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Fatty Acid Interaction with Mitochondrial Uncoupling Proteins

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The phenomena of fatty acid interaction with mitochondrial integral membrane proteins, namely uncoupling proteins (UCPs), are reviewed to emphasize the fatty acid cycling mechanism that has been suggested to explain the UCP function. Fatty acid-induced uncoupling is suggested to serve in bioenergetic systems, to set the optimum efficiency, and to tune the degree of coupling of oxidative phosphorylation. Fatty acid interaction with the "classic" uncoupling protein (UCP1) from mitochondria of thermogenic brown adipose tissue (BAT) is well known. UCP1 is considered to mediate purine nucleotide-sensitive uniport of monovalent unipolar anions, including anionic fatty acids. The return of protonated fatty acid leads to H^+ uniport and uncoupling. Experiments supporting this mechanism are also reviewed for plant uncoupling mitochondrial protein (PUMP) and ADP/ATP carrier. The fatty acid cycling mechanism is predicted, as well for the recently discovered uncoupling proteins, UCP2 and UCP3.

KEY WORDS: Uncoupling of mitochondria; uncoupling proteins; UCP1; UCP2; UCP3; plant uncoupling mitochondrial protein; ADP/ATP carrier; fatty acids; proteoliposomes; brown adipose tissue mitochondria.

INTRODUCTION

Fatty acid-induced uncoupling was observed early in the birth of bioenergetics (for reviews see Wojtczak and Schönfeld, 1993; Skulachev, 1998). In later decades, it was correlated only in one case, with the function of a single protein, the "classic" uncoupling protein (thermogenin, UCP1) in mitochondria of a specific tissue specialized for thermogenesis, the brown adipose tissue (BAT, Nicholls, 1979; Nedergaard and Cannon, 1992; Ježek *et al.*, 1998; Ježek and Garlid, 1998). BAT was recognized as an organ of nonshivering thermogenesis in newborns, cold-acclimated and hibernating mammals, and in overfed rodents (Nedergaard and Cannon, 1992). The tissue is well suited for the thermogenic role, because BAT cells are loaded with mitochondria containing the tissue-specific UCP1. Each cell is innervated and tissue has a rich microvasculature since the majority of blood flow passes through BAT. As a consequence, UCP1 provides an efficient and regulated nonshivering thermogenesis to the whole body. Its expression and cell differentiation in preadipocytes is acutely regulated by norepinephrine via β_3-adrenergic receptor. β_3 activation also leads to the initiation of the lipolytic cascade and stimulates UCP1 function.

Existence of the purine nucleotide-inhibited H^+ translocation physically ongoing in the UCP1 structure, but only in the presence of fatty acids as an activating factor, became the "common dogma" in the field (Klingenberg, 1990). The only contradictory fact to this dogma was the finding that UCP1 translocates monovalent unipolar anions (Ježek and Garlid, 1990), namely halides (Nicholls and Lindberg, 1973), non-physiological anions, such as alkylsulfonates and monovalent phosphate analogs (Ježek and Garlid, 1990), and also physiological ketocarboxylates, such as pyruvate (Ježek and Borecký, 1998). Skulachev (1991, 1998) has independently formulated the fatty acid (FA) cycling hypothesis, when carriers such as

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ADP/ATP carrier are considered to conduct FA anion (Fig. 1a), while the neutral FAs return back across the lipid bilayer and carry H^+. This mechanism has later been supported by some experimental evidence and has lead to the identification of additional carrier proteins directly interacting with FAs in mitochondria.

