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Review

Homologues of the uncoupling protein from brown adipose tissue (UCP1): UCP2, UCP3, BMCP1 and UCP4

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1. Introduction

In this contribution we aim to summarise our work of recent years and, without seeking to be exhaustive, to describe our thinking about the 'uncoupling proteins' (UCP). We make no apology for our somewhat partisan point of view, including in the choice of references, which we expect will be counterbalanced by other contributors to this special issue.

Mitochondria oxidise substrates and a large part of the energy released by this process is used by these organelles to phosphorylate ADP into ATP. The term coupling summarises the property of mitochondria to adapt their oxidation rate to the energy demand (ATP production). In vitro, the respiratory rate of a mitochondrial preparation when phosphorylation of ADP is occurring (state 3) is several times higher than the rate when ADP is exhausted and therefore no more ATP is produced (state 4). The respiratory control ratio (state 3 rate/state 4 rate) quantifies the coupled state of mitochondria. Damaged mitochondrial preparations show uncoupling and their ATP production is severely compromised. In vivo, uncoupled mitochondria are expected to be of 'no value' and even worse to be deleterious since they would dissipate metabolic energy and compromise ATP production. Yet they would produce heat and oxidise reduced coenzymes, two activities that could have a positive value per se. In living organisms, fermentation suggests the need for oxidation, whereas the adaptive thermogenesis occurring in the brown adipose tissue of mammals illustrates the need for adaptive thermogenesis.

The term UCP was introduced to describe the 32 kDa protein found in brown adipose tissue mitochondria, now the term UCP1 is used (see below). In the presence of UCP1, a high respiratory rate occurs in the absence of ATP production, and this could be explained by the high permeability of the mitochondrial inner membrane to protons when UCP1 is active. However, UCP1 is also able to transport various anions [1] and is not exclusively a proton transporter (reviewed in [2], see also [3]). This led to disagreement about the exact mechanism used by UCP1 under physiological conditions [4–6]. Some authors consider that UCP1 is not a proton transporter at all and that proton transport is an indirect consequence of anion transport [4] (Fig. 1). Nevertheless, the presence of large quantities of UCP1 enables a rapid backflow of protons through the mitochondrial inner membrane, and therefore proton pumping by the mitochondrial respiratory chain cannot generate a proton electrochemical gradient high enough to suppress substrate oxidation. Respiratory control is therefore lost, and large quantities of substrates are burned without energy conservation.

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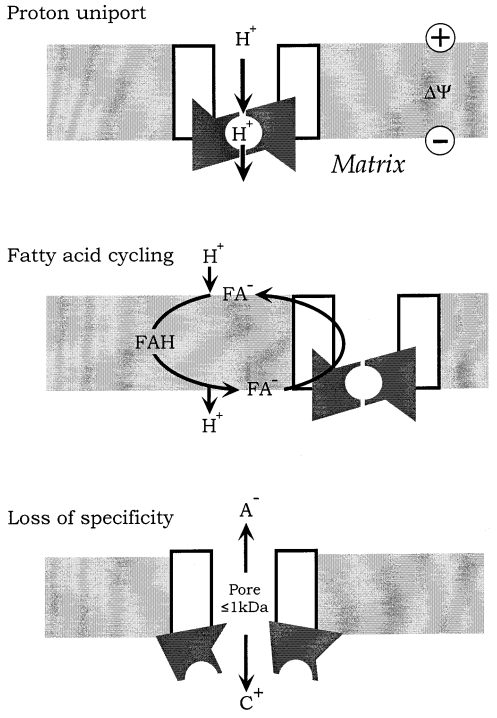

Fig. 1. Different mechanisms proposed to explain situations where uncoupling of mitochondria is observed. The mitochondrial anion carrier protein inserted in the mitochondrial inner membrane (grey rectangle) is shown with two functional domains (see Fig. 2). The orientation of the membrane potential is indicated. To uncouple mitochondrial respiration, the protein could behave as a proton transporter (top). In the fatty acid cycling model (middle), the protonated fatty acids that move freely in the lipid phase of the membrane undergo de-protonation on the inner side of the membrane. The anionic form of the fatty acid is then driven back to the outer side by the mitochondrial carrier, where this fatty acid anion can be protonated, and a new cycle starts. Finally, a loss of the specificity of transport (bottom), likely to involve the gating domain of the carrier, could lead to uncoupling. In the more extreme case, modification of the structure of the carrier allows solutes of 1000 Da to cross the membrane through the pore constituted by the modified carrier.

Therefore, heat production in the brown adipocytes is intense. A stringent control on UCP1 gene expression, and a direct control of the activity of the protein itself, ensures that this energy dissipative pathway operates when thermogenesis is needed. UCP1 is able to bind nucleotides and nucleotide binding inhibits its uncoupling (transport) activity, whereas free fatty acids increase it. This is physiologically apt, since 'at rest' in brown adipocytes endogenous nucleotides would keep UCP1 inactive, whereas stimulation of adipocytes triggering lipolysis would release fatty acids used as fuels for the uncoupled respiration resulting from UCP1 activation. These issues were addressed in the 70s and early 80s, reviewed in [7,8]. More recently the construction of mice knocked-out for the *ucp1* gene has proved the crucial role of this protein in the regulatory thermogenesis necessary for mice to cope with a low ambient temperature [9].

