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Review

Mitochondrial proton leak and the uncoupling protein 1 homologues

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Abstract

Mitochondrial proton leak is the largest single contributor to the standard metabolic rate (SMR) of a rat, accounting for about 20% of SMR. Yet the mechanisms by which proton leak occurs are incompletely understood. The available evidence suggests that both phospholipids and proteins in the mitochondrial inner membrane are important determinants of proton conductance. The uncoupling protein 1 homologues (e.g. UCP2, UCP3) may play a role in mediating proton leak, but it is unlikely they account for all of the observed proton conductance. Experimental data regarding the functions of these proteins include important ambiguities and contradictions which must be addressed before their function can be confirmed. The physiological role of the proton leak, and of the uncoupling protein 1 homologues, remains similarly unclear.

Keywords: Mitochondrion; Proton conductance; Basal metabolic rate; Respiration; UCP1; UCP2; UCP3; BMCP1; Fatty acid; Brown adipose tissue; Thermogenesis; Zebrafish; Carp

1. Introduction

1.1. Mitochondrial proton conductance

The mitochondrial electrochemical proton gradient, formed as electrons are passed down the respiratory chain, is the primary energy source for cellular ATP synthesis. However, not all of the energy available in the electrochemical gradient is coupled to ATP synthesis. Some is consumed by 'proton leak' reactions, whereby protons pumped out of the matrix are able to pass back into the mitochondria through proton conductance pathways in the inner membrane which circumvent the ATP synthase [1–4].

1.2. Physiological significance of the proton leak

These non-productive proton conductance pathways are physiologically important. They account for a surprisingly high proportion of cellular metabolic rates, e.g. 25 and 52% of the resting respiration rate of isolated rat hepatocytes and perfused (but not contracting) rat hindquarter muscles, respectively [3,5]. However, the proton leak reactions and the ATP synthase compete for the same driving force, and the question arises whether the contribution that proton leak makes to metabolic rate remains significant at higher metabolic rates, when flux through the ATP synthase must increase. Rolfe et al. [6] determined the proportional contribution of the proton leak to metabolic rate as respiration rate was increased 2-fold, by stimulating gluconeogenesis and ureagenesis in hepatocytes, and by induc-

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Table 1
Contribution of mitochondrial proton leak to the respiration rate of hepatocytes isolated from endothermic and ectothermic vertebrates and invertebrates

	Organism	Respiration (%)	Reference
Vertebrates			
Endotherms	Mouse	~19%	[75]
	Rat	19–26%	[6,39]
	Ferret	~19%	[75]
	Sheep	~27%	[75]
	Pig	~15%	[75]
	Horse	~16%	[75]
Ectotherms	Lizard	up to 30%	[22]
	Lamprey	up to 40%	[76]
	Frog	up to 25%	[77]
Invertebrate			
	Snail[a]	up to 25%	[77]

[a]Hepatopancreacytes

ing contraction in perfused rat hindquarters. They showed that the contribution of mitochondrial proton leak does decrease as workload increases, but only slightly. Proton leak is measured in these studies as 22 and 34% of the energy budget of stimulated hepatocytes and skeletal muscle, respectively. Therefore, proton leak remains an important component of the rat metabolic rate, even at higher metabolic rates. The best estimates from all available data suggest that proton leak accounts for about 20% of rat SMR [6], making it the largest single contributor to mammalian SMR.

Numerous studies have established that the significant contribution proton leak makes to cellular metabolic rate is a ubiquitous phenomenon, apparently a property of all eukaryotic cells. In addition to liver and muscle, mitochondrial proton leak has been demonstrated in kidney [7], thymocytes [8], and brain [7,9]. Across a wide range of ectothermic and endothermic vertebrates of different body mass, the proton leak accounts for about 20–25% of total hepatocyte oxygen consumption (Table 1). Thus, the proportional contribution of proton leak to total hepatocyte metabolic rate appears to be conserved in all species, regardless of phylogenetic position or body mass. An important corollary of this observation is that endothermy is apparently not achieved by simply increasing the proportional contribution of proton leak to SMR.

1.3. Characteristics of proton conductance

The proton permeabilities of phospholipid membranes are anomalously high relative to other ions. Proton permeability coefficients of planar lipid membranes and liposomes range from 10^{-6} to 10^{-3} cm s^{-1} [10], values which are six to eight orders of magnitude larger than those for other small ions [11–14]. In liposomes made from liver mitochondrial phospholipids, proton flux has been measured at 29.4 nmol H^+ min^{-1} (mg phospholipid)$^{-1}$ (at pH 7.2, 37°C and an imposed membrane potential of 160 mV). This corresponds to a permeability coefficient of 2.5×10^{-3} cm s^{-1} (calculated by assuming that 1 mg of phospholipid occupies 0.309 m^2 bilayer [15]), indicating that mitochondrial phospholipid bilayers are in the high end of this proton permeability range. This permeability coefficient is essentially conserved in liposomes made from liver mitochondrial inner membrane phospholipids of a wide range of vertebrate species [16].

While the protein-free mitochondrial phospholipid bilayers are particularly permeable to protons, the mitochondrial inner membrane in its native state has an even greater proton conductance. A mitochondrial proton permeability coefficient can be calculated from the data of Brookes et al. [17], Krishnamoorthy and Hinkle [18] and O'Shea et al. [19]. At the same electrical potential of 160 mV, it ranges between 1.7×10^{-1} and 2×10^{-2} cm s^{-1}, with an average value of 7.6×10^{-2} cm s^{-1}, depending on body mass, thyroid status and phylogenetic position of the organism. Thus, the inner membrane in its native state can be more than an order of magnitude more permeable to protons than the phospholipid bilayer.