Moreover, novel uncoupling proteins have been discovered. First, it was the plant uncoupling mitochondrial protein PUMP (Vercesi *et al.*, 1995) and, subsequently, two new mammalian UCPs, UCP2 and UCP3. Human UCP2 was originally found by Tartaglia and colleagues by screening the expressed sequence tag (EST) libraries as a sequence coding for protein 59% identical with hUCP1 (Gimeno *et al.*, 1997). Its mRNA has been identified in the heart, BAT, white fat, skeletal muscle, kidney, lung, placenta, and tissues of the immune system, including leukocytes and macrophages (Fleury *et al.*, 1997). Although its uncoupling role is still hypothetical, UCP2 has been proposed to regulate body weight, to be involved in fever (Faggioni *et al.*, 1998), and in the defense against generation of reactive oxygen species (for review see Ježek and Garlid, 1998). UCP3, 57% identical with UCP1 in humans, was found by reverse transcription PCR (Boss *et al.*, 1997) and was recognized to be specific for skeletal muscle and BAT, thereby becoming a potential candidate for epinephrine-induced muscle nonshivering thermogenesis. In the light of these new findings, it is extremely helpful to summarize previous observations with homologous UCP1. This is the main scope of the present article.

STRUCTURAL PREREQUISITES FOR FATTY ACID CYCLING ON UCP1

UCP1 sequence is about 30% homologous to the sequences of the ADP/ATP carrier, phosphate carrier, and many other mitochondrial anion carriers which together form the gene family of homologous mitochondrial anion carrier proteins (MACP; Klingenberg, 1990; El Moualij *et al.*, 1997). The only carrier-mediating cations within this family known to date is the carnitine carrier. In a predicted structure of the *transmembrane* α-helices of UCP1, residues with the ability to form an array of H-bonds are missing (Klingenberg, 1990; Ježek and Garlid, 1998.) Photoaffinity labeling and studies of UCP1 mutants have indicated that the regulatory purine nucleotide-binding domain, although spread along several transmembrane α-helices, is

