), UCP1 is effectively a fatty acid/fatty acid anion "exchanger," in which long chain fatty acids are transported in either a protonated or nonprotonated state but remain bound to the protein/membrane because of their hydrophobicity, leading to a net proton transport only.

Through the use of new purification procedures, we have uncovered the true composition of native UCP1. As such, there is now robust experimental evidence demonstrating that the two most studied mitochondrial carriers, UCP1 and the ADP/ATP carrier, are monomeric and bind cardiolipin, which sets a precedent for the rest of the protein family.

Methods

Purification of UCP1 by New Methods. Mitochondria were isolated from brown adipose tissue of newborn lambs (see *SI Materials and Methods*) and the membranes enriched by alkaline treatment (53). Mitochondria (∼600 mg of protein) were suspended in 360 mL of alkaline lysis buffer (100 mM Na_2CO_3 pH 11.5, 1 mM EDTA) and incubated for 30 min with gentle stirring at 4 °C. Membranes were pelleted by centrifugation (200,000 × *g* for 40 min) and resuspended by homogenization in 360 mL of membrane wash buffer [20 mM Tris·HCl pH 7.4, 1 mM EDTA, 10% (vol/vol) glycerol]. The suspension was recentrifuged and the pellets resuspended in membrane storage buffer [10 mM Tris·HCl pH 7.4, 10% (vol/vol) glycerol] by using a small glass homogenizer to a final protein concentration of ∼20 mg/mL before storage in liquid nitrogen. Enriched membranes (50–60 mg) were thawed, harvested by centrifugation (250,000 × *g* for 15 min) and suspended in solubilization buffer [3–4% (wt/vol) 10MNG, 300 mM NaCl, 20 mM Tris pH 8.0 with Complete protease inhibitor minus EDTA] to ∼10 mg/mL protein. The sample was gently agitated for 1 h (<10 °C), centrifuged (250,000 × *g* for 20 min) to remove insoluble material, and the supernatant desalted using PD-10 columns (GE Healthcare). UCP1 was purified by passage through a Vivaspin S Maxi H cation exchange spin column (Sartorius), supplemented with 50–80 mM NaCl and further purified by passage through a Vivapure Q Maxi H anion exchange spin column. The purified protein was supplemented with 150 mM NaCl and 1 mM EDTA and mixed with thiopropyl Sepharose 6B (Sigma; 100–150 mg dry per mg of UCP1, prerinsed in deoxygenated water) for 1 h with gentle agitation in an empty PD-10 column (<10 °C). The column was packed and eluted by gravity flow and washed with 50–75 mL per mg of protein of deoxygenated TPS buffer (50 mM Tris·HCl pH 8.0, 150 mM NaCl, 1 mM EDTA) containing 0.05% 10MNG or 0.2% 10M, ±0.1 g lipid per g detergent (the flow rate was ∼5 mL/min). The damp resin was mixed with 2.5 mL TPS buffer containing 150 mM DTT for 15 min (<10 °C) and the column was centrifuged (500 × *g*, 5 min) to recover UCP1. The eluate was exchanged into storage buffer (10 mM Tris·HCl pH 7.4, 0.01% 10MNG or 0.1% 10M, ±0.1 g lipid per g detergent) by using a PD-10 desalting column and concentrated to 5–8 mg/mL using a centrifugal device (50K MWCO Amicon Ultra) before storage in liquid nitrogen. Details of the other methods used in this study are given in *SI Materials and Methods*.

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1. Nedergaard J, Bengtsson T, Cannon B (2007) evidence for active brown adipose tissue in adult humans. *Am J Physiol Endocrinol Metab* 293(2):E444–E452.
2. Harms M, Seale P (2013) Brown and beige fat: Development, function and therapeutic potential. *Nat Med* 19(10):1252–1263.
3. Cypess AM, et al. (2009) Identification and importance of brown adipose tissue in adult humans. *N Engl J Med* 360(15):1509–1517.
4. Saito M, et al. (2009) High incidence of metabolically active brown adipose tissue in healthy adult humans: Effects of cold exposure and adiposity. *Diabetes* 58(7): 1526–1531.
5. Bartelt A, et al. (2011) Brown adipose tissue activity controls triglyceride clearance. *Nat Med* 17(2):200–205.
6. Nedergaard J, Cannon B (2010) The changed metabolic world with human brown adipose tissue: Therapeutic visions. *Cell Metab* 11(4):268–272.
7. Hirschberg V, Fromme T, Klingenspor M (2011) Test systems to study the structure and function of uncoupling protein 1: A critical overview. *Front Endocrinol (Lausanne)* 2:63.
8. Divakaruni AS, Brand MD (2011) The regulation and physiology of mitochondrial proton leak. *Physiology (Bethesda)* 26(3):192–205.