Since 1997 genes encoding proteins closely related to the 32 kDa protein of brown adipose tissue mitochondria were discovered [10–14], and therefore the term UCP has been changed into UCP1 to refer to the brown adipose tissue mitochondrial UCP, whereas the terms UCP2, UCP3 have been introduced for the closest homologues. It was proposed that these proteins share also functional similarities with UCP1, and therefore would also be able to induce uncoupling of respiration.

2. Molecular studies on UCP1

2.1. Cloning

UCP1 was found to have the interesting property of being expressed only in specialised cells called brown adipocytes, constituting the brown adipose tissue of mammals. This peculiarity made the study of this protein and of its gene exceptionally attractive and, moreover, easier in several respects. The early cloning of a cDNA for UCP1 was possible because of its exceptionally high expression in brown fat, and with the help of the inducible character of its mRNA [15]. The cDNA indicated the complete sequence UCP1 [16], and direct amino acid sequencing unraveled UCP1's primary structure [17].

2.2. Sequence analysis

Analysis of its amino acid sequence showed that UCP1 is organised as a triplicated structure [17]. This was initially observed with a partial sequence of the ADP/ATP translocator [18]. Many transporters found in the mitochondrial inner membrane share this property, and they constitute a large family of homologous proteins [19,20]. More specific sequences attracted our attention in UCP1. A stronger homology with the ADP/ATP carrier was found in the

third (C-terminal) domain of the protein. Since UCP1 is able to bind nucleotides, and the ADP/ATP carrier transports them, it was proposed that the C-terminal domain is involved in the interaction between UCP1 (or the translocator) and nucleotides [16]. Thereafter it was noticed that a short sequence (amino acids 261–269 in rat UCP1) was shared between UCP1, the translocase and the DNA binding domain of several transcription factors [21]. These intriguing homologies prompted us to initiate a recombinant expression programme with the aim of studying the possible involvement of these amino acids in the regulation of the activity of UCP1, using site-directed mutagenesis.

2.3. Setting up a bacterial expression system

Since the primary aim was to determine whether a limited domain of UCP1 would be sufficient to promote nucleotide binding, we started with an expression system where subdomains of UCP1 could be produced and purified. We chose to use mal-E-based fusion proteins produced in *Escherichia coli*. From this source mal-E fusion proteins could be easily produced and purified, but binding experiments would prove inconclusive. However, it became apparent that these fusion proteins were readily recognised by anti-UCP1 antibodies and could be used for a topological study of UCP1 in the mitochondrial inner membrane [22], and thus a fairly complete topological map of UCP1 was produced [23]. This work on UCP1 is probably still the strongest experimental evidence in support of the theoretical model proposed for mitochondrial carrier proteins with six transmembranous α helices (Fig. 2).

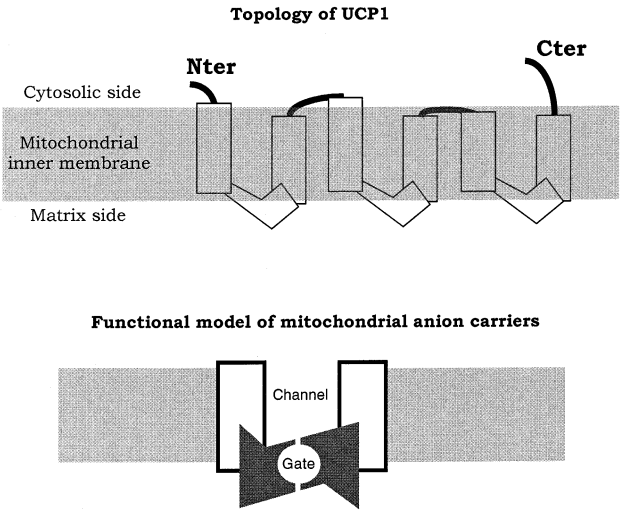

Fig. 2. Two different representations of a mitochondrial carrier. Topology (top): UCP1 is shown in the inner membrane, the regions recognised by non-permeant macromolecular probes like antibodies are shown protruding above or below the grey rectangle representing the mitochondrial inner membrane. Rectangles represent α helices. Functional domains (bottom): the channel domain (open rectangles) would constitute a pathway through the membrane whereas the gating domain (dark grey polygon) would determine the specificity of transport.

2.4. Recombinant mammalian cell line: UCP1 in mitochondria

An expression vector for UCP1 was introduced in a mammalian cell line in order to observe whether the introduction of UCP1 into mitochondria is sufficient to reproduce the uncoupled phenotype observed in brown adipose tissue mitochondria. However, in contrast with many studies involving transfection of mammalian cell lines, transient transfection procedures could not be used since the study of mitochondrial preparations required homogenous starting material, and therefore the tedious isolation of stable transformants was unavoidable. As expected, introduction of the UCP1 expression vector in CHO cells produced a new cell line expressing UCP1 in mitochondria. When these mitochondria were studied in vitro, they showed a spontaneously uncoupled state, which was restored to a coupled state by addition of GDP. Therefore, introduction of UCP1 in mammalian cells by genetic manipulation was sufficient to reproduce the mitochondrial alteration observed in brown fat [24]. This observation complemented the work done with purified UCP1 in reconstitution experiments designed to demonstrate its activity as a passive proton transporter [25–28]. This first establishment of a recombinant expression system for a functional UCP1 opened the way to the use of site-directed mutagenesis studies on UCP1.