1.4. Mechanism(s) of proton conductance

Aspects of the phospholipid fatty acid composition of the inner membrane are strongly correlated with the proton conductance of the membrane [17,20,21]. However, variation in the fatty acid composition of the mitochondrial inner membrane phospholipids reconstituted in liposomes does not appear to account for the phylogenetic or allometric differences in proton conductance [16,17]. And indeed, the experiments with liposomes made from inner membrane

phospholipids suggest that the proton conductance of the protein-free membrane would support only 5% (in rat [16]) to about 25% (in a lizard [15,22]) of total proton leak. It is therefore clear that other aspects of the inner membrane play an important role in determining bilayer proton permeability.

Three possible explanations for the much greater proton permeability of the native inner membrane relative to the phospholipid liposome are [15]: (1) bilayer asymmetry or other complex phospholipid ordering phenomena; (2) non-specific effects of an interaction between phospholipids and proteins in the inner membrane; or (3) the existence of specific proteins catalysing some or most of the proton leak.

A number of experiments have examined these mechanisms, but provide few clear insights. O'Shea et al. [19] found that the incorporation of cytochrome oxidase into liposomes had no effect on proton permeability. Mimms et al. [23] reported that incorporation of glycophorin into liposomes had little effect on cation permeability (however, see [24]). P.M. Stevenson and M.D. Brand (unpublished) found a linear correlation between cytochrome oxidase content of liposomes and proton leak, but no effect of detergent-free cytochrome P450 incorporation on leak. Oliver and Deamer [25] have found that α-helical hydrophobic long-chain polyalanine and polyleucine incorporated into liposomes were able to conduct protons but not potassium ions. Short-chain polyamino acids or long-chain polyhistidine had little or no effect on liposomal cation permeabilities. Other synthetic amphipathic helices have also been found to produce ion-conducting defects in lipid bilayers (see [25]), among them the 21-residue peptide: H_2N-$(LSLLLSL)_3$-$CONH_2$, which formed highly proton-selective channels [26]. Taken together, these studies indicate that specific protein characteristics could in principle be involved in proton conductance.

The evidence for the participation of specific proteins in proton leak is equally mixed. Brown and Brand [2] found that little or no proton leak appears to be mediated by the proton-pumping respiratory complexes. Similarly, the adenine nucleotide translocase (ANT) does not appear to catalyse a significant basal proton leak since yeast mitochondria overexpressing ANT are not more uncoupled than controls [27]. However, Skulachev (reviewed in [28]) reports that the ANT and other members of the mitochondrial carrier family can catalyse a fatty acid dependent proton conductance. The uncoupling protein 1 (UCP1) homologues have been proposed as candidates to catalyse the mitochondrial proton leak. They have high amino acid sequence identity with UCP1, perhaps the only proven protein uncoupler of oxidative phosphorylation.

2. The uncoupling protein and its homologues

2.1. UCP1 – a protein-catalysed proton conductance

Perhaps the best-studied example of a protein-catalysed proton conductance is found in mitochondria from brown adipose tissue (BAT), the specialised adipose tissue which occurs primarily in small mammals. BAT mitochondria contain thermogenin, or UCP1, a 32 kDa inner membrane protein that catalyses the net transfer of protons from the mitochondrial intermembrane space to the matrix (see [29] for review). The physiological function of UCP1, and of BAT, is heat production (see [30] for review). Small mammals adapt to cold by inserting more UCP1 into their BAT mitochondria [31,32], which (under β-adrenergic stimulation) become more permeable to protons (see [30] for review). This results in an augmentation of the futile cycling of protons in a matrix–intermembrane space-matrix circuit that allows heat to be produced at a greater rate, but does no more useful work. Arteriovenous anastomoses in BAT provide an effective means of exporting the heat throughout the circulation, thus allowing small mammals to achieve homeothermy in the face of low environmental temperature.

UCP1 has some physiologically significant, and experimentally useful, properties: it can be 'turned on' and 'turned off'. Fatty acids are required for maximal UCP1 activity. UCP1 activity can also be fully inhibited by purine nucleoside di- and tri-phosphates such as guanosine diphosphate (GDP) (see [33] for review). Physiologically, these properties may be important in allowing BAT to respond to acute changes in the animal's need to produce body heat. Experimentally, they are useful in allowing the measurement of UCP1 activity, and in allowing a distinction to be made between the UCP1 proton

Fig. 1. Alignment of a new zebrafish UCP (zUCPx) with a previously identified zebrafish UCP2 (zUCP2), and human UCP2 (hUCP2) and UCP3 (hUCP3). The zebrafish UCPx was assembled from translated ESTs identified by tFASTA searches of the European Molecular Biology Laboratory nucleotide sequence databases. The full UCPx sequence was assembled from nine translated ESTs, with accession IDs AW305965, AW422188, AW280377, AW305876, AW419703, AI497417, AI497345, AW419878, AI497227. The zebrafish UCPx is approximately 70% identical to hUCP2, 70% identical to hUCP3 and 69% identical to zUCP2.

conductance pathway and other pathway(s) of mitochondrial inner membrane proton conductance.

