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Minireview

The mechanism of proton transport mediated by mitochondrial uncoupling proteins

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Abstract The effort to understand the mechanism of uncoupling by UCP has devolved into two models – the fatty acid protonophore model and the proton buffering model. Evidence for each hypothesis is summarized and evaluated. We also evaluate the obligatory requirement for fatty acids in UCP1-mediated uncoupling and the question of fatty acid affinity for UCP1. The structural bases of UCP transport function and nucleotide inhibition are discussed in light of recent mutagenesis studies and in relationship to the sequences of newly discovered UCPs.

Key words: Uncoupling protein; Fatty acid; H^+ transport; Chloride transport; Mutagenesis

1. Introduction

Uncoupling protein (UCP1) catalyzes electrophoretic proton back-flux across the inner membrane of BAT mitochondria, dissipating redox energy and providing heat to mammals. Knowledge of the mechanism by which UCP1 catalyzes proton conductance has achieved new importance with the discovery of new and rather ubiquitous mammalian uncoupling proteins, UCP2 and UCP3, whose transport functions have so far been inferred only by virtue of their strong sequence identities with UCP1 [1–4]. However, the mechanism of H^+ transport by UCP1 has remained controversial, and this disagreement inevitably raises a barrier to progress in understanding the function of all UCPs. This review will therefore focus on the biophysical basis of UCP1-mediated uncoupling.

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

The FA protonophore model, diagrammed in Fig. 1, was introduced by Skulachev [5] and Garlid et al. [6]. Subsequent reports have appeared both in support of the model [7–11] and in opposition to the model [12,13]. As shown in Fig. 1, UCP catalyzes flip-flop of the anionic head group of FA from the matrix leaflet 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 carboxyl 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.

A striking feature of this model is that UCP1 does not conduct protons at all. In the FA protonophore model, UCP transports anions, and proton flux occurs independently, by non-ionic diffusion. The physiological substrates of UCP1 are FA, but a wide variety of anions are transported [14] and are hypothesized to share parts of the same pathway used by FA anions, as will be discussed later.

The FA protonophore hypothesis arose from the discovery that alkylsulfonates are transported by UCP1 and that both the V_{max} and the apparent affinity ($1/K_m$) increase with increasing alkyl chain length [14]. This of course was a major clue, because alkylsulfonates and FA are identical, except for their head groups.

The strongest evidence for the hypothesis was provided by a comparison of two close analogues: laurate and undecanesulfonate. Undecanesulfonate anion is transported by UCP1 with K_m very similar to the K_m for laurate-induced H^+ transport. Undecanesulfonate is also a competitive inhibitor of laurate-induced H^+ transport, and both anions are competitive inhibitors of Cl^- transport. These analogues differ in two important respects: undecanesulfonate does not catalyze UCP-mediated H^+ transport and cannot support non-ionic diffusion across the bilayer. The latter property is due to the fact that sulfonates are very strong acids. From these facts, we deduced that undecanesulfonate transport reflects a half-cycle of the physiological transport mechanism. Inasmuch as the anionic head group of undecanesulfonate, which resembles laurate in its kinetic properties, is transported by UCP1, there is no physicochemical basis for excluding 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 non-ionic diffusion. An extensive study turned up no such exceptions [10,11]. Moreover, absence of proton-

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Abbreviations: UCP, uncoupling protein; FA, fatty acids; PN, purine nucleotides; BAT, brown adipose tissue

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Table 1
K_m values for fatty acid-dependent, UCP-mediated proton flux

Fatty acid	K_p [reference]	K_m (Total)	K_m (Memb)	K_m (Aqueous)
Lauric	5×10^3 [32]	8 μM	11.4 mM	2.3 μM
Oleic	3.6×10^5 [18]	5 mM	9.9 mM	28 nM
Palmitic	3.7×10^5 [18]	5.3 μM	10.5 mM	28 nM

K_m (Total) values refer to total FA in the assay, and were determined as in [5]. K_m (Memb) and K_m (Aqueous) were determined according to Eqs. 1 and 2, in the text.

ated FA flip-flop was found to correlate with inability to support UCP1-mediated H^+ transport.