2.5. The yeast expression system

The expression of wild-type UCP1 was well tolerated by the CHO cell line. This was ascribed to the fact that within these cells endogenous levels of nu-

cleotides would keep UCP1 partially or completely inhibited and therefore cellular bioenergetics were not compromised by this UCP. However, with a view to producing mutant proteins with altered properties, it was postulated that a facultative aerobic organism like yeast would be of value. As a microorganism, yeast would produce more of the protein, which was expected to reach yeast mitochondria and fold naturally, unlike in bacteria where recombinant proteins accumulate as inclusion bodies. We therefore accepted with enthusiasm the proposal of Dr Rial to collaborate on the recombinant expression of UCP1 in yeast. Yeast offered another crucial advantage over other eukaryotic cells: very good inducible/repressible vectors were available and this is of crucial importance in selecting and maintaining recombinant strains containing an expression vector encoding a potentially deleterious protein.

2.6. The YUP (YMUC) incident

Although the basis of the yeast expression system was sound, an unexpected complication arose. Experiments designed to check the effects of nucleotides on yeast mitochondria revealed the existence of an endogenous uncoupling pathway [29], the activity of which could result in a complete uncoupling of respiration in vitro. To overcome this complication, we studied this pathway's characteristics in detail, and found that it (called YUP for yeast uncoupling pathway) is an uncoupling pathway sensitive to the ATP/ADP+Pi ratio [30,31]. ATP promotes uncoupling; ADP and inorganic phosphate are inhibitors. Consequently, media containing high phosphate concentrations (5–10 mM) had to be used to obtain yeast mitochondria where YUP is inhibited and where UCP1 activity could be studied. The essential characteristics of YUP regulation have been confirmed by others, although they called it 'YMUC' [32,33].

2.7. UCP1 at work in yeast

Once YUP is inhibited by phosphate, it is possible to observe the activity of UCP1 in recombinant yeast mitochondria. This was true with isolated mitochondria [34] and with permeabilised spheroplasts (M. Goubern and F. Bouillaud, unpublished data). Many mutants of UCP1 have been studied in this system, including mutants of the domain mentioned beforehand. Although this domain was shown to be required for nucleotide inhibition [35], it became obvious that it has some more general importance in UCP1. Removal of this domain produced a mutant protein which allows the passage of very large solutes (up to 1500 Da). This limit is very similar to that of the so-called mitochondrial transition pore, a structure in which the ADP/ATP translocator is thought to play a key role, reviewed in [36]. Therefore it is of interest to notice that deletion of this domain, which is similar in UCP1 and in the ADP/ATP translocator, led to the conversion of UCP1 into such a pore. UCP1 (like other carriers) has a triplicated structure, there are therefore two other related domains in the protein. In subsequent mutagenesis studies it was shown that these three domains participate similarly in the control of transport by UCP1 [37]. Fig. 3 outlines how such similarity may be found between the structure of mitochondrial carriers and the functional division of the carrier activity into channel and gating domains.

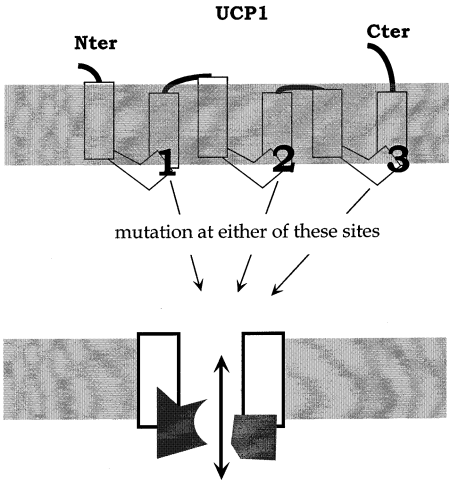

Fig. 3. The localisation of mutations introduced in UCP1 is shown on the topological domain, whereas the interpretation in functional terms is given at the bottom. This obviously suggests that the topological map and the functional domains coincide to some degree, which is made apparent by the similarity of the schemes used for topological and functional representations.

Experiments using plates or in liquid culture showed that induction of expression of UCP1 in yeast resulted in a lower growth rate [35,37,38]. It was of interest to observe that the addition of fatty acids to the culture medium further increased the generation time and that this increase was dependent

on mutations in the UCP1 sequence [38]. The fatty acid bromopalmitate was used as a non-metabolisable derivative, but experiments using plates showed that other fatty acids like linoleic acid (Rial et al., unpublished data) could be used.