When UCP1 is fully inhibited by GDP, or when UCP1 expression has been knocked out, the proton conductance of BAT mitochondria is not essentially different from that of mitochondria from other tissues [34,35]. These observations tell us that UCP1 activity accounts for only part of the total proton conductance of BAT mitochondria. And, as UCP1 is not expressed in other tissues, it cannot account for the proton conductance observed in all mitochondria. However, UCP1 can provide, and indeed has provided, a theoretical framework for the identification and study of other candidate uncoupling proteins.

2.2. The UCP1 homologues

The UCP1 homologue labelled UCP2 by Fleury et al. [36] is clearly a candidate uncoupling protein that might account for the basal uncoupling of mitochondria from many sources, especially since UCP2 mRNA is found widely in mammalian tissues. Other UCP1 homologues, like UCP3 [37] and the plant UCP [38] are more limited in their distribution. Together these proteins (and perhaps some others) form a family of proteins identifiable on the basis of between 50 and 60% amino acid sequence identity with UCP1. Could the UCP family be responsible for the observed basal level of uncoupling observed in all mitochondria?

Several observations suggest that they are not. The uncoupling proteins are either not present or present at vanishingly low levels in normal mammalian hepatocytes. However, hepatocyte mitochondria even within cells show a typical uncoupling of oxidative phosphorylation [39]. Also, in a number of eukaryotic organisms whose full genome sequence is now known we can search for members of the UCP family. In *Saccharomyces cerevisiae* and *Caenorhabditis elegans* no protein sequence with greater than about 35% identity to any of the UCPs can be identified [40]. A similar observation can be made in the recently available *Drosophila melanogaster* genome sequence. This level of amino acid sequence identity is similar to that which is shared amongst all members of the mitochondrial transporter family (about 25–35%). Thus, UCP1 homologues are apparently absent in these organisms. Because UCP1 homologues are not found in all organisms, nor in all tissues within an organism, they cannot account for the observed proton conductance of all mitochondria (though other, more distantly related, proteins might still fulfil this function).

These observations suggest that the UCP1 homologues do not catalyse the basal proton conductance of mitochondria. Nonetheless, they may provide an inducible pathway through which proton conductance can be increased in some mitochondria, as UCP1 does in BAT. Indeed, the thermogenic function of UCP1 appears to have influenced many of the hypotheses regarding the physiological roles of the UCP homologues. Perhaps because they were initially identified in mammals, it was thought that

UCP2 and UCP3 may be involved in mammalian thermogenesis. The proteins have been found in all mammals examined to date.

However, a number of observations suggest a disconnection between the UCP1 homologues and endothermy (Table 2). UCP1 homologues are found in ectotherms. An mRNA encoding a protein with 82% amino acid sequence identity with the mammalian UCP2s has been identified in carp and zebrafish [41]. A second UCP1 homologue, with about 70% sequence identity to mammalian UCP2 can be identified through the assembly of expressed sequence tag (EST) sequences from zebrafish (Fig. 1). Neither zebrafish nor carp are capable of even regional endothermy.

The plant UCP has been suggested by some to be involved in heat production [38]. At least six plant species, including potato, tomato, rice, soybean, aspen and corn express UCP, though none of these are known to employ endothermy as a freeze-avoidance strategy. Similarly, the tissue expression of the plant UCP does not fit well with the hypothesis of regional endothermy. The UCP is expressed in virtually all plant tissues including roots, stems, leaves and bark (Table 2).

Even in mammals, no clear connection between

Table 2
The occurrence of mRNA encoding UCP1 homologues in tissues from endothermic and ectothermic organisms

	Tissue	Expression details	Sequence identity	Accession ID
Mammals				
Mouse	urogenital ridge	foetus	97% over 148 aa	AI256818
Mouse	kidney	adult	90% over 146 aa	AI115988
Mouse	mammary gland	4 weeks old	94% over 144 aa	AA929720
Mouse	macrophage	WEHI-3 cell line	96% over 118 aa	AI097961
Mouse	colon cancer cell line			AI316588
Mouse	thymus	4 weeks old		AI019378
Pig	white adipose tissue		94% over 309 aa	AF036757
Human	skeletal muscle		100% over 309 aa	U82819
Human	colon tumour			AA633093
Human	uterus	serous papillary carcinoma	89% over 116 aa	AI884479
Human	fibroblast			AA065221
Human	endometrium	adenocarcinoma	82% over 121 aa	AI671640
Human	B-lymphocyte			AA298140
Human	proliferating erythroid cells	cultured mononuclear cells	100% over 59 aa	AI630458
Ectothermic vertebrates				
Carp	peritoneal exudate cells	48 h alginate induction	100% over 310 aa	AJ243486
Zebrafish	regenerating fin	day 0 post fin excision	94% over 310 aa	AJ243250
Carp	leukocyte		99% over 91 aa	AU052042
Zebrafish	embryos and adult livers	shield stage	73% over 187 aa	AI497227
Cephalochordate	larval stage	2 days	67% over 165 aa	Z83259
Plants				
Potato			100% over 295 aa	Y11220
Tomato	leaf	4 weeks old	91% over 117 aa	AI779574
Cotton	immature fibre	6 days post anthesis	72% over 143 aa	AI731438
Aspen	cambial region	1.5 m actively growing tree	80% over 127 aa	AI162254
Corn	kernel	10 days post fertilisation	82% over 115 aa	AI677128
Arabidopsis	pooled tissues	seedlings, tissue culture roots	99% over 93 aa	AA713080
Soybean			68% over 94 aa	AI899961