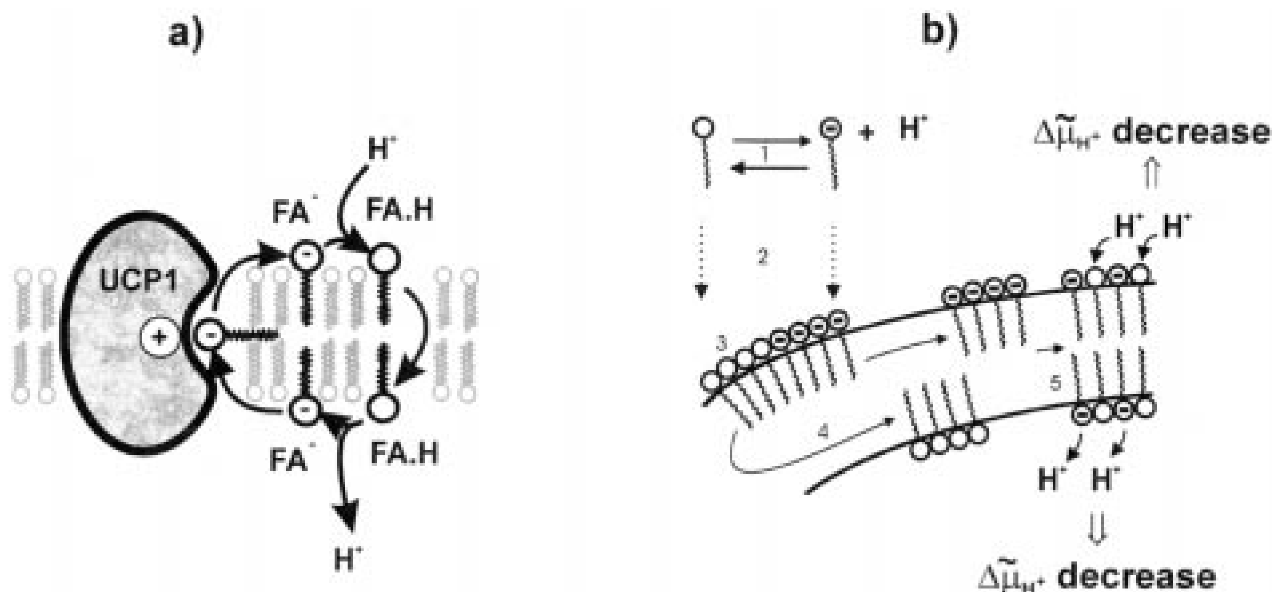

Fig. 1 Role of fatty acids in bioenergetics. (a) Fatty acid cycling enabled by uncoupling proteins and other carriers; (b) flip-flop acidification, i.e, transient FA-induced acidification of matrix or intraliposomal lumen upon FA release (addition) from the external compartment. Acid–base equilibrium (1) in the external aqueous phase and immediate FA partitioning (2) into the external lipid leaflet (3) set a nonequilibrium state from the point of view of membrane. If all species were able to move across the membrane, the approach to membrane equilibrium would not require any dissociation of neutral FAs inside. However, since H^+ release inside the liposomal lumen is observed upon the FA addition (Ježek *et al.*, 1997a,b), one must consider that only neutral FA molecules are able to flip into the inner lipid leaflet (4). This is followed by reestablishment of the acid–base equilibrium inside and outside (5), leading to the H^+ release inside.

located in the third matrix segment and half of the sixth α-helix (see Ježek *et al.*, 1998; Ježek and Garlid, 1998, for review). Purine nucleotide binding from the cytosolic side, decreasing with increasing pH and by Mg^{2+}, represents a key physiological regulator of UCP1 (Klingenberg, 1990; Nedergaard and Cannon, 1992; Ježek *et al.*, 1998; Ježek and Garlid, 1998). Recently, the uncoupling function of UCP1 was also predicted that originates because of the residues of the second matrix segment (Bienengraeber *et al.*, 1998.) Hence, it seems that the functional part of UCP1 is concentrated in the matrix "bottom" of the protein (Fig. 2). Consequently, the anion movement over a thin polypeptide barrier representing perturbation in the lipid bilayer seems to be just the most probable model for the molecular mechanism of UCP1 function.

FUNCTIONAL PREREQUISITES FOR FATTY ACID CYCLING ON UCP1

The interacting residues of the anion pathway are most likely surrounded by the lipid bilayer core. This is compatible with the finding that more hydrophobic anions are translocated faster and have higher affinity toward this site (Ježek and Garlid, 1990; Garlid *et al.*, 1996). Consequently, it has been suggested that a unifying principle of UCP1 translocation mechanism is the anion uniport that includes the uniport of fatty acid anions as well (Garlid *et al.*, 1996). The charged fatty acid interacts in the same binding domain as do the various anions, as documented by many kinetic, binding, and photoaffinity labeling experiments (Garlid *et al.*, 1996; Ježek *et al.*, 1995, 1996a; Růžička *et al.*, 1996). There is no reason to consider the hydrophobic anions, such as hexane sulfonate or undecane sulfonate, to be translocated by UCP1 with their charges and not to consider the same for the fatty acid anions. Translocation of FA anions then leads to FA cycling (Skulachev, 1991; Garlid *et al.*, 1996; Ježek *et al.*, 1998), when anionic FA is protonated on the other side of the membrane (the cytosolic side *in vivo*) and, as a neutral FA, returns by flipping back across the lipid bilayer (Fig. 1a). Just a finding of "inactive" fatty acids, unable to flip-flop and simultaneously being unable to induce H^+ transport with the reconstituted UCP1, belongs to the strong experimental evidences favor the FA cycling mechanism (Ježek *et al.*, 1997a,b). In the following section, I shall discuss these experiments that lead to the support of the FA cycling mechanism.

FATTY ACID-INDUCED UNCOUPLING IN BAT MITOCHONDRIA

Two types of experiments represent an assay for UCP1 function in BAT mitochondria. Type I, when overall coupling parameters such as respiration, membrane potential, and ΔpH are monitored. The excellent studies of Nicholls (1974a, 1977; Heaton and Nicholls, 1976; Nicholls and Bernson, 1977) are referred for the details. BAT mitochondria are uncoupled when isolated, unless a large amount of BSA and up to millimolar purine nucleotide di- or triphosphates (Heaton *et al.*, 1978) are added. Such a concerted effect of both factors, the fatty acid removal and the presence of GDP (ATP, GTP, ADP), was recognized to lead to the maximum coupling in BAT mitochondria (Nicholls, 1974a). Employing the carnitine cycle for combustion of endogenous FAs (by addition of CoA and L-carnitine in the presence of ATP) is an alternative way to study the uncoupling effect of the subsequently added FAs (Locke *et al.*, 1982; Ježek *et al.*, 1989).

