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How do uncoupling proteins uncouple?

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Abstract

According to the proton buffering model, introduced by Klingenberg, UCP1 conducts protons through a hydrophilic pathway lined with fatty acid head groups that buffer the protons as they move across the membrane. According to the fatty acid protonophore model, introduced by Garlid, UCPs do not conduct protons at all. Rather, like all members of this gene family, they are anion carriers. A variety of anions are transported, but the physiological substrates are fatty acid (FA) anions. Because the carboxylate head group is translocated by UCP, and because the protonated FA rapidly diffuses across the membrane, this mechanism permits FA to behave as regulated cycling protonophores. Favoring the latter mechanism is the fact that the head group of long-chain alkylsulfonates, strong acid analogues of FA, is also translocated by UCP.

Keywords: Mitochondria; Uncoupling protein; Fatty acid; Nucleotide; Ion transport; Reconstitution

1. Introduction

The uncoupling proteins (UCP) catalyze fatty acid (FA)-dependent, electrophoretic proton flux across the inner membrane of mitochondria. Understanding the transport mechanism of UCP has proved to be an interesting and challenging quest, and this review will focus on some of the inevitable controversies that have arisen during these endeavors. The following discussion also applies to the transport properties of UCP2 and UCP3, because they are qualitatively identical in every detail to those of UPC1 [1]. We will not deal here with questions surrounding UCP regulation, which have been treated in recent reviews [2,3].

2. The fatty acid buffering mechanism of UCP-mediated H^+ flux

Klingenberg has long held that UCP is a proton-transporting protein. The FA buffering model was introduced, in part, to accommodate the finding that UCP-mediated H^+ transport exhibited an absolute requirement for FA [4,5]. It is postulated that protons move through an aqueous pathway in UCP, and that FA head groups are lined up along the pathway as buffering cofactors that operate in conjunction with resident H^+-conducting amino acids, such as histidines. This arrangement requires a somewhat awkward transverse alignment of the FA tails within the bilayer [2,5–7].

Klingenberg and coworkers [6] have recently shown that mutation of two histidine residues in UCP1 cause loss of H^+ transport. The authors' interpretation is that His145 and His147 comprise part of the proton conducting pathway, and they have

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extrapolated their findings to the new UCPs: because UCP2 contains neither histidine, they conclude that it does not conduct H^+ ions. Because UCP3 contains only one histidine, they conclude that it must conduct protons only weakly.

New evidence appears to refute this hypothesis: UCP2 and UCP3 catalyze fatty acid-dependent, electrophoretic proton flux as well as electrophoretic flux of the FA analog, undecanesulfonate. In short, their transport properties are qualitatively identical to those of UCP1 [1]. Moreover, plant uncoupling protein (PUMP) also contains no histidines in this region, but it too catalyzes FA-dependent H^+ flux [8].

3. The fatty acid protonophore mechanism of UCP-mediated H^+ flux

The FA protonophore model was introduced by Garlid et al. [9] and its main features are summarized diagrammatically Fig. 1. UCP catalyzes flip-flop of the anionic head group of FA from the inner to the outer leaflet of the inner membrane. Transport of the anion is driven by the high, inside-negative membrane potential ($\Delta\Psi$). After the carboxylic head group has crossed the membrane, it picks up a proton, and the protonated FA spontaneously and rapidly flip-flops back to the matrix side, where deprotonation completes the cycle. The net result of the cycle is delivery of protons with charge to the matrix. Thus, FA behave as cycling protonophores by virtue of the fact that UCP permits the anionic charge to move across the inner membrane.

In the FA protonophore model, UCPs do not conduct protons at all. Rather, like all other members of this gene family, they transport anions. Proton flux occurs independently of UCP, by non-ionic diffusion of the protonated head group across the bilayer. Note that both legs of the FA transport pathway are largely restricted to movement of the head group across the hydrophobic part of the barrier. This is because the equilibrium position of the FA head group is probably at the level of the acyl–glycerol linkage for both steps. Thus, the FA head groups cycle *within* the membrane, releasing protons to, and accepting protons from, the bulk aqueous phases at the two interfaces [9].