Data represent a sample of UCP occurrence taken from tFASTA searches of the European Molecular Biology Laboratory's nucleotide database. tFASTA compares an amino acid sequence with translations, in all six open reading frames, of nucleotide sequences. Mammalian sequences were tFASTAed with the human UCP2 sequence. Fish and cephalochordate sequences were tFASTAed with the carp UCP2 sequence. Plant sequences were tFASTAed with the potato UCP sequence. Much of the data is taken from EST libraries, and in these cases an incomplete sequence has been aligned with a full sequence. The length of aligned regions and their (translated) amino acid sequence similarities are shown under 'Sequence Identity'.

UCP1 homologue expression and thermogenesis is obvious (see Table 2). UCP2 protein is apparently most highly expressed in lung, intestine and spleen [42], none of which are thought to play an important role in thermogenesis. In UCP1 knockout mice, BAT UCP2 mRNA levels rise about 14-fold. Yet these mice are cold intolerant, indicating that these higher levels of UCP2 have not replaced the thermogenic capacity of UCP1 (see below). Similarly, in starved rats held at thermoneutrality, UCP3 levels increase [43], though this is not reflected by altered mitochondrial proton conductance [44] (see below).

The distribution and expression patterns of the UCP1 homologues suggest that they do not account for the ubiquitous basal proton conductance of mitochondria. Similarly, their function is probably not thermogenesis. They may nonetheless catalyse a proton conductance. Many researchers have attempted to test this hypothesis by genetically manipulating the expression of the UCP1 homologues and measuring the effect on mitochondrial function.

2.3. Functional studies of the UCP1 homologues and proton conductance

2.3.1. Heterologous expression of UCPs in yeast

Direct studies of the function of the UCP1 homologues have been done most often using the yeast model. Expression of UCP2 and UCP3 in *S. cerevisiae* appears to confirm their uncoupling activities: membrane potential is lowered and state 4 rates are increased. Three criticisms can, however, be made of most of these experiments: (1) in many cases an inhibition of fully FCCP-uncoupled rates of respiration is apparent. There is no reason, related to a putative uncoupling activity of a UCP, for this to occur. It indicates a disruption of mitochondrial substrate oxidation; (2) the presence and function of the protein are in some cases inferred from the appearance of an uncoupling phenotype. This approach, especially where UCP2 or UCP3 expression has not been quantified, has the danger of being self-selecting – expression conditions may be altered until an uncoupling phenotype is observed, thus apparently confirming that enough of the protein has been made and it is active; (3) absolute levels of UCP1 homologues expressed in yeast have, in all studies, remained undetermined. Similarly, absolute levels of UCP2 and UCP3 protein in mammalian tissues are unknown. Each of these criticisms is addressed in detail below.

Expression of UCP2 or UCP3 in yeast typically results in small, often non-significant, increases in the state 4 (or state 2) respiration rates of isolated mitochondria [45]. These state 4 rates may then be expressed as a percent of the rates obtained with a concentration of artificial uncoupler assumed to give maximal respiration. However, in some experiments [45,46] UCP expression in yeast can be seen to reduce the fully uncoupled respiration rates. As a result, the estimate of respiratory control (RC), made by taking the ratio of fully uncoupled/state 4 rate, is lowered. This has been interpreted as confirmation of uncoupling. However, there is no reason why uncoupling resulting from UCP expression should affect fully uncoupled respiration rates, and it is inappropriate to standardise state 4 rates to fully uncoupled rates that have been so altered. The fully uncoupled rate is not a measure of the amount of yeast mitochondria, but rather it is a measure of respiratory chain function. A significant difference in the value of the RC may as readily indicate respiratory chain dysfunction as increased leak rate.

The various analytical difficulties imposed by alterations of respiratory chain activity can be circumvented by determination of the proton leak kinetics. Estimates of the proton conductance of the inner membrane (which is the basis of uncoupling) can be made by simultaneously measuring membrane potential ($\Delta\Psi$) and respiration rate as the activity of the respiratory chain is titrated in the presence of oligomycin (which inhibits proton flux through the ATP synthase). This exercise allows a proton leak curve to be constructed for UCP-containing and control mitochondria. The proton conductances can then be compared at a given value of $\Delta\Psi$.

This approach was taken with the expression of mouse brain mitochondrial carrier 1 (mBMCP1) expressed in yeast [47]. In this study, yeast spheroplast preparations expressing BMCP1 exhibited greater respiration at a given $\Delta\Psi$ (assessed via tetraphenylphosphonium ion uptake), suggesting increased proton leak relative to empty vector controls. However, in these experiments respiration was normalised to the fully uncoupled rates to try to compensate for any differences in respiratory chain per spheroplast. This normalisation involves the assumption that

BMCP1 expression has no effect on fully uncoupled rates. However, fully uncoupled rates are approximately 25% lower in BMCP1 spheroplasts (see figure 6 in [47]). This may reflect inhibition of respiratory chains rather than decreased number of chains, since it is also observed in isolated mitochondria when UCP1 [48] or UCP3 [46] is overexpressed. Thus, the normalised respiration rates may overestimate the proton cycling rate in BMCP1 spheroplasts, and the extent of uncoupling by BMCP1.