Type II experiments are probing for H^+ translocation as such, either monitoring proton ejection induced in nonrespiring mitochondria by valinomycin (Kopecký *et al.*, 1984, 1987) or swelling in K-acetate induced by valinomycin in nonrespiring mitochondria (Ježek, 1987; Ježek *et al.*, 1994, 1996a). Experiments of type II have revealed that almost no H^+ movement exists in the nearly zero levels of fatty acids (Ježek *et al.*, 1988, 1994, 1996a), adjusted by the presence of a large amounts of BSA. In addition, H^+ translocation monitored by the type II experiment in BAT mitochondria was always completely inhibited by GDP (Kopecký *et al.*, 1984, 1987; Ježek, 1987; Ježek *et al.*, 1988). The same principle of valinomycin-induced K^+ diffusion potential-driven uniport was employed to induce Cl^- transport via UCP1 and, again, it was fully inhibited by GDP (Nicholls and Lindberg, 1973; Kopecký *et al.*, 1984, 1987; Ježek *et al.*, 1988), and likewise the uniport of many other monovalent unipolar anions (Ježek and Garlid, 1990). H^+ extrusion proceeded also in K_2SO_4, which was a sign that Cl^- and H^+ movements are nearly independent and this lead to the proposal that two channels exist within the structure of UCP1 (Kopecký *et al.*, 1984). Today, we know that FA anions have, by one order of magnitude, higher affinity toward UCP1 when compared to Cl^- (Garlid *et al.*, 1996) and, therefore, FA cycling can proceed in the media containing Cl^-.

Both type I and II assays were applied later to measure the function of plant uncoupling mitochon-