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

Protons are directly transported by UCP in the buffering model of uncoupling, introduced by Winkler and Klingenberg [15]. In recognition of the requirement for FA [15,16], it was postulated that FA are buffering cofactors that operate in conjunction with resident H^+-conducting amino acids, such as histidines [12,15].

The isolated fact that alkylsulfonates are competitive inhibitors of FA-induced H^+ transport via UCP1 [6,16] is consistent with this model, because alkylsulfonates are strong acids and cannot buffer. On the other hand, the fact that the anionic charge on alkylsulfonates is *transported across the membrane* is a serious problem for the buffering model. As stated above, there is no known physicochemical mechanism that would permit alkylsulfonate anion transport and prohibit fatty acid anion transport.

In an important study, Klingenberg and coworkers [12] have recently shown that mutation of two histidine residues

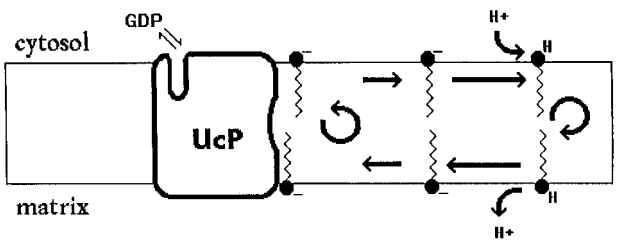

Fig. 1. The UCP-catalyzed protonophoretic cycle. The diagram shows an inner membrane segment containing UCP1. The complete uncoupling cycle consists of six steps: (i) FA anion partitions in the lipid bilayer with its head group at the level of the acyl glycerol linkages and *below* the surface of the phospholipid head groups. This location is shielded from the aqueous, which causes the pK_a values of FA in membranes to be 3–4 units higher than their values in solution [19]. There is no significant flux of FA anion, because the bilayer energy barrier is too high [30]. (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 [14]. (iii) The energy barrier to FA anion transport is lowered by a weak binding site located about halfway through the UCP transport pathway [20]. 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 [14] 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 [31], then diffuses laterally away from the conductance pathway. (v) The FA is protonated and the protonated FA rapidly flip-flops again, delivering protons electroneutrally to the mitochondrial matrix and completing the cycle. (Reproduced with permission from [6].)

in UCP1 cause loss of H^+ transport. The authors' interpretation of this result is that His^{145} and His^{147} comprise part of the proton-conducting pathway, and they have 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. It should be pointed out, however, that plant uncoupling protein (PUMP) catalyzes FA-dependent H^+ flux [9] and contains no histidines in this region (see Table 2). Moreover, preliminary evidence from our laboratories shows that UCP2 and UCP3 also catalyze Fa-dependent H^+ flux (Garlid, K.D., Jabůrek, M. and Ježek, P., unpublished results).

4. Are fatty acids required for UCP activity?

On the basis of experiments in BAT mitochondria and liposomes, the laboratories of Klingenberg [12,15] and Garlid [6,16] agree that FA are obligatory for UCP1 activity. This requirement has been called into question in a recent paper 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 [13]. It must be stressed that the BSA-insensitive rate is only about 10–15% of the V_{max} in the presence of palmitate. This small degree of uncoupling is incompetent to achieve the physiological role of UCP1, so the relevance of the effect is not clear. It is also not clear that the effect is outside the range of measurement error[2].