In none of the experiments with transfected yeast has the expression levels of the UCP1 homologues been measured. Successful expression of the protein has in some cases been inferred from the appearance of the uncoupling phenotype itself. This approach assumes that any observed uncoupling results from protein activity, rather than from some more general disruption of mitochondrial physiology that might result from the expression of any exogenous protein. Two studies have attempted to control for possible non-specific effects of inserting a foreign protein into the inner mitochondrial membrane by transfecting yeast with the oxoglutarate carrier. Among the members of the mitochondrial carrier family that have no known uncoupling activity, the oxoglutarate carrier is most similar in sequence to UCP2 and UCP3. This control was used by Sanchis et al. [47] but surprisingly there are no data presented on the amount of oxoglutarate carrier expressed or the effects of this carrier on respiration rates or proton leak kinetics. Zhang et al. [45] reported that oxoglutarate carrier expression in yeast did not affect growth, mitochondrial state 4 rates, or uncoupled rates. These observations provide a somewhat stronger argument that the effects observed with UCP3 are not related to a general non-specific effect of protein insertion into mitochondrial inner membrane. Unfortunately, the data are limited by the fact that the estimates of amounts of UCP3 and oxoglutarate carrier expressed were determined with influenza virus hemagglutinin epitope-tagged constructs which were different from the UCP expression constructs used in the functional studies of the mitochondria.

A better control for the effects of protein expression on mitochondrial inner membrane integrity is UCP1. UCP1 has the advantages of being readily quantifiable (by Western blot) and having an activity that can be 'turned on' or 'turned off' with palmitate or GDP, respectively. This allows a distinction to be made between proton conductance due to UCP1 activity and that which occurs even when the protein is rendered inactive. Recently, experiments on UCP1 expression in yeast suggest that the expression of UCP1 in mitochondria can affect mitochondrial properties in ways not related to its native activity [48]. From these experiments, it is clear that at moderate levels of UCP1 expression, UCP1 activity is completely GDP inhibitable, but as UCP1 expression levels are increased, a GDP-insensitive proton conductance is apparent. This suggests that an uncoupling of yeast mitochondria can occur as an artifact of the expression of members of the UCP family.

The third important criticism of studies where UCPs have been expressed in yeast concerns the amounts of protein expressed and how this relates to the amount of protein that may be present in, for example, mammalian mitochondria. Any conclusion that UCP2- or UCP3-mediated uncoupling in yeast is proof of the physiological uncoupling roles of these proteins would disregard the possibility that orders of magnitude more protein may have been expressed in yeast than are present in mammalian mitochondria. For any given activity, this could have the effect of rendering the uncoupling observed in yeast physiologically irrelevant. For example, if a UCP is expressed at 1 μg/mg mitochondrial protein in transfected yeast, but present at only 10 ng/mg in mammalian tissue, the total amount of proton conductance catalysed in mammalian mitochondria would be 100-fold less than that observed in yeast. This could readily account for the discrepancies observed between yeast mitochondria and mitochondria from genetically unaltered mammalian tissues.

2.3.2. Studies of UCPs reconstituted in liposomes

An alternative approach taken to study function and regulation of UCP2 and UCP3 has been to reconstitute these proteins in liposomes after expression in *Escherichia coli* [49,50]. Mitochondrial carrier proteins expressed in *E. coli* accumulate in large quantities in inclusion bodies. Following isolation and solubilisation of inclusion body proteins, the goal is to correctly renature the protein in the presence of phospholipids to recover native function. Confirmation of native function in such a system is inherently nebulous when native function is un-

known, as with UCP2 and UCP3. However, a reasonable way of approaching this problem is to reconstitute UCP1 under identical conditions. If the conditions permit recovery of function for UCP1, then any activity recovered with UCP2 or UCP3 could be argued as reflecting in vivo activity.

Echtay et al. [49] reconstituted a purine nucleotide-sensitive chloride transport from bacterially expressed UCP1. Anion transport by UCP1 is a well-known biochemical phenomenon with no obvious physiological significance. Reported K_i values were 0.4–1.5 μM, similar to values reported for inhibition of BAT mitochondrial proton and anion conductance, where UCP1 is present in its native form. Proton transport activity was not recovered with bacterially reconstituted UCP1, but was demonstrated for UCP1 reconstituted from hamster BAT mitochondria. Under the same conditions, bacterially expressed UCP3 exhibited a chloride transport that was more sensitive to inhibition by purine nucleotides (K_i from 0.08 to 0.34 μM) than for bacterially expressed UCP1. No proton transport activity was demonstrated with UCP3, although it was implied that this protein functions as an uncoupler.

Jaburek et al. [50] reconstituted a fatty acid-dependent proton transport activity from bacterially expressed UCP2 and UCP3. The specific activities reported are very similar to those reported in earlier studies on UCP1 reconstitution from BAT [51–53] but only 8–25% of values from more recent experiments [49]. These activities were inhibited by purine nucleotides; reported K_i values were about 3000 times greater than those determined by Echtay et al. [49] for inhibition of UCP3 chloride transport. Unfortunately, Jaburek et al. [50] did not use bacterially expressed UCP1 as a control to demonstrate firstly that its known uncoupling activity could be reconstituted under conditions identical to those used for UCP2 and UCP3, and secondly that its inhibition by nucleotides is similar to that which occurs in BAT mitochondria. This critical point introduces reasonable doubt about the methods employed to reconstitute UCP2 and UCP3.