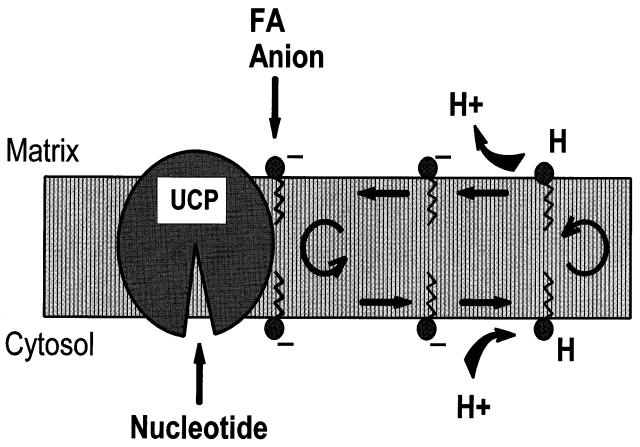

Fig. 1. The UCP-catalyzed protonophoretic cycle. The diagram shows an inner membrane segment containing UCP. The complete uncoupling cycle consists of six steps: (i) FA anion partitions in the lipid bilayer with its head group at the level of the acyl glycerol linkages and *below* the surface of the phospholipid head groups. This location is shielded from the aqueous phase, which causes the pK_a values of FA in membranes to be 3–4 units higher than their values in solution [23]. There is no significant flux of FA anion, because the bilayer energy barrier is too high [21]. (ii) The FA anion diffuses laterally in the bilayer to reach a subsurface binding site on UCP that is shielded from the bulk aqueous phase [12]. (iii) The energy barrier to FA anion transport is lowered by a weak binding site located about halfway through the UCP transport pathway [24]. The electric field created by redox-linked proton ejection drives the anionic head group to the energy well. The preference of UCP for hydrophobic anions [12] indicates that the hydrophobic FA tail remains in the bilayer during transport. (iv) The FA carboxylate group moves to the other side of the membrane by a flip-flop mechanism [25], then diffuses laterally away from the conductance pathway. (v) The FA anion is protonated. (vi) The protonated FA flip-flops rapidly and spontaneously, delivering protons electroneutrally to the mitochondrial matrix and completing the cycle. (Reproduced, with permission, from [1].)

4. Anion transport by UCP

The discovery that Cl^- ions are transported by UCP1 [10] raised an important mechanistic issue. Given that $\Delta\Psi$ in mitochondria is high and negative, a Cl^- uniport would accomplish nothing and could play no physiological role in the cell. This is a 'case of the dog that didn't bark', suggesting to us that anion transport would provide the clue to the elusive transport mechanism of UCP1. Accordingly, we decided to study anion transport and its relationship to FA and H^+ transport.

After characterizing UCP-mediated Cl^- flux using

reconstituted UCP [11], we turned to inhibitors of Cl^- flux, where we found several surprises. Many anionic inhibitors were identified, but it turned out anions that inhibited were also transported by UCP [12]. Conversely, those that did not inhibit were not transported. It would not have been surprising to have found non-transported anions that competed for a hydrophilic surface site, but this was not the case. Cl^- transport was only inhibited by *transported* anions, such as amphiphilic sulfonates. Cl^- transport was unaffected by non-transported anions, such as hydrophilic sulfonates. This led to the concept that any substrate binding site on UCP must be buried below the phospholipid head groups and therefore shielded from the aqueous environment.

A second surprise was the correlation between alkylsulfonate hydrophobicity and transport parameters. Both the V_{max} and the apparent affinity ($1/K_m$) increased with increasing alkyl chain length [12]. This finding suggested that the transport pathway for anions within UCP is a hydrophobic pathway, an idea that was consistent with the shielding concept. It should be noted that the concept of a hydrophobic pathway, introduced in 1990 [12], implies that FA travel along the *outside* of UCP, at the protein–lipid interface.

It was immediately apparent that this conjecture had a bearing on the mechanism of FA-activation of H^+ flux through UCP. Accordingly, we began studies on long-chain (C11–C16) alkylsulfonates, which are essentially identical with FA, except for their head groups. We measured the transmembrane charge transfer, because there is no fluorescent probe for sulfonates. This method is a reliable measure of UCP-mediated charge transport, because we showed that charge transport and directly measured, FA-induced H^+ flux were identical [9].