As commonly prepared, BAT mitochondria also remain partially uncoupled in the absence of nucleotides and the presence of BSA [17]. This finding also led to the conclusion that mitochondria are uncoupled in the absence of fatty acids. We carried out a series of experiments that raise doubts about this interpretation. When prepared in the usual way, BAT mitochondria exhibited a high GDP-sensitive rate of uncoupling that could not be reversed by BSA, even by lengthy incubations with high BSA concentrations. On the other hand, when BSA (2–5 mg/ml) was added to the BAT tissue *during homogenization*, and maintained in the wash medium during isolation, uncoupling was reduced to undetectable levels [16]. This hysteresis in the BSA effect emphasizes the fact that BSA cannot remove all endogenous FA once they are bound to mitochondria. We believe that three factors contribute to BSA hysteresis: (i) when present during tissue homogenization and release of FA from stores, BSA can efficiently adsorb FA; (ii) if BSA is not present at this time, mitochon-

[2] It is impossible to evaluate whether the BSA-insensitive rates are statistically different from zero. No state 4 respiration rates whatsoever are provided in the paper, nor are statistics provided. Instead, the effects of BSA are compared on the basis of respiratory control ratios from two different populations of mitochondria.

Table 2
Comparison of amino acid sequences among the uncoupling proteins

	135						140					145							150					155					160		
UCP1 134	E	V	V	K	V	R	L	Q	A	Q	S	H	L	H	G	I	–	–	K	P	R	Y	T	G	T	Y	N	A	Y	R	I
UCP2 138	D	V	V	K	V	R	F	Q	A	Q	A	R	A	G	G	G	–	–	R	Y	R	Q	S	–	T	V	N	A	Y	K	T
UCP3 138	D	V	V	K	V	R	F	Q	A	S	I	H	L	G	P	S	R	S	D	R	K	Y	S	G	T	M	D	A	Y	R	T
PUMP 140	D	L	V	K	V	R	L	Q	A	E	G	K	L	P	A	G	V	P	R	–	R	Y	S	G	A	L	N	A	Y	S	T

The sequences given are found in the matrix loop segment between the third and fourth transmembrane helices. The sequences for UCP1, UCP2, UCP3, and PUMP are from [34], [2], [3], and [33], respectively. PUMP has been analyzed by MALDI-mass spectroscopy (Jezek et al., unpublished data) and more than 30% of its sequence was found identical to the *St*UCP gene cloned from potato gene library [33]. The matched sequences included the matrix loop segment, confirming that FA-translocating PUMP contains no histidines in this region.

dria become abnormally exposed to FA that bind with high affinity to mitochondria (partition coefficients surpass 10^5, even for those FA that are readily removed by BSA [18]); (iii) once FA have been allowed to partition into the inner membrane they will be more difficult to remove – they must leave the inner membrane, cross the intermembrane space to reach the outer membrane, and then leave the outer membrane to bind to BSA. Taking these factors into account, together with the experimental phenomenon of hysteresis, we are skeptical of claims [13,17] that brief exposure to BSA in the assay medium is equivalent to complete FA removal.

The obligatory requirement of UCP1 for FA is most apparent in reconstitution experiments, in which BioBeads are employed to remove detergent and form liposomes [6,15]. When *frozen* BAT mitochondria were used, we invariably observed a residual H^+ flux in proteoliposomes. This effect was eliminated by washing the thawed mitochondria with 5 mg/ml BSA prior to extraction [6]. When *fresh* BAT mitochondria were used, BioBeads alone apparently removed all of the endogenous FA, because residual proton flux was nearly identical to proton leak in protein-free liposomes [5]. From these experiments, and those of Winkler and Klingenberg [15], it is clear that UCP1-mediated H^+ flux exhibits an absolute requirement for FA in the reconstitution system.

5. 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 [5]. It is evident that FA interaction with UCP takes place in the lipid phase. We reported K_m values based on total FA, $[FA]_{Tot}$. These can readily be converted to K_m values based on membrane FA, $[FA]_{Memb}$ through recently available partition coefficients in liposomes [18]:

$$[FA]_{Memb} = \frac{K_p[FA]_{Tot}}{\{1 + K_p \cdot V_m/V_a\}} \quad (1)$$

where K_p is the partition coefficient and V_m/V_a is the lipid:aqueous volume ratio, taken to be 0.5×10^{-3} in these experiments (0.5 mg lipid/ml). The corresponding aqueous concentration, $[FA]_{Aq}$, is given by

$$[FA]_{Aq} = [FA]_{Memb}/K_p \quad (2)$$

Table 1 contains K_m data for three FA studied in liposomes containing reconstituted UCP1. The aqueous K_m values vary by a factor of 10^3, whereas membrane K_m values are remarkably similar at 10–11 mM, consistent with a common binding site on UCP1.