Echtay et al. [49] imply that UCP3 functions as an uncoupler, while Jaburek et al. [50] state that their results directly demonstrate that UCP2 and UCP3 are in vivo uncouplers. Both studies are limited by the fact that (a) inclusion body preparations are invariably contaminated with bacterial proteins that could potentially introduce artifactual activities into the final liposomal preparations; and (b) the artificial lipids used to reconstitute the proteins are not representative of the lipid composition of the mitochondrial inner membrane, thus potentially influencing these proteins in unknown ways [54]. Additionally, the former study is clearly limited by the fact that reconstituted UCP3 did not exhibit proton transport, while the vastly lower nucleotide affinities reported for UCP2 and UCP3 in the latter study may indicate that these proteins are incorrectly refolded. Such a suggestion stems from the argument that reconstitution of a high affinity binding event must reflect a more accurate protein refolding than reconstitution of a very low affinity binding event for the same nucleotides. Thus, if UCP2 and UCP3 are not correctly folded, then there must be some doubt regarding the reliability of the reconstitution procedures.

The argument that UCP2 and UCP3 are incorrectly folded in the study by Jaburek et al. [50] because of the low nucleotide affinities could be refuted on the grounds that (a) there is no a priori reason to believe that UCP2 and UCP3 should exhibit high affinity nucleotide binding as observed by Echtay et al. [49]; and (b) the high affinity nucleotide effects on UCP3 reported by Echtay et al. [49] do not reflect true function because UCP3 did not exhibit proton transport and therefore must not be in a correctly folded form. The latter argument can be dismissed on the grounds that reconstitution of a proton transport activity does not indicate that the protein is folded in a form that reflects its in vivo function. The former argument is one that cannot be dismissed, but the fact that Echtay et al. [49] successfully reconstituted nucleotide binding to bacterially expressed UCP1 in a manner consistent with its in vivo affinity suggests that, in vivo, UCP3 may also bind nucleotides with similar affinity. Taken together, these reconstitution experiments do not conclusively demonstrate that UCP2 and UCP3 function as uncouplers in vivo.

2.3.3. *Transgenic overexpression and gene knockout studies*

Transgenic mice overexpressing uncoupling proteins and knockout models have been produced as a direct approach to investigate the physiological

function of UCP1 and UCP1 homologues. Matthias et al. [35] and Monemdjou et al. [34] studied the bioenergetics of BAT mitochondria from UCP1-ablated mice originally developed by Kozak and coworkers [55] Interestingly, they found that UCP2 mRNA levels are increased up to 14-fold in the BAT of these mice (though it is not known whether UCP2 protein levels were also increased), but that this was not associated with increased mitochondrial proton conductance. Mitochondria from UCP-1 knockout mice are innately in an energised state as evidenced by a high membrane potential and a low respiration rate. The UCP1-ablated mice are highly cold intolerant, and this suggests that the heat production lost by UCP1-ablation is not compensated for by other processes. Importantly, neither UCP2 nor UCP3 have replaced the proton conductance or thermogenic capacity lost by UCP1-ablation in these mice.

Transgenic mice that overexpress human UCP3 in skeletal muscle (UCP3tg mice) have been created recently by Clapham and coworkers [56]. Total UCP3 expression (mRNA) was increased 66-fold in skeletal muscle with little or no expression in other tissues (other than BAT). Despite an increase in food intake, UCP3tg mice weighed less than controls. More importantly, proton conductance was increased 2–3-fold in mitochondria from UCP3tg mice [57]. These results are all consistent with a role of UCP3 in energy expenditure.

The simplest interpretation of these data would be that UCP3 uncouples in vivo and therefore in mice overexpressing the protein more energy is dissipated as heat than in control mice. This would explain why UCP3tg mice remain lean in spite of an observed hyperphagia. However, it is important to consider that a 66-fold increase in UCP3 mRNA levels, and the corresponding unknown increase in protein, could lead to alterations in the mitochondrial membrane integrity which might account for the observed increase in proton leak. Whether all the extra protein synthesised is integrated into the mitochondrial membrane and, if this is the case, whether it is correctly folded is a question that remains to be answered. In light of a recent report of artifactual uncoupling coincident with high levels of UCP1 expression in yeast mitochondria [48], data from models in which overexpression is achieved by genetic manipulation should be interpreted with caution.

Perhaps more compelling are two recent studies of UCP3 knockout mice [58,59]. Gong et al. [58] reported UCP3 (−/−) mice that are not obese and have a phenotype similar to control mice. Therefore, the lack of UCP3 is not associated with obesity. In these mice UCP2 is not up-regulated in muscle to compensate for the lack of UCP3. The proton leak is reduced in isolated mitochondria from skeletal muscle of these mice suggesting that UCP3 accounts for some of the proton leak in skeletal muscle [58]. Similar results were found by Vidal-Puig et al. [59] in UCP3 knockout mice. They are neither obese nor cold sensitive and there is no up-regulation of other UCP mRNAs which could explain the absence of a particular phenotype. Muscle mitochondria from UCP3 knockouts are more coupled, as measured by an increase in RC ratio (state 3/state 4 rates) (but see above for criticism of this assay). Mitochondria lacking UCP3 show an increased production of reactive oxygen species (ROS) as determined by measurement of superoxide production and aconitase activity [59]. It has previously been shown that mitochondrial membrane potential regulates the generation of ROS [60,61]. Thus, a partial uncoupling, and its associated decrease in membrane potential, could lead to a decreased superoxide production. These results support a role for UCP3 in preventing excessive oxidative damage in skeletal muscle.