The strongest evidence for the FA protonophore hypothesis was provided by a comparison of laurate and undecanesulfonate (C11-sulfonate). The C11-sulfonate head group is transported by UCP1 with K_m very similar to the K_m for laurate-induced H^+ transport. C11-sulfonate is a competitive inhibitor of laurate-induced H^+ transport, and both anions are competitive inhibitors of Cl^- transport, with similar K_i values [9].

Importantly, C11-sulfonate differs from laurate in that it cannot support nonionic diffusion across the bilayer. This failure is due simply to its strong acid character. In addition, C11-sulfonate does not catalyze UCP-mediated H^+ transport. From these facts, we deduced that C11-sulfonate transport reflects a half-cycle of the physiological transport mechanism – UCP can translocate the charge, but the sulfonate anion is not competent to complete the protonophoretic cycle, because it cannot move the protons back across the bilayer [9].

These findings, it seems to us, render untenable the FA buffering model of UCP-mediated proton flux [2,6,7]. C11-sulfonate closely resembles laurate in all its kinetic properties. Inasmuch as the anionic head group of C11-sulfonate is demonstrably *transported across the membrane* by UCP1, there is no physicochemical basis for exclusion of the FA anionic head group from this pathway.

We devoted considerable effort to experiments designed to falsify the hypothesis by identifying a FA (or analogue) that induced UCP-mediated H^+ transport but could not deliver protons by nonionic diffusion. An extensive study turned up no such exceptions [13,14]. Moreover, absence of protonated FA flip-flop was found to correlate with inability to support UCP1-mediated H^+ transport.

Finally, we return to Cl^- transport. Nicholls and Lindberg [10] and Rial et al. [15] observed no effect of BSA on Cl^- permeability in brown adipose tissue mitochondria. Using an improved light scattering technique, we were able to show both an increase in GDP-sensitive Cl^- flux with BSA and complete inhibition of Cl^- flux with palmitate [4]. Moreover, laurate and C11-sulfonate are competitive inhibitors of UCP-mediated Cl^- flux in the reconstituted system [9]. We predict that all anions transported by the UCPs, including FA, are mutually competitive inhibitors. The manner in which Cl^- and other hydrophilic anions share parts of the transport pathway used by FA anions has been discussed previously [9,16].

The FA protonophore model is the only model that is consistent with the anion-transporting function of the gene family to which UCP belongs and, moreover, is the only model to achieve a mechanistic integration of proton and anion transports through UCP. These aspects are highly satisfactory, but aesthetic arguments do not constitute scientific proof,

and our evidence is essentially an argument based on Occam's razor [9].

5. Specific questions raised about the FA protonophore model

Nicholls and Rial [2] point out that UCP1 is active in transporting protons at pH 8.0 – we have shown this as well [17] – and that the proportion of protonated FA falls from 84% to 24% as the pH increases from 6.7 to 8.0. They then suggest that this drop in protonated FA would limit its transport at pH 8. This is by no means the case. Everyone agrees that UCP-mediated proton uniport is limited by $\Delta\Psi$; in the FA protonophore model, this means that FA *anion* transport is rate-limiting. Everyone agrees that flip-flop of protonated FA is many orders of magnitude faster than proton flux through UCP (see [9], for example) and *not* rate-limiting. Fick's law permeation is governed by the product of *two* terms: a permeability coefficient and a concentration gradient. In the case of protonated FA, the permeability coefficient is so large that UCP-catalyzed proton flux by FA cycling would not be impeded at any achievable pH.

The ad hoc statement was made that C11-sulfonate inhibition of laurate-dependent H^+ flux is due to 'competitive removal of FA' from the membrane [2,7]. This seems unlikely on theoretical grounds, because the mole fraction of FA in the membrane is only 0.01 at the K_m for FA [16]. We have recently addressed this problem experimentally. First, we measured the FA pH jump in liposomes in 0, 50, and 100 μM C11-sulfonate (total assay concentrations). C11-sulfonate had no effect on the extent of pH jump, which it presumably would have had if it were displacing FA from the membrane. Secondly, we find that charge transport is not reduced in mixtures of laurate and C11-sulfonate at constant total concentration of the two anions. Since C11-sulfonate is a competitive inhibitor of laurate-induced proton transport, it follows that laurate must be a competitive inhibitor of C11-sulfonate. This result is also inconsistent with membrane displacement of FA.