A recent paper [13] contains a confused discussion of the apparent affinity of UCP1 for palmitate. The authors compared *aqueous* K_m values with *total* K_m values, despite an explicit statement that the latter values include the partition coefficients [5]. The erroneous conclusion that one set of values is more 'physiological' than the other [13] arises entirely from this inappropriate comparison. When properly compared, the observed K_m values for FA are very similar in all published experiments. Thus, the K_m for palmitate is about 15 nmol/mg protein (80 nM free FA) in intact mitochondria [13], and about 10 nmol/mg lipid (21 nM free FA) in proteoliposomes (Table 1).

6. Is UCP1 a FA channel or carrier?

A minimum model of anion transport through UCP1 will include binding sites on both surfaces and a central binding site to facilitate transport through the bilayer. In fact, the 'surface' binding sites are shielded from the aqueous medium and are therefore subsurface, as is evident from the fact that transport of alkylsulfonates is completely unaffected by hydrophilic sulfonates [14]. The remarkable observation that any competitive inhibitor of FA or alkylsulfonate transport is also transported by UCP1 [14] also indicates that the binding sites are subsurface. They are probably located near the phospholipid acylglycerol linkages, which is also the equilibrium position of the FA carboxylic moiety [19].

The existence of a single energy well located near the center of the membrane was deduced from flux-voltage analysis of non-ohmic Cl^- flux [20]. The FA protonophore model states that Cl^- cannot access the subsurface binding sites for FA, and that Cl^- is transported by virtue of thermal bombardment of the membrane. Thus, Cl^- uses only part of the physiological conductance pathway for FA anions [6]. 'Random' access to the central energy well in UCP1 is reflected in the very low affinity for Cl^- ($K_m \simeq 140$ mM) [6].

The fact that V_{max} values for alkylsulfonates increase with increasing chain length [14] places a further constraint on the transport pathway – the hydrophobic tail of FA must remain at all times in contact with the lipid bilayer core and must turn and reverse direction within this environment as the FA head group moves from one side to the other.

Within these constraints, the transport pathway in UCP1 may be an anion carrier/gated pore [21] or a hydrophobic, single-file, anion conductance pathway [20]. These alternatives cannot be distinguished at the present time. In either case, polar or cationic residues should reside in the subsurface region to serve as initial binding sites for the FA head group.

Movement across the membrane would be facilitated by additional polar or cationic groups that are buried near the center of the membrane.

7. Location of the fatty acid transport pathway in UCPs

Mutations of the seven cysteines in UCP1 to serine had no effect on function or regulation of UCP1 [22,23]; however, Gonzalez-Barroso et al. [24] found that mutation of Cys^{304} to glycine increased the apparent affinity of UCP1 for palmitate about 2-fold [13]. A 2-fold change in affinity reflects a very small change in binding energy, about 0.38 kcal/mol. This is most likely a consequence of minor conformational changes in the mutated protein and not due to involvement of Cys^{304} in or near the transport site.

In contrast, mutation of histidines 145 and 147 had a dramatic effect [12]. Single mutations reduced laurate-induced H^+ flux by 85–90%, and the double mutation nearly abolished H^+ transport. Loss of function after mutation can be difficult to interpret; however, a very nice aspect of this work was the demonstration that Cl^- transport and nucleotide binding were unaffected by the amino acid substitutions. The mutations were thus specific for H^+ transport (alkylsulfonate transport was not evaluated).

These findings, which strongly imply that H145 and H147 are located in the FA anion translocation pathway, are fully consistent with the FA protonophore model. In view of the preceding discussion, the fact that His mutations block FA transport without affecting Cl^- transport implies that these residues reside near the subsurface binding site for FA at the matrix lipid-water interface. We would predict further that one or both of these histidines protrudes partially into the membrane to be near the level of the acylglycerol linkages.