It is surprising that UCP3 knockout mice appear to have the same SMR as controls [58,59]. However, given estimates of a 15% contribution of muscle mitochondrial proton leak to whole animal SMR [4–6], SMR should decrease by 15% or less, which may be undetectable. If SMR really is unchanged, two explanations are possible. Either the estimates of the contribution of proton leak to SMR are too great, or the contribution of UCP3 to muscle mitochondrial proton conductance in vivo is less than that suggested by experiments on UCP3 knockout mitochondria in vitro.

2.3.4. *Evidence from physiologically perturbed natural models*

While data from genetically manipulated systems support an uncoupling function for the UCP1 homo-

logues, experiments on animal models that have been physiologically perturbed do not. In starved rats, levels of UCP2 and UCP3 mRNA in muscle tissue increase [43], despite the fact that thermogenesis is known to be depressed in this state [62]. Also, despite increases in UCP2 and UCP3 mRNA levels of more than 5-fold and 4-fold, respectively [43], and of UCP3 protein levels by 2-fold, mitochondrial proton conductance remains unchanged [44]. In mice, a starvation-induced 3.5-fold increase in soleus muscle UCP3 mRNA expression [63], did not increase soleus muscle heat production in vitro. The fact that changes in environmental temperature do not affect UCP3 mRNA expression in skeletal muscle but starvation does, supports the hypothesis that this increase in UCP3 gene expression could be mediated by free fatty acids, whose circulating levels increase during fasting [43,64].

Lanni et al. [65] examined muscle UCP3 mRNA levels and proton leak in hypo- and hyperthyroid rats, and found a correlation between UCP3 mRNA and proton leak. However, this disappeared when bovine serum albumin (BSA) was included in the incubation medium. The simplest interpretation of this result is that the differences in proton conductance between hypo- and hyperthyroid rats are related to differences in contaminating free fatty acids, which are known mitochondrial uncouplers. BSA chelates the free fatty acids and therefore abolishes the effect. A similar differential fatty acid uncoupling has been observed in liver mitochondria from obese rats. These mitochondria exhibited greater proton conductance than controls in the absence of BSA, but no difference in the presence of BSA, due to the uncoupling of obese rat mitochondria by contaminating fatty acids [17]. It might be argued that the differences in proton conductance are observed only in the presence of fatty acids because they are required cofactors for UCP3 activity. However, Cadenas et al. [44] found that fatty acids were equally effective at increasing proton conductance in mitochondria from starved and control rats, despite elevated UCP3 (protein) levels in the former instance. Thus, the study of Lanni et al. [65] provides another example (in the presence of BSA) of an increase in UCP3 mRNA but no change in proton conductance of isolated mitochondria.

The study of Jekabsons et al. [66], in which, despite altered UCP2 and UCP3 mRNA expression, non-phosphorylating respiration of skeletal muscle mitochondria is unaffected also casts doubt on UCP2 and UCP3 involvement in a physiological uncoupling. It should be noted, however, that in Lanni et al. [65] and Jekabsons et al. [66], expression levels of UCP2 and/or UCP3 are inferred from mRNA levels, which may not reflect protein levels.

Experiments with lipopolysaccharide (LPS)-injected mice are also inconsistent with an uncoupling role for the UCP1 homologues [67]. Mouse core body temperature changes in response to LPS injection, but skeletal muscle UCP3 mRNA levels correlate poorly with these changes. Core temperature increases are paralleled by decreases in muscle UCP3 mRNA. Also, no differences in muscle mitochondrial proton leak are found, despite altered UCP2 and UCP3 mRNA levels. These data support the dissociation of UCP3 expression and thermogenesis. They also suggest that UCP3 expression does not affect mitochondrial proton conductance (though, again, mRNA and protein levels may be dissociated).

In summary, the physiological manipulations of UCP1 homologue expression are not associated with the predicted uncoupling phenotypes. However, there is only one instance where actual protein levels have been measured. Future studies must tie observations of function to protein, and not mRNA, expression. This will necessitate the production of reliable antibodies, capable of detecting what are likely to be very low levels of the proteins.

2.4. *The regulation of proton conductance mediated by the UCP1 homologues*

Purine nucleoside di- and tri-phosphates recouple isolated BAT mitochondria. This was the fundamental discovery that facilitated identification of UCP1 as the component responsible for the high mitochondrial proton conductance in this tissue. Extensive nucleotide binding studies using a variety of approaches has led to the conclusion and general acceptance that the proton conductance that is inhibited by purine nucleotides in UCP1-containing mitochondria (or liposomes) defines the uncoupling activity mediated by UCP1.

2.4.1. Nucleotide effects on mitochondria and liposomes containing UCP2 and UCP3

As discussed above (Section 2.3.1), addition of purine nucleotides to UCP1-expressing yeast mitochondria or UCP1-containing liposomes is the only reliable way to quantify the amount of uncoupling that is specific to the activity of this protein. The same criterion is necessary for artificial systems containing UCP2 or UCP3. That is, the view that UCP2 and UCP3 have a (physiologically) relevant uncoupling activity would be strong if the effects of expressing/inserting these proteins in artificial systems were reversed by an inhibitor that also was effective at inhibiting a proton leak in mitochondria where the proteins are naturally present. Purine nucleotides are candidate inhibitors given that the extensive nucleotide binding domain of UCP1 is 75–80% conserved in UCP2 and UCP3.