Another ad hoc criticism was that "... the C11-sulfonate did not pass through UCP1 but was driven through the membrane... in a ternary complex formed with valinomycin and K^+" [2,7]. Since our laboratory described the first ion-pair transport in mitochondria [18], we are well aware of this possibility, and we always carry out control studies in liposomes. It is simple to show directly that C11-sulfonate is not transported in liposomes by ion-pair transport with the concentrations of valinomycin that we routinely use. We have also studied UCP1-mediated transport of C11-sulfonate in the absence of valinomycin, using CCCP-mediated H^+ transport as the counter-flux, together with a pH gradient. C11-sulfonate was transported normally in a GDP-dependent fashion.

In brown adipose tissue (BAT) mitochondria, short-chain alkylsulfonates increase the K_i for GDP [5]. It was claimed [1] that this conflicts with the proposed lack of overlap between the GDP binding site and FA transport domain [4]. However, Klingenberg's group also views these domains as functionally separate, and it is his laboratory that showed that various anions are weak competitive inhibitors of nucleotide binding to UCP1 [2]. Among the strongest is sulfate, and it is not surprising that sulfonates are also weakly competitive for this site. We view the effect as a weak 'side-reaction' of the functional head group having nothing to do with transport. This effect is seen largely with short-chain alkylsulfonates, which must be used at high concentrations because their K_m for transport is high. With long-chain alkylsulfonates, the effect is, as expected, even weaker: the K_i for GDP inhibition of C11-sulfonate transport is about 30 μM at pH 7.2.

It is not clear on what basis the claim was made that C11-sulfonate is required at a 50-fold higher concentration to inhibit H^+ transport than the dose of laurate used to activate [2], but this too is incorrect. The published K_i value is about 70 μM for inhibition and 8–20 μM for laurate-induced activation [9].

This leaves only one serious objection to the FA protonophore model. Klingenberg [2,7] mentions unpublished experiments showing that ω-glucopyranoside palmitic acid induces a weak proton transport through UCP1. Because of the hydrophilic group at the tail, this FA derivative would not be expected to

be able to flip-flop with protons. The likely explanation for this result is that the compound is very unstable, and its chemical structure lends itself to hydrolysis (loss of the glucose moiety). Thus, the observed proton transport is likely due to the presence in the assay of free palmitate. This issue could readily be resolved with simple controls to determine whether or not this compound can catalyze nonionic proton delivery across the liposomal membrane. Our protocols would reveal this directly, because the two steps of UCP-mediated proton uniport are temporally separated: FA addition causes the proton jump due to nonionic equilibration, and subsequent addition of valinomycin causes the electrophoretic movement of FA head group [1,9,20].

6. The apparent K_m for fatty acid-induced uncoupling by UCP1

In the protonophore model, FA diffuse laterally within the membrane until they reach a weak binding site on UCP that serves to concentrate the FA in the conductance pathway (Fig. 1 [9]). FA interaction with UCP takes place in the lipid phase, and it is necessary to consider K_m values based on concentrations in this phase. Although we have reported K_m values based on total [FA] in the assay [9], these can easily be converted to membrane and aqueous K_m values using partition coefficients. We find that laurate, oleate and palmitate all have similar *membrane* K_m values of 10–12 nmol/mg lipid, or about 1 mol% [16].

A recent paper erroneously compares *aqueous* K_m values with *total* K_m values, which differ by orders of magnitude, and concludes that UCP1 does not function as a FA anion transporter [19]. In fact, K_m values from our two laboratories are similar when properly compared: using aqueous K_m values, Gonzalez-Barroso et al. [19] obtain 80 nM for palmitate, and we find 28 nM [16]. Klingenberg and Huang [2] agree with our contention that UCP1 recruits FA primarily from the lipid phase, and this is supported by the relative independence of K_m[membrane] on chain length [16]. K_m values based on aqueous concentrations vary over three orders of magnitude [16], and we consider them to be irrelevant for understanding FA interaction with UCP.