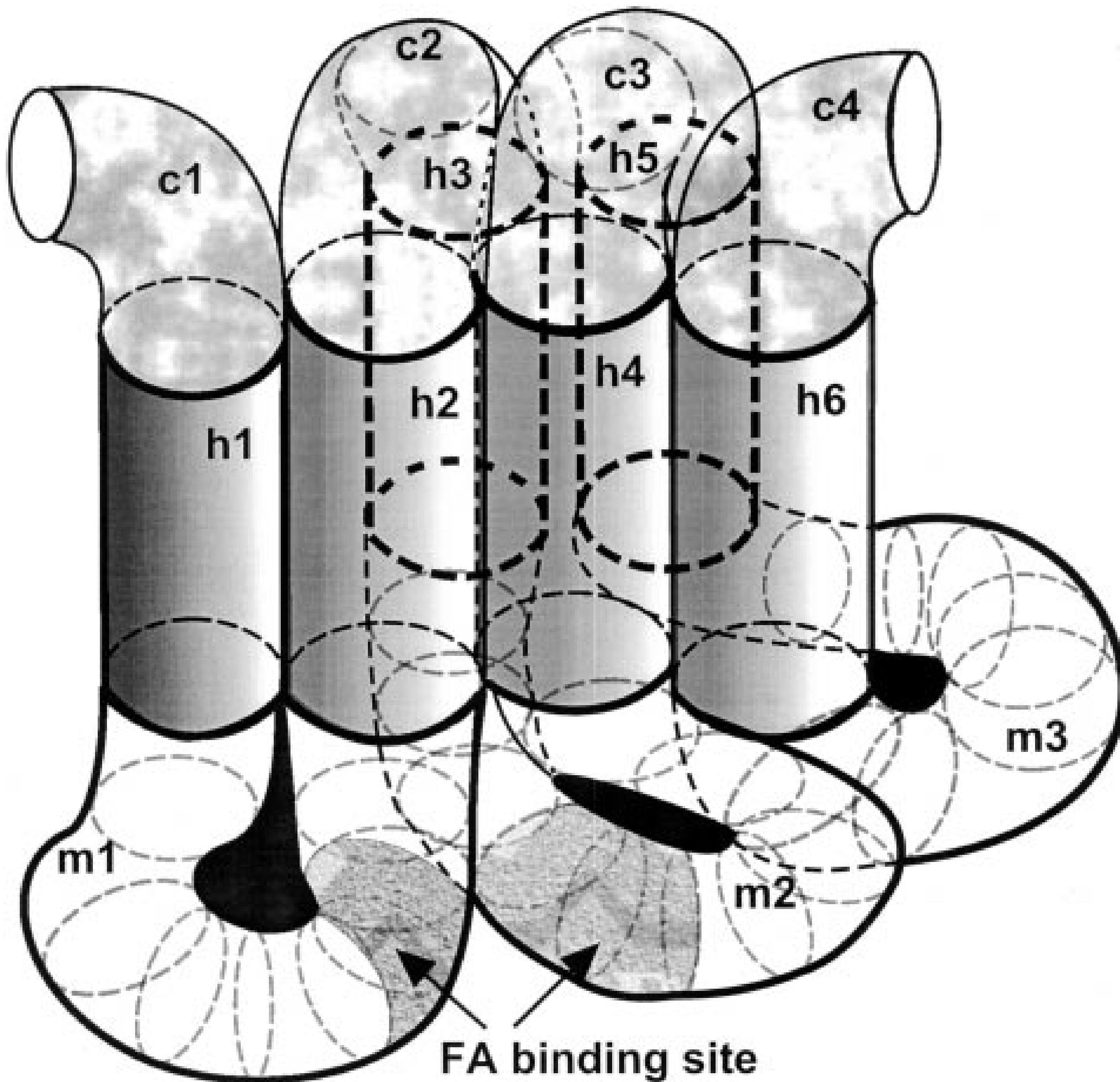

Fig. 2 Possible topological model of uncoupling protein-1 with a probable location of fatty acid binding site. c1–c4, cytosolic segments (parts of protein exposed into the intermembrane space between the inner and outer mitochondrial membrane), h1–h6, membrane spanning α-helixes; m1–m3, matrix segments (parts of the protein exposed into the matrix space). Topological model illustrates α-helixes as cylinders indicating maximum space location of the side-chain amino acid residues. The model is based on the transmembrane folding model of Klingenberg (1990) and is assumed that the predicted α-helixes are in contact, namely, that the α-helixes h2, h4, and h6 form an "inner cycle," whereas the α-helixes h1, h3, and h5 are located behind them. The connecting cytosolic and matrix segments are illustrated to indicate their relative mass with regard to the α-helixes. The matrix segments have approximately twice as many amino acid residues, while the cytosolic segments have nearly the same amount. Since UCP1 is expected to function as a dimer (Klingenberg, 1990), the figure represent a one half of its actual dimeric structure.

The binding site for fatty acids is illustrated as hypothetically placed to the common area formed as a "bottom" of the protein structure. In this region, on the second matrix segment, two histidines were found in the UCP1 structure, the substitution of which abolished FA-induced H^+ uniport in proteoliposomes (Bienengraeber *et al.*, 1998). In spite of fact that these histidines do not exist in the PUMP structure, the region is a good candidate for interaction with fatty acids. However, the FA-binding site might cover the first matrix segment as well.

The binding site for purine nucleotides is most probably formed by an aqueous space located between the fourth and sixth α-helix and the third matrix segment. (In order to preserve clarity of the topological model, the putative aqueous space perturbance of the membrane is not marked.) Arginines of the second α-helix are supposed to interact with nucleotides as well (Modrianský *et al.*, 1997).

drial protein (PUMP; Vercesi *et al.*, 1995; Ježek *et al.*, 1996b). Most surprising in these studies was that potato or tomato mitochondria behaved, in some situations, almost exactly in the same way as BAT mitochondria. The two exceptions were found for PUMP: first, the affinity of purina nucleotides is by one to two orders of magnitude lower and, second, that PUMP does not translocate Cl^- and pyruvate (the smallest translocated anion found was hexane sulfonate; Ježek *et al.*, 1996b, 1997c).