Changes in yeast mitochondrial membrane potential due to UCP1 activity were examined using fluorescent probes (i.e. DiOC(6)3) which accumulate in the organelle in response to membrane potential. It should be noted that yeast needs concentrations of the DiOC(6)3 probe (100 nM) that are two orders of magnitude higher than the concentrations recommended for mammalian cells [39]. This is due to the presence of the cell wall, which constitutes a barrier to probe diffusion. Expression of UCP1 is accompanied by a decrease in labelling with the DiOC(6)3 probe. Induction with galactose lasted for several hours before analysis by flow cytometry, and so there is a risk that what we observed is a delayed consequence of the induction, including modification of mitochondrial structure rather than a mere recording of membrane potential. Note that there are cases of recombinant expression of mitochondrial carriers that do not disturb yeast growth or DiOC(6)3 labelling, examples being the oxoglutarate carrier ([40] and Fleury et al., unpublished data). This is also true for UCP1 mutants, although during tests in vitro they still show a fatty acid stimulation and nucleotide inhibition of proton leak [37]. Disturbance of yeast bioenergetics is therefore not an obligatory consequence of recombinant expression of a mitochondrial carrier, and mutated forms of UCP1 could be completely inhibited under the intracellular conditions. Decreased growth rate and reduced DiOC(6)3 labelling strongly suggest that the transport activity of the newly introduced protein alters mitochondrial membrane potential and/or mitochondriogenesis. Good correlation was observed between the fluorescence decrease measured by flow cytometry, growth rate modification and the uncoupling efficiency of the protein observed in isolated yeast mitochondria ([35,37] and Fleury et al., unpublished data).

2.8. The control of ucp1 gene expression

The *ucp1* gene is expressed exclusively in brown adipocytes, and its expression is highly inducible. Cyclic AMP [41] and retinoic acid [42,43] are two powerful inducers of *ucp1* gene transcription. An increase in cyclic AMP results from adrenergic stimulation of brown adipocytes which occurs upon cold exposure, but the physiological role of retinoic acid is more obscure. Studies combining in vivo (cell transfection, transgenic mice) and in vitro (gel shift assays, DNA footprinting) experiments have been undertaken to unravel the genomic regions controlling the expression of the *ucp1* gene specifically in brown fat, and its response to neurohormonal stimulation. It was shown that the 4.5 kb located in front of the transcription start site of the *ucp1* gene were able to drive the expression of a reporter gene with characteristics identical to the endogenous *ucp1* gene [41]. Further investigation showed that the qualitative characteristics of the rodent *ucp1* gene are contained within a 211-bp element located about 2 kb upstream from the transcription site [44]. This property is illustrated in transgenic mice where the 211-bp element is inserted in front of a viral promoter driving the transcription of a reporter gene [45]. This reporter gene is expressed exclusively in brown fat where it responds to stimuli like the endogenous *ucp1* gene. Since in these experiments the 211-bp element confers on the viral promoter the essential properties of the *ucp1* gene promoter region, it is concluded that most of the relevant properties of the *ucp1* promoter are clustered within this 211-bp element, and future studies will therefore aim to define the *trans*-activating factors interacting with this 211-bp DNA sequence.

2.9. UCP1 in humans

The human *ucp1* gene was cloned, and this allowed probing for the expression of UCP1 mRNA in humans [46], as well as genetic studies to examine to what extent the *ucp1* gene is implicated in energy expenditure and body weight control in man. A polymorphic site was discovered in the human *ucp1* gene. This is due to a single base change in the promoter sequence of the *ucp1* promoter sequence leading to the disappearance of a *Bcl*I restriction site, this allelic form is present in about 25% of the human *ucp1* genes [47]. Data from the study of this polymorphism suggest that the *ucp1* gene is not a major obesity gene, but may participate with other genes in

predisposing to fat accumulation [48]. Increase in body weight over a 12-year period was correlated with this *Bcl*I polymorphism of the *ucp1* gene [47]. This is wholly in line with the idea that a marginal increase/decrease in energy expenditure due to UCP1 would result over time in a smaller/larger increase in body weight, rather than being a determinant of the body weight itself. As a mirror image of this study, a correlation was found between this polymorphism of the *ucp1* gene and the resistance to weight loss during hypocaloric diet [49]. The human *ucp1* gene promoter has been isolated and its transcriptional regulation has been studied. This revealed a situation similar to that of rodents since retinoic acid, norepinephrine (cyclic AMP), and thiazolidinedione are activators of transcription. However, in contrast with the rodent (rat, mice) *ucp1* gene, the transcriptional activation of the human *ucp1* promoter by cyclic AMP was found to be strictly dependent on retinoic acid presence [M.M. Gonzalez-Barroso, C. Pecqueur, C. Gelly, D. Sanchis, M.C. Alves-Guerra, F. Bouillaud, D. Ricquier, A.M. Cassard-Doulcier, J. Biol. Chem. 275 (2000) 31722–31732].