7. Are fatty acids required for UCP activity?

The laboratories of Klingenberg [2,5–7] and Garlid [4,9] agree that FA are obligatory for UCP1 activity in proteoliposomes, and our laboratory has reached the same conclusion in BAT mitochondria [4]. This requirement was challenged by experiments showing that a residual, GDP-sensitive uncoupling remains in the presence of BSA, leading the authors to conclude that UCP1 can conduct protons in the absence of FA [19]. First, we stress that the data show the BSA-insensitive rate to be only about 10% of the V_{max} in the presence of palmitate. This small degree of uncoupling is incompetent to achieve the physiological role of UCP1. Whereas all laboratories may agree that this FA-independent uncoupling is physiologically irrelevant, the interesting question remains whether or not it is *mechanistically* relevant. If UCP1 can conduct protons in the absence of FA, then presumably both mechanistic models are wrong.

We [16] and Klingenberg and Huang [2] have raised the possibility that BSA may not have removed all of the FA – as little as 1% of maximal FA levels could result in the observed uncoupling. If we assume that there is a residual, FA-independent proton flux, can it arise in a manner consistent with either of the two mechanistic models? The critical point, properly emphasized by Rial and coworkers [3,19], is that this phenomenon is observed in intact mitochondria, at high values of $\Delta\Psi$ that are not achieved in proteoliposomes. At high $\Delta\Psi$, it is possible that the residual, FA-insensitive H^+ flux is simply due to H^+ leak. We have pointed out that ion leak across biomembranes occurs almost exclusively next to membrane proteins, which may form weak seals with the bilayer [21], and BAT mitochondria contain an excess of proteins due to UCP1 itself. We are currently investigating the possibility that FA-independent uncoupling is due to non-specific ion leak.

8. Quantitative aspects of UCP1-mediated proton transport

We routinely observe a V_{max} for laurate transport of about 20 μmol/(mg UCP1 per min) at 25°C [9]. V_{max} values from Klingenberg's laboratory have re-

cently increased to 100 μmol/(mg UCP1.min) at 10°C [2]. If the activation energy for UCP1 is about 80 kJ/mol, this corresponds to 550 μmol/(mg UCP1 per min) at 25°C, about 25-fold higher than our value. As Klingenberg and Huang have pointed out [2], this value is not consistent with what is known about UCP1 activity in BAT mitochondria.

The reason for this large discrepancy may have to do with the different assay methods used. We use a direct method that reports the total acid transported, which is the desired quantity for H^+ transport [17]. Klingenberg's laboratory uses an indirect method of measuring external pH changes, originally with a pH electrode and then with pyranine [22], which produces the extremely high rates. We considered and rejected using pH probes to measure proton transport. The pH method is indirect and subject to error, sensitivity requires very low buffering, and pH changes necessary for the measurement may affect transport. The bottom line is that the protons are transported, pH is not.

Our V_{max} value of 20 μmol/(mg UCP1.min) is in good agreement with measurements in intact BAT mitochondria. Nicholls and Rial [3] measured a conductance of 16 nmol H^+/(mg mitochondrial protein/min per mV) at 25°C. Assuming that UCP1 comprises 10% of total protein and that $\Delta\Psi$ is about 120 mV in our assay, this works out to 19.2 μmol/(mg UCP1 per min).

9. Summary

This review has focused on the two competing hypotheses that describe the mechanism of uncoupling by UCP. We have addressed in some detail the major questions raised about the FA Protonophore Model, and we can summarize the major factors favoring it. (i) It is an anion transport model, and the UCPs are members of the gene family of anion transporters. (ii) Our finding that UCP2 and UCP3 are qualitatively identical with UCP1 with respect to FA-induced proton flux and C11-sulfonate flux [1] favors the FA protonophore model, because the FA buffering model predicts that they do not transport protons [6]. (iii) The FA protonophore model is strongly supported by the behavior of FA analogues, the long-chain alkylsulfonates: their head groups are transported by the UCPs, their transport is blocked by nucleotides, they compete with FA (and vice versa), and they inhibit Cl^- transport similarly to FA [9].

The long-chain alkylsulfonates are very useful probes of the half-cycle of FA anion transport that occurs via UCP. We are now completing a more extensive study of the transport behavior of these important compounds. We are also attempting to address the origin of the small residual proton flux that occurs in BAT mitochondria in the presence of BSA.

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