Assay in respiring mitochondria is difficult for ascribing the single effect to a single protein. Fatty acids interact with numerous mitochondrial proteins, namely, the proteins of the MACP family. Distinction of the contribution of a single protein from monitoring modulating or protective effects of specific ligands of the protein is not always decisive. However, some attempts in this direction were successful. For example, FA-induced uncoupling (cycling) is reported to be prevented by carboxyatractyloside (CAT) on the ADP/ATP carrier (Skulachev, 1991; Brustovetski and Klingenberg, 1994; Schönfeld *et al.*, 1996), namely, in alkaline pH around 7.8 (Samartsev *et al.*, 1997), and by glutamate on glutamate/aspartate carrier, namely, at pH 7.0 (Samartsev *et al.*, 1997). The pH dependencies of both phenomena most probably reflect the participation of arginine (ADP/ATP carrier) and histidine (glutamate/aspartate carrier) in the observed FA cycling.

Concerning UCP1 heterologously expressed in yeast, Gonzales-Barroso *et al.* (1998) made claims that uncoupling exists at nearly zero FA levels and that is solely mediated by UCP1. This was deduced from the finding of residual H^+ conductance (10–15% of V_{max} for palmitate) monitored as state-4 respiration rate which was 33% sensitive to GDP and which existed at about 0.5 nmol of endogenous FAs milligram of mitochondrial protein. They made attempts to refute the FA cycling mechanism. First, their data did not exclude the existence of the FA cycling mechanims at higher FA content. Second, they have unjustifiably compared aqueous-free FA concentrations with the K_m values for FA-induced uncoupling expressed in total amount of FA. Their data would be much stronger if the residual content of FAs was measured analytically and not derived from the measured respiration rates. The reported amount of endogenous FAs (0.5 nmol/mg protein) accounts approximately to the phospholipid-to-FA molar ratio of 1900 and most probably also to a significant molar ratio relative to expressed UCP1. For comparison, in intact BAT mitochondria, combustion of endogenous FAs by β-oxidation via a carnitine cycle was able to reduce the FA levels down to 1.4 nmol/mg protein (Heaton and Nicholls, 1976). Complete removal of FAs from the sites located at the matrix "bottom" of the integral membrane proteins is rather difficult. When a FA molecule originally resides in the inner leaflet of the inner membrane, the process of reestablishing FA binding equilibrium upon the addition of external BSA involves flipping of FAs across two membranes (inner and outer) and the intermembrane space between them. Binding equilibrium, when all FAs are bound on BSA and no molecules reside inside the membrane or on a binding site of a competing protein, cannot theoretically exist even at a higher amount of BSA.

The discussion of Gonzales-Barroso *et al.* (1998) failed to consider many aspects involved in the complicated system of yeast mitochondria with the artificially inserted UCP1. For example, the effect of endogenous pyruvate, the cycling of which was recently suggested to exist (Ježek and Borecký, 1998), has not been excluded. In addition, the effect of undecane sulfonate, competing with FAs, was not tested; possible inhibitory effects of FAs on mitochondrial proteins were not considered. The effects of pH, CAT, and glutamate were not thoroughly investigated. Actually, the CAT effect was described as missing, in spite of its reported existence in the other yeast strains (Polčic, *et al.*, 1997; Ježek *et al.*, 1998).

However, one good argument was noted in the paper of Gonzales-Barroso *et al.* (1998), that the proton conductance and FA effects at extremely high membrane potentials are not examined by the type II experiments in mitochondria and by the assays in the reconstituted systems based upon driving ion fluxes by the K^+ diffusion potential. Potentials occurring as a result of respiratory chain H^+ pumping are higher, in some cases, than those established in proteoliposomes. Hence, the FA-induced uncoupling at extreme potentials needs to be further investigated. Nevertheless, the

The overall topology of UCP1 (and other homologous UCPs) resembles the Greek temple architecture. Note the big ground mass in the matrix side bottom, the columns-α-helices, and the "attica," represented by the cytosolic segments. Columns are immersed into the lipid "sea," however, there should exist the aqueous perturbance, at least between the fourth and sixth α-helix, in order to accommodate water-soluble nucleotides into the preformed cavity of the nucleotide-binding site.