3. Cloning new homologues of UCP1

3.1. UCP2 and UCP3

Evidence gradually accumulated for the existence of close homologues of UCP1. For various reasons this was not examined for a long time, probably because these reports invariably led their authors to conclude that UCP1 was not just expressed in brown fat [50,51], although this proved erroneous in some cases [52]. We decided to seek homologues of UCP1 in a mouse muscle cDNA library and found a clone called UCP-like, which was later renamed UCP2 [10]. Other groups meanwhile had cloned UCP2 [14] and/or UCP3 [11–13]. The sequences of UCP1, UCP2 and UCP3 were highly similar, suggesting common properties in addition to being members of the 'mitochondrial anion carrier family' (Fig. 4). For example, alignment analysis suggests that the distance between the UCP1, UCP2 and UCP3 sequences is shorter than the distance between mammalian and yeast adenine nucleotide translocases (Fig. 4). Conservation of the different sequences is slightly variable, and according to the Unigene database (www.ncbi.nlm.nih.gov/UniGene) the percentage similarity between human and rodent (rat and mouse) UCP1 sequences is 79%; it is 85% for UCP3 and it rises to 95% for UCP2. Conclusions concerning the biochemical activities of UCP2 and UCP3 have to be supported by experimental data. At this point it should be borne in mind that the transport properties of these proteins examined in reconstituted or recombinant systems do not auto-

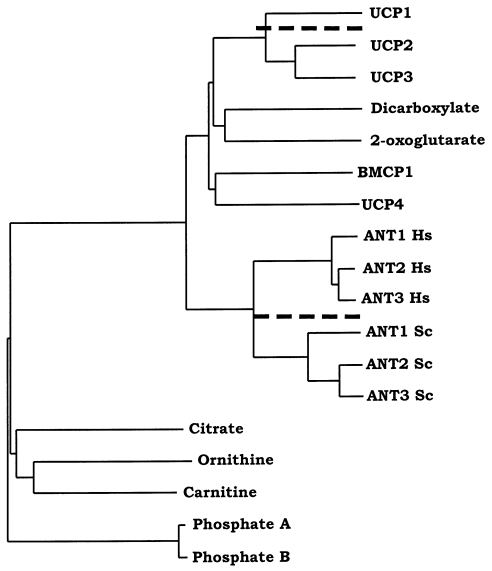

Fig. 4. This tree indicates the similarities between several mitochondrial carriers. The length of horizontal branches is proportional to the divergence between amino acid sequences of the different proteins. The sequences were collected at the National Center for Biological Information (NCBI) database (www.ncbi.nlm.nih.gov). Human sequences of the dicarboxylate, 2-oxoglutarate, citrate, ornithine, carnitine and phosphate carriers as well as isoforms of the ADP/ATP translocator (ANT1–3 Hs) were compared to human UCP1–4 and BMCP1 sequences. There are two isoforms of the phosphate carrier (A and B) produced by an alternative splicing. The Clustal W was used to generate alignment and the guide file (dnd) used to draw the tree with the NJplot software. To estimate a maximum distance between proteins with the same biochemical activities in two different organisms the ADP/ATP translocases of the yeast *Saccharomyces cerevisiae* (ANT1–3 Sc) have also been introduced. The dotted segment corresponds to the divergence between human and yeast ADP/ATP translocases. This same segment is represented close to the UCPs. The purpose of this figure is only to compare these distances, not to define the evolutionary relationships between carriers, because the introduction of yeast ANT sequences modifies significantly the shape of the tree in comparison with the shape obtained with human sequences only.

matically lead to more pertinent conclusions concerning their physiological role. For example, chloride transport by UCP1 is supported by much solid experimental evidence noted with brown adipose tissue isolated mitochondria [1,2] or in reconstituted systems [3,53], but is of little help understanding the physiological relevance of UCP1. The physiological relevance of the potentially various activities of such proteins is a complicated issue: for example interactions with various intracellular regulators could cancel out some activities observed in vitro or reveal others not yet observed in artificial systems.

3.2. BMCP1 and UCP4

The existence of a further homologue expressed in the central nervous system was postulated on the basis of expressed sequence tags and according to several hybridisation signals. As can be seen in Fig. 4, this additional homologue is more distantly related to UCP1 than are UCP2 and UCP3, and for this reason we finally called it BMCP1 [54]. Another homologue termed UCP4 is also expressed in brain [55].

3.3. Expression patterns and chromosomal location

To date, five genes have been identified in mammals: UCP1, UCP2, UCP3, BMCP1, and UCP4 (Fig. 4). Gene mapping shows that the *ucp2* and *ucp3* genes are a few kb apart [56], and probably emerged from a gene duplication event. The chromosomal localisation of the *ucp2 ucp3* cluster is 11q13 between D11S916 and D11S911 in humans and chromosome 7 in mice [10]. BMCP1 is located on the X chromosome (Xq25–26) in man, between the genetic markers DXS1206 and DXS1047, and has been cloned from clone dJ20I3 from the Sanger Centre (D. Sanchis, personal communication). UCP4 has been mapped on 6p11.2–q12 close to the genetic marker SHGC-34952 [55].