9. Fedorenko A, Lishko PV, Kirichok Y (2012) Mechanism of fatty-acid-dependent UCP1 uncoupling in brown fat mitochondria. *Cell* 151(2):400–413.
10. Klingenberg M, Huang SG (1999) Structure and function of the uncoupling protein from brown adipose tissue. *Biochim Biophys Acta* 1415(2):271–296.
11. Garlid KD, Jabúrek M, Jezek P (1998) The mechanism of proton transport mediated by mitochondrial uncoupling proteins. *FEBS Lett* 438(1–2):10–14.
12. Shabalina IG, Jacobsson A, Cannon B, Nedergaard J (2004) Native UCP1 displays simple competitive kinetics between the regulators purine nucleotides and fatty acids. *J Biol Chem* 279(37):38236–38248.
13. Divakaruni AS, Humphrey DM, Brand MD (2012) Fatty acids change the conformation of uncoupling protein 1 (UCP1). *J Biol Chem* 287(44):36845–36853.
14. Esteves TC, Echtay KS, Jonassen T, Clarke CF, Brand MD (2004) Ubiquinone is not required for proton conductance by uncoupling protein 1 in yeast mitochondria. *Biochem J* 379(Pt 2):309–315.
15. Shabalina IG, et al. (2006) UCP1 and defense against oxidative stress. 4-Hydroxy-2-nonenal effects on brown fat mitochondria are uncoupling protein 1-independent. *J Biol Chem* 281(20):13882–13893.
16. Lin CS, Klingenberg M (1980) Isolation of the uncoupling protein from brown adipose tissue mitochondria. *FEBS Lett* 113(2):299–303.
17. Lin CS, Hackenberg H, Klingenberg EM (1980) The uncoupling protein from brown adipose tissue mitochondria is a dimer. A hydrodynamic study. *FEBS Lett* 113(2): 304–306.
18. Lin CS, Klingenberg M (1982) Characteristics of the isolated purine nucleotide binding protein from brown fat mitochondria. *Biochemistry* 21(12):2950–2956.
19. Nedergaard J, Cannon B (1985) [3H]GDP binding and thermogenin amount in brown adipose tissue mitochondria from cold-exposed rats. *Am J Physiol* 248(3 Pt 1): C365–C371.
20. Jakobs P, Braun A, Jezek P, Trommer WE (1991) Binding of ATP to uncoupling protein of brown fat mitochondria as studied by means of spin-labeled ATP derivatives. *FEBS Lett* 284(2):195–198.
21. Winkler E, Klingenberg M (1992) Photoaffinity labeling of the nucleotide-binding site of the uncoupling protein from hamster brown adipose tissue. *Eur J Biochem* 203(1-2):295–304.
22. Klingenberg M, Appel M (1989) The uncoupling protein dimer can form a disulfide cross-link between the mobile C-terminal SH groups. *Eur J Biochem* 180(1):123–131.
23. Klingenberg M (2008) The ADP and ATP transport in mitochondria and its carrier. *Biochim Biophys Acta* 1778(10):1978–2021.
24. Krämer R, Palmieri F (1989) Molecular aspects of isolated and reconstituted carrier proteins from animal mitochondria. *Biochim Biophys Acta* 974(1):1–23.
25. Pebay-Peyroula E, et al. (2003) Structure of mitochondrial ADP/ATP carrier in complex with carboxyatractyloside. *Nature* 426(6962):39–44.
26. Nury H, et al. (2005) Structural basis for lipid-mediated interactions between mitochondrial ADP/ATP carrier monomers. *FEBS Lett* 579(27):6031–6036.
27. Ruprecht JJ, et al. (2014) Structures of yeast mitochondrial ADP/ATP carriers support a domain-based alternating-access transport mechanism. *Proc Natl Acad Sci USA* 111(4): E426–E434.
28. Klingenberg M (2009) Cardiolipin and mitochondrial carriers. *Biochim Biophys Acta* 1788(10):2048–2058.
29. Beyer K, Klingenberg M (1985) ADP/ATP carrier protein from beef heart mitochondria has high amounts of tightly bound cardiolipin, as revealed by 31P nuclear magnetic resonance. *Biochemistry* 24(15):3821–3826.
30. Winkler E, Klingenberg M (1992) An improved procedure for reconstitution of the uncoupling protein and in-depth analysis of H+/OH- transport. *Eur J Biochem* 207(1): 135–145.
31. Winkler E, Klingenberg M (1994) Effect of fatty acids on H+ transport activity of the reconstituted uncoupling protein. *J Biol Chem* 269(4):2508–2515.
32. Bamber L, Slotboom DJ, Kunji ER (2007) Yeast mitochondrial ADP/ATP carriers are monomeric in detergents as demonstrated by differential affinity purification. *J Mol Biol* 371(2):388–395.