reconstituted PUMP (Ježek *et al.*, 1997c) and UCP1 (Ježek *et al.*, unpublished data) also show steep exponential dependencies of fatty acid-induced H^+ fluxes on the applied diffusion potential, similar to those measured in respiring liver mitochondria (Nicholls, 1974b), whereas the corresponding potential dependencies for hexane sulfonate uniport via PUMP (Ježek *et al.*, 1997c) and Cl^- uniport via UCP1 are less pronounced. The latter is compatible with a double-peak-energy barrier model, i.e., with a possible existence of a weak binding site located centrally in the lipid bilayer core (Garlid, 1990; Garlid *et al.*, 1989).

FATTY ACID-INDUCED UNCOUPLING IN PROTEOLIPOSOMES WITH THE RECONSTITUTED UCP1

A consensus became evident from the numerous attempts to reconstitute UCP1 into liposomes that the protein, indeed, requires the presence of fatty acids in order to observe H^+ translocation (Strieleman *et al.*, 1985a; Klingenberg and Winkler, 1985; Ježek *et al.*, 1990a; Winkler and Klingenberg, 1992, 1994). It has been also reported that the same protein is able to mediate Cl^- uniport across the liposomal membrane (Strieleman *et al.*, 1985b; Ježek *et al.*, 1990b). Chemical modifications of arginines lead to the decreased GDP inhibition, but did not influence H^+ conductance in the reconstituted system (Katiyar and Shrago, 1989), thus showing again that GDP-binding site resides apart from the uncoupling function. Subsequent mutagenesis determined R276 as the interacting arginine (Murdza-Inglis *et al.*, 1994). Various protocols and/or preparations differed in the reported orientations of UCP1 molecules. Klingenberg has claimed that in his experimental system the uniform orientation was attained, as judged from the degree of GDP inhibition (Klingenberg and Winkler, 1985; Winkler and Klingenberg, 1992, 1994). On the contrary, we have consistently observed a nearly equal orientational distribution, i.e., 50% distribution with the nucleotide-binding site inward vs outward. Consequently, in our system, the external GDP inhibited up to 50% and the complete inhibition was observed only when the proteoliposomes were loaded by GDP and external GDP was also added (Ježek *et. al.*, 1990b, Garlid *et al.*, 1996). A similar situation was observed in the case of PUMP (Ježek *et al.*, 1997c).

All anions, recognized on the basis of mitochondrial assays to be UCP1 substrates, were also found to be competitive inhibitors of Cl^- uniport (Ježek and Garlid, 1990). This competition includes FAs as well (Ježek *et al.*, 1994, 1997b; Garlid *et al.*, 1996). The "external" translocation sites of UCP1 are shielded, as demonstrated by the failure of nontransported substrate analogs to inhibit transport (Ježek and Garlid, 1990). All these findings were prerequisites for the validity of the FA cycling hypothesis. The competition was mutual, since lauric acid-induced H^+ translocation in proteoliposomes containing UCP1 was found to be inhibited by nitrate or hexane sulfonate (Ježek *et al.*, 1994) and by undecane sulfonate (Garlid *et al.*, 1996). The finding that undecane sulfonate competes with FA-induced H^+ transport provided the first strong evidence supporting the FA cycling mechanism (Garlid *et al.*, 1996). Undecane sulfonate was found to be translocated by UCP1 in a charged form (evidently representing the anion uniport) and this transport was nearly completely inhibited by GDP present in both intraliposomal lumen and external medium (Garlid *et al.* 1996). Unlike laurate, undecane sulfonate is strong acid existing in the neutral unionized form below pH 2. This explains why undecane sulfonate does not cause the flip-flop acidification (Fig. 1b) as do the fatty acids upon their addition to the liposomes because of the reestablishment of both membrane and acid–base equilibrium (Ježek *et al.*, 1997a, 1998). Undecane sulfonate does not induce H^+ translocation, even in