The expression pattern of these genes, based on mRNA detection, has been evaluated in various publications and reviewed [57–59]. Briefly, UCP3 mRNA is found in skeletal muscle and also in brown adipose tissue. BMCP1 and UCP4 are predominantly expressed in neural tissues, namely the brain [54,60]. UCP2 is more ubiquitous but mRNA levels differ greatly from one organ to another. UCP2 is highly expressed in spleen, for example, but levels are low in liver. Organs involved in immune defence, or rich in macrophages, express abundant UCP2 mRNA. Several lines of evidence point to specificity of UCP2 expression in several cell types but not in others, see for example [61,62]. Variations in UCP2 and UCP3 mRNA levels have been correlated with different physiological states, reviewed in [57–59]. Numerous studies indicate that expression of UCP2 and UCP3 is stimulated by thyroid hormones, and also in the presence of high levels of fatty acids. This leads to patterns of expression inconsistent with a role in promoting energy expenditure, since starvation, which promotes the use of fatty acids but decreases energy expenditure, induces an increase in mRNA levels of UCP2 and UCP3. It should be recalled, however, that almost all publications are based on mRNA levels, which are not always consistent with levels of mitochondrial proteins [63] or possibly of UCPs [64] (Pecqueur et al., in preparation). Furthermore, we now feel that the specificity of antibodies used [64–69] may not have been examined carefully enough (Pecqueur et al., in revision).

3.4. A plant UCP

A plant homologue of UCP1 obtained from *Solanum tuberosum* (StUCP) has been studied in the yeast expression system [70]. A fascinating characteristic of StUCP is its induction by cold exposure of plants, which is reminiscent of the control of UCP1 expression in brown adipose tissue [70]. The existence of such a protein had been postulated on the grounds of experimental evidence obtained with isolated mitochondria from *S. tuberosum* tubers [71], and the authors of this report used the term PUMP (for plant uncoupling mitochondrial protein). Despite several attempts, we were unable to reproduce the experiment presented in the original paper [71] describing a re-coupling of mitochondria with nucleotides, as this re-coupling, if present, was masked by the activation of mitochondrial respiratory chain by nucleotides (M. Goubern and F. Bouillaud, unpublished data), suggesting a problem in the choice of the variety of plants used. The activity and physiological relevance of the plant UCP (PUMP) is being studied by other authors in parallel with the activity of the alternative oxidase found in plants, since both

are expected to participate in re-oxidation of coenzymes independently of ATP generation [72].

3.5. Others

As is apparent from the work with plants, such sequences are not limited to mammals or homeotherms. Homologues have been described in protozoa [73], fungi [74], and fishes [75], although the molecular characterisation is not always complete. This widespread occurrence certainly questions the role of such proteins in thermogenic processes, but it would be an over-simplification to consider the uncoupling of mitochondria solely in terms of heat production.

4. Activities of these homologues

4.1. A quick review of the role of 'uncoupling'

It has long been postulated that some mitochondrial uncoupling may be advantageous for eukaryotic cells (for a recent review [76]). Some mitochondrial uncoupling is taken to mean a proton conductance of the inner membrane clearly above the minimum observable value. It should be recalled that in state 4, the membrane potential would rise to 200 mV, thereby generating an electrical field of almost 10^5 V per cm. A non-specific leak of protons across inner membrane is therefore not unlikely. Proton conductance is increased by hyperthyroidism [77] and phylogeny [78], for example. These observations thus argued for some mechanism able to increase the proton conductance of the inner membrane in a regulated manner. The unexpected occurrence of the YUP phenomenon in yeast [29] alerted us to the existence of such mechanisms.

Why should such regulated mechanisms be beneficial to the organism when they would lead to energy loss? Possible roles included thermogenesis, regulation of metabolic processes (a possible dissociation between re-oxidation of coenzymes and oxidative phosphorylation), and last but not least the control of oxygen radical production in the mitochondrial respiratory chain. In state 4 (no ATP production), the reduced respiratory chain and the high membrane potential would increase oxygen radical production at the level of the Q cycle. Accordingly it has been proposed that mild uncoupling would be a mechanism to reduce the membrane potential and therefore superoxide ion production at the mitochondrial level [76]. In this respect, partial uncoupling and the accompanying loss of energy would be 'a price worth paying' to increase 'metabolic flexibility' or to avoid poisoning by oxygen radicals when the ATP demand is low.

Several suggestions have been made for the possible mechanisms involved in this process. A role of the fatty acid composition of phospholipids of the inner membrane was shown to be unlikely [79], and proteins are therefore likely to be involved. Experimental evidence shows that proteins normally acting in an 'energy conservative manner' could under certain experimental conditions lead to uncoupling and energy waste [76,80,81]. The best example would be the adenine nucleotide translocase, which promotes the fatty acid cycling mechanism and is likely involved in the mitochondrial permeability transition pore. However, the extent to which these activities observed in vitro are recruited in the cellular context is a matter of debate. The discovery of sequences so close to a 'physiologically relevant' UCP, and showing such activity in the functional tests conducted so far [10,40,82,83], made the hypothesis of specialised genes being involved in such control of the proton permeability of the inner membrane very attractive. Accordingly, it was proposed very early that UCP2 is involved in the control of oxygen radical production [61].