The marginally higher state 4 rates of yeast mitochondria expressing UCP2 or UCP3 are unaffected by 1 mM purine nucleoside di- and tri-phosphates [45,46,68,69]. In contrast, and as discussed above (Section 2.3.3), purine nucleotides inhibit these proteins reconstituted into artificial liposomes either with high [49] or very low [50] affinity. Despite high expression of UCP3 in skeletal muscle, proton leak of the isolated mitochondria is not inhibited by 1 mM purine or pyrimidine nucleoside di- or triphosphates [70,71]. BAT from UCP1 knockout mice exhibits 5–14-fold higher UCP2 mRNA [35,55]. Yet BAT mitochondria isolated from UCP1-deficient mice display similar (10 μM) GDP binding and inner membrane energisation as liver mitochondria, where UCP2 mRNA is only weakly expressed. Furthermore, the proton leak kinetics of these mitochondria are insensitive to 1 mM GDP [34]. Thus, purine nucleotides neither reverse the effects of UCP1 homologue expression in yeast mitochondria nor inhibit the basal proton conductance of skeletal muscle or UCP1 knockout BAT mitochondria despite the finding that the activities associated with the reconstituted proteins are nucleotide sensitive.

2.4.2. Fatty acid effects on mitochondria and liposomes containing UCP2 and UCP3

When reconstituted from BAT or yeast mitochondria, UCP1 requires exogenous fatty acids for its activity [52]. Whether fatty acids are necessary for UCP1 activity in isolated mitochondria (BAT or yeast) is an unresolved issue (e.g. [35,72,73]). Although UCP1 can be activated by fatty acids of different chain lengths, peak activation occurs with laurate (C_{12}) and myristate (C_{14}), while the more commonly used palmitate (C_{16}) is less effective [74].

Using bacterially expressed and reconstituted UCP2 and UCP3, fatty acids either have no effect on stimulating proton transport activity [49] or, as with UCP1 reconstituted from BAT or yeast, are required in order to observe this activity [50]. In the latter study, different fatty acid preferences are noted between UCP2 and UCP3. In contrast, respiration of yeast cells or mitochondria expressing UCP2 or UCP3 is not stimulated by concentrations of 2-bromopalmitate or palmitate that stimulate UCP1 expressing yeast [46,69]. All-*trans*-retinoic acid stimulates respiration of yeast mitochondria expressing UCP1 and UCP2 but not UCP3 [46]. Although fatty acids stimulate uncoupling in mammalian mitochondria where UCP2 and UCP3 are naturally expressed, they are equally effective at uncoupling mitochondria where UCP2 and UCP3 expression is very low or absent (e.g., liver, UCP1 knockout BAT mitochondria). To emphasise this point, oleate is equally effective in uncoupling skeletal muscle mitochondria isolated from fed and starved rats, despite upregulation of UCP2 and UCP3 mRNA and UCP3 protein [44]. Other mitochondrial carriers with physiological functions unrelated to uncoupling (e.g., ANT) may mediate the observed fatty acid effects [28]. Thus, there are no data to indicate that the uncoupling effects of fatty acids or *trans*-retinoic acid in artificial UCP expression systems is related to the uncoupling effect of fatty acids in mitochondria where UCP2 and UCP3 are naturally expressed.

3. General conclusions

The mechanisms by which mitochondrial proton leak occurs in animal mitochondria remain incompletely understood. Despite a significant accumulation of data from various model systems, the physiological and biochemical functions of the UCP1 homologues are insufficiently proven. No physiological change in UCP2 or UCP3 levels in an animal

model has led to changes in proton conductance. This has been attributed to a lack of effector molecules in the case of isolated mammalian mitochondria. However, clearly this should apply also to mitochondria isolated from yeast and from transgenic mouse strains, where uncoupling phenotypes are readily observed. Similarly, molecules (e.g. laurate, palmitate, retinoic acid, nucleotides) which apparently stimulate or inhibit activity in some experimental models often fail to stimulate the activity in another system. The existence of artifactual uncoupling under some conditions of UCP expression, and the inhibition of substrate oxidation that can accompany it, both suggest that genetic manipulation of protein expression can affect the mitochondrion in secondary ways that manifest as the very phenotypes hypothesised for the UCP1 homologues.

UCP2 and UCP3 knockout studies provide the best evidence available that these proteins may be involved in proton leak. However, they are also important in confirming that neither UCP2 nor UCP3 is responsible for all of the proton conductance observed in mammalian mitochondria. And as other mechanisms must therefore also be involved in the proton leak, it is necessary to consider whether removal of any individual protein from the inner membrane is able to affect remaining proteins or structures in ways that manifest as altered proton conductance.

Perhaps the most significant omission in the data regarding the UCP1 homologues is the expression levels of the proteins themselves in natural systems. It is possible that in transgenic and heterologous expression systems, the UCP1 homologues are being expressed at levels that are orders of magnitude greater than those which occur in mitochondria naturally. Indeed, the anecdotal evidence seems to suggest that these levels in mammals may be extremely low. It remains essential therefore to establish a connection between the amount and observed 'activity' of these proteins.

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