33. Bamber L, Harding M, Monné M, Slotboom DJ, Kunji ER (2007) The yeast mitochondrial ADP/ATP carrier functions as a monomer in mitochondrial membranes. *Proc Natl Acad Sci USA* 104(26):10830–10834.
34. Bamber L, Harding M, Butler PJ, Kunji ER (2006) Yeast mitochondrial ADP/ATP carriers are monomeric in detergents. *Proc Natl Acad Sci USA* 103(44):16224–16229.
35. Nury H, et al. (2008) Mitochondrial bovine ADP/ATP carrier in detergent is predominantly monomeric but also forms multimeric species. *Biochemistry* 47(47): 12319–12331.
36. Crichton PG, Harding M, Ruprecht JJ, Lee Y, Kunji ER (2013) Lipid, detergent, and Coomassie Blue G-250 affect the migration of small membrane proteins in blue native gels: Mitochondrial carriers migrate as monomers not dimers. *J Biol Chem* 288(30): 22163–22173.
37. Stuart JA, Harper JA, Brindle KM, Jekabsons MB, Brand MD (2001) A mitochondrial uncoupling artifact can be caused by expression of uncoupling protein 1 in yeast. *Biochem J* 356(Pt 3):779–789.
38. Zoonens M, et al. (2013) Dangerous liaisons between detergents and membrane proteins. The case of mitochondrial uncoupling protein 2. *J Am Chem Soc* 135(40): 15174–15182.
39. Chae PS, et al. (2010) Maltose-neopentyl glycol (MNG) amphiphiles for solubilization, stabilization and crystallization of membrane proteins. *Nat Methods* 7(12): 1003–1008.
40. Ocloo A, Shabalina IG, Nedergaard J, Brand MD (2007) Cold-induced alterations of phospholipid fatty acyl composition in brown adipose tissue mitochondria are independent of uncoupling protein-1. *Am J Physiol Regul Integr Comp Physiol* 293(3): R1086–R1093.
41. Alexandrov AI, Mileni M, Chien EY, Hanson MA, Stevens RC (2008) Microscale fluorescent thermal stability assay for membrane proteins. *Structure* 16(3):351–359.
42. Crichton PG, et al. (2015) Trends in thermostability provide information on the nature of substrate, inhibitor, and lipid interactions with mitochondrial carriers. *J Biol Chem* 290(13):8206–8217.
43. Huang SG, Klingenberg M (1995) Fluorescent nucleotide derivatives as specific probes for the uncoupling protein: Thermodynamics and kinetics of binding and the control by pH. *Biochemistry* 34(1):349–360.
44. Kunji ER, Crichton PG (2010) Mitochondrial carriers function as monomers. *Biochim Biophys Acta* 1797(6-7):817–831.
45. Rodríguez-Vico F, Martínez-Cayuela M, Zafra MF, García-Peregrin E, Ramírez H (1991) A procedure for the simultaneous determination of lipid and protein in biomembranes and other biological samples. *Lipids* 26(1):77–80.
46. Azzu V, Parker N, Brand MD (2008) High membrane potential promotes alkenal-induced mitochondrial uncoupling and influences adenine nucleotide translocase conformation. *Biochem J* 413(2):323–332.
47. Modriansky M, Murdza-Inglis DL, Patel HV, Freeman KB, Garlid KD (1997) Identification by site-directed mutagenesis of three arginines in uncoupling protein that are essential for nucleotide binding and inhibition. *J Biol Chem* 272(40):24759–24762.
48. Klingenberg M, Echtay KS (2001) Uncoupling proteins: The issues from a biochemist point of view. *Biochim Biophys Acta* 1504(1):128–143.
49. Robinson AJ, Kunji ER (2006) Mitochondrial carriers in the cytoplasmic state have a common substrate binding site. *Proc Natl Acad Sci USA* 103(8):2617–2622.
50. Robinson AJ, Overy C, Kunji ER (2008) The mechanism of transport by mitochondrial carriers based on analysis of symmetry. *Proc Natl Acad Sci USA* 105(46):17766–17771.
51. Winkler E, Wachter E, Klingenberg M (1997) Identification of the pH sensor for nucleotide binding in the uncoupling protein from brown adipose tissue. *Biochemistry* 36(1):148–155.
52. Vozza A, et al. (2014) UCP2 transports C4 metabolites out of mitochondria, regulating glucose and glutamine oxidation. *Proc Natl Acad Sci USA* 111(3):960–965.
53. Fujiki Y, Hubbard AL, Fowler S, Lazarow PB (1982) Isolation of intracellular membranes by means of sodium carbonate treatment: Application to endoplasmic reticulum. *J Cell Biol* 93(1):97–102.