4.2. The uncoupling activity of these proteins

The first issue to examine is whether these proteins have an uncoupling activity. Although simple in principle, this is a difficult issue in practice. As noted above, the observation of uncoupled mitochondria strongly suggests damage of these organelles during preparation, and in refuting such an explanation it is advisable to present data indicating that some physiologically relevant parameters would regulate the uncoupling state. A first simplification would be to consider that the uncoupling activity has to be regulated like the activity of UCP1. Accordingly, mitochondria where such a UCP is expected to be present are examined for some fatty acid-induced uncoupling and/or nucleotide-induced re-coupling. In fact such

tests are full of complications because it has long been known that the uncoupling effects of fatty acids on mitochondria are likely to be explained at least partially by interaction between fatty acids and members of the mitochondrial carrier family other than UCPs, reviewed in [76,80]. A carrier heavily involved in these effects is the adenine nucleotide translocase, and further complicates matters since this protein obviously also interacts with nucleotides. It is still a matter of debate whether the fatty acid cycling due to such a mitochondrial carrier is of any physiological relevance. This indicates that the uncoupling observed after addition of compounds like fatty acids might be a side effect of a 'non-UCP' carrier protein conserved by natural selection for other purposes. Therefore, reconstitution of the cycling phenomenon in phospholipid vesicles containing UCP2 and UCP3 is not that informative regarding their physiologically relevant activity [84]. On the other hand, when mitochondrial preparations are used, the assumption that fatty acid stimulation of mitochondrial respiration and/or its inhibition by nucleotides is possibly attributable to the UCP homologue thought to be present in the mitochondrial membrane deserves more thorough examination. Controversial data have been reported concerning correlation between the expression level of UCP2 or UCP3 and the proton leak of the inner membrane [68,85,86].

Although the yeast expression system was used mainly for historical reasons, we think it has several advantages compared to 'natural mitochondria' and to 'artificial vesicles' where a purified protein is introduced. Recombinant yeasts have the great advantage of providing close control, since mitochondria from yeast grown under the same conditions but containing an empty expression vector are used as control, and therefore we expect to compare mitochondria differing only by the presence of a UCP. However, we are still in a mitochondrial context, and so the high and sustained membrane potential generated by the respiratory chain is present. Experiments should be conducted within physiological limits to keep mitochondria active and at least partially coupled.

4.3. UCP2, UCP3 and BMCP1 in recombinant yeast

Several groups have successfully expressed the UCPs in yeast and their results are consistent with the in vivo and in vitro uncoupling activity of these homologues [10,40,82,83,87]. In our hands, the induction of UCP2 expression in yeast resulted in a lower growth rate and decreased staining with the DiOC(6) probe, indicating a likely decrease in mitochondrial membrane potential and at least demonstrating a mitochondrial effect [10]. Studies with isolated mitochondria showed that when UCP2 is present, mitochondria are less coupled than in its absence [87]. The simultaneous measurement of respiratory rate and of mitochondrial membrane potential in permeabilised yeast spheroplasts showed that the proton conductance of the mitochondrial inner membrane is increased when BMCP1 is expressed [54]. Qualitatively similar results were obtained with UCP2 (M. Goubern and F. Bouillaud, unpublished data). A semi-quantitative analysis indicates that the effects of UCP3 on yeast in vivo are greater than the effect of UCP1, the reverse being true with isolated mitochondria [40], a situation that is also likely to be the case for UCP2 (compare [10] and [87]). This could be explained by various facts, such as the constitutive inhibition of the wild-type UCP1 by endogenous nucleotides, the presence in yeast of endogenous activators of UCP2 or UCP3, and a different specificity for the transported solutes. We should stress also that our experiments with yeast mitochondria were done in the presence of a high phosphate concentration and this may alter the activity of a 'UCP' in the not so unlikely eventuality that such a protein's activity is influenced by the ATP/ADP+Pi ratio. It remains to determine whether the uncoupling activity observed in yeast is maintained/relevant in a mammalian cell. Unfortunately, there are as yet very few published data on recombinant mammalian cells stably expressing UCP1 homologues [88].

4.4. Regulation of the uncoupling activity

It is very likely that the uncoupling activity of these proteins is under control within the cell. This is probably obligatory in the case of a protein like BMCP1 since the proton permeability increase conferred by this protein on the yeast inner membrane is so high [54] that it seems to us incompatible with neuronal survival. Mechanisms have therefore to be proposed to maintain the uncoupling activity under

control inside the cell. A first explanation would be that all the uncoupling effects observed so far are experimental artefacts, a point of view that would lead us to consider with suspicion quite a significant part of the literature describing reconstituted or recombinant expression systems. A second explanation would be that the uncoupling effects are no more and no less significant than the other uncoupling effects of mitochondrial carriers explored so far. In this respect UCPs would be highly specialised transporters (since they are not present in all mitochondria), perhaps more prone to induce uncoupling given their higher homology with UCP1, which is specialised for uncoupling and thermogenesis. Keeping these considerations in mind, several mechanisms could be proposed and need to be explored to confirm or refute the physiological relevance of their uncoupling effects. For example, the uncoupling effect is likely to be heavily influenced by the amount of protein present, and in this respect the significant or dramatic uncoupling effect recorded in yeast could be ascribed to a high expression level, not far from (but still lower than) the amount of UCP1 present in brown adipose tissue (E. Rial, I. Arechaga, personal communication). In brown adipose tissue, UCP1 could account for as much as 5% of mitochondrial proteins, although there is probably more UCP1 than is needed to uncouple brown fat mitochondria. Quantitative measurement of UCPs in tissues is in its infancy, and where UCP levels are much lower than the UCP1 level in brown fat, the metabolic relevance of the UCPs would have to be downgraded proportionally (Pecqueur et al., in preparation). This would not rule out a significant role of the uncoupling activity in specialised processes.