H145 and H147 are located in the matrix loop between transmembrane helices 3 and 4 of UCP1. As shown in Table 2, each of the UCPs contain 3–4 positive charges in the stretch corresponding to H145 and R152 in UCP1. These cationic residues may provide subsurface binding sites for hydrophobic anions and may also serve to anchor this segment to the membrane phospholipid head groups. To provide internal anion binding sites, the loop must protrude to a considerable extent into the membrane. In fact, this is in accord with the folding model of Klingenberg and Nelson [25] in which a β-loop is inserted between each pair of transmembrane helices. The eight downstream residues between R152 and R161 in UCP1 are rich in hydrogen-bonding side chains (see Table 2), and these residues could form the central energy well of the anion conductance pathway.

Unlike the buffering model, which requires dissociable residues for H^+ transport [12], the FA protonophore model merely requires weak binding sites for anions, which may be provided by histidine, lysine, or arginine. Similarly, the energy well sites may be provided by residues that are cations or H-bond donors. The sequences in Table 2 show that all UCPs contain such sites.

Additional experiments with the His mutants may help to distinguish between the two UCP transport models. The buffering model envisions a separate anion transport pathway in UCP1, and hence predicts that alkylsulfonate transport would be unaffected by the mutations. In the FA protonophore model, transport of Cl^- and short-chain sulfonates would be unaffected, because they do not use the surface binding sites. On the other hand, transport of long-chain sulfonates would be abolished by the His mutations, like FA. More precisely, the model predicts that the mutations would increase the K_m to levels exceeding the solubility of both long-chain FA and sulfonates.

8. Regulation of UCP1

There is considerable agreement about the nature of nucleotide regulation of UCP1. Purine nucleotide (PN) inhibition of UCP-mediated H^+ flux is purely allosteric, as confirmed by non-competitive kinetics, by the lack of FA influence on the K_i for PN inhibition [16], and by EPR studies with 5-doxylstearic acid [26]. These studies show that FA anion transport and nucleotide inhibition are distinct features of UCP involving two distinct binding domains.

The nucleotide binding pocket in UCP1 has been mapped by photoaffinity labelling and site-directed mutagenesis, and is very extensive. Nucleoside phosphates interact with the three arginines that are located on helices 2, 4, and 6 [27], and the sugar base reacts with the matrix segment D233-E261 that connects helices 5 and 6. The latter interaction is presumed to confer nucleotide specificity [28].

Nucleotide binding and inhibition take place in three steps [27]. First, binding of the sugar base moiety and the binding of one β-phosphate group result in a loose-binding conformation of UCP1. In the second step, protonation of E190 makes R83 available for binding to the second charge on the β-phosphate of diphosphates and the γ-phosphate of triphosphates. An unidentified histidine is thought to bind to the second charge on the γ-phosphate of triphosphates [28]. These events, which cause tight binding of PNs [29], have been shown to be insufficient for inhibition [27]. To reach the inhibited state, α-phosphate must bind to R276. This final step induces a conformational change that modifies the anion transport pathway, causing inhibition of transport [27].

It is noteworthy that the three phosphate binding arginines are conserved in UCP2, UCP3, and PUMP, suggesting that these proteins are also regulated by nucleotides. On the other hand, there are strong differences in the nucleotide-binding matrix loop between the fifth and sixth transmembrane domains, suggesting that nucleotide specificities may differ.

9. Summary

Two competing hypotheses describe the mechanism of uncoupling by UCP – the FA protonophore model and the H^+ buffering model. Both models employ the experimental observation that FA are obligatory for UCP1-mediated uncoupling. It may be possible to discriminate between the models when the transport functions of UCP1 and UCP2 are known. The FA protonophore model predicts that they will catalyze FA-dependent proton flux, whereas the buffering model predicts that they will not.

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