4.5. *Retinoids and proton transport by UCP1 and UCP2*

The description of certain retinoids as positive regulators of the UCP2 uncoupling activity in a pH-dependent manner [87] strengthened the idea of this uncoupling activity being more than an in vitro artefact. Its specificity fits with the characteristics of protein catalysis and rules out a mere damaging of the mitochondrial inner membrane by a foreign protein, and the pH effect is well in line with the intracellular effect of a pH rise leading to an increase in metabolic activity [89]. According to the results obtained in the yeast expression system, UCP2 proved to be regulated differently than UCP1. In contrast with what was observed with UCP1, the presence of UCP2 did not markedly increase the sensitivity of yeast mitochondria to fatty acid uncoupling. Moreover, the basal or retinoid-stimulated uncoupling effect of UCP2 could not be inhibited by nucleotides.

The discovery of retinoids as activators of UCP2 stems from the work on UCP1 and *ucp1* gene transcription. Since retinoic acid was a strong inducer of *ucp1* gene expression, it was considered that it may also activate proton transport by UCP1. Retinoic acid signalling would therefore constitute a second complete stimulation pathway for brown adipose tissue thermogenesis, alternate or supplementary to the noradrenaline/cyclic AMP pathway. Analogies have been drawn between UCP1 and bacteriorhodopsin on the grounds of the mechanism of proton or chloride transport [26], and therefore the activation of UCP1 and UCP2 by retinoids would support further the hypothesis of some similarity in the molecular mechanisms underlying ion transport in these proteins. This retinoid sensitivity of UCP1 and UCP2 could be seen as a primitive property of both proteins, UCP1 having evolved towards a broader specificity so that fatty acids became activators, and this was associated with a much higher sensitivity. This means on the other hand that UCP2 is relatively insensitive, which could mean that proper activators are still to be found. In fact, our experiments may suggest that the maximal rate obtained in the presence of UCP2 is higher than with UCP1, see figure 3B in [87]. This interaction between UCP2 and retinoids might also reveal a role in intracellular cell signalling [87].

Still these experiments do not tell us whether or not this uncoupling activity is of any help/importance in the mammalian organism. Again the various transport activities recorded for UCP1 illustrate the difficulty in drawing physiological conclusions from these in vitro experiments, although they are of invaluable help in understanding the molecular mechanisms underlying ion transport by membranous proteins. The study of genetically engineered mammals where *ucp* genes would be either specifically destroyed or overexpressed is expected to shed more light on their physiological relevance. Alterna-

tively, human genetics data may indicate the relevance of these loci to different diseases or to quantitative variations in genetically heritable traits.

4.6. Human genetics, gene microarrays, and knock-out (KO) mice

A genetic study involving the use of microsatellites in humans showed that the chromosomal region containing *ucp2* and *ucp3* genes is linked to the basal metabolic rate [90]. This is certainly not a demonstration of their uncoupling activity but on the other hand it does support the hypothesis of the relevance of these genes in the genetic determination of the basal metabolic rate. Many other reports (reviewed in [59]) have sought to relate metabolic disorders like obesity or diabetes to the *ucp2 ucp3* locus. As no clear pattern emerged from these studies, it is anticipated that *ucp2* and *ucp3* genes do not play a major role in the determination of body weight. The linkage between *ucp2 ucp3* locus and anorexia nervosa indicates either how circumstantial these linkage studies may be, since the chromosomal region studied contains *ucp2*, *ucp3*, as well as many (tens?) of other genes, or alternatively it may suggest that the relevant traits have yet to be discovered.

A second piece of evidence suggests that the importance of UCP2 might be different from what we expected in the first instance. Two cell lines were derived from the same B lymphoma tumour: one is resistant and the other is sensitive to radiation-induced apoptotic cell death. Using microarrays to detect genes transcribed differently in response to irradiation in both cell lines, it appeared that the sensitive cells induce expression of the UCP2 mRNA whereas resistant cells do not [91]. This expression is an early event occurring before any mitochondrial dysfunction could be detected. This observation makes *ucp2* a gene likely to be involved in the cellular response to stress, either in an ultimate attempt to survive, or by participating in the programme of apoptotic cell death.

Developments in the field of KO mice are very recent. Mice knocked-out for UCP3 have been produced in Dr Reitman's [92] and Dr Lowell's [93] laboratories. Both reports are consistent with the original hypothesis that UCP3 behaves as a proton transporter since it was shown that the proton conductance of the inner membrane of isolated muscle mitochondria is decreased in UCP3 KO mice [92], or that the respiratory control and the production of oxygen are higher in the muscle mitochondria of UCP3 KO mice [93]. This obviously does not rule out the possibility of UCP3 having another transport activity, but this activity seems dispensable or efficiently replaced by another gene product since mice are viable.

5. Prospects

Much future work will focus on the physiology of KO mice, and should clarify the physiological role of these proteins. We anticipate several unexpected consequences of this work. Relevant activities could then be explored further using reconstituted or recombinant expression systems. At this point we hope that the knowledge accumulated will not only stimulate debate about the mechanisms of transport across membranes but also lead to applications beneficial for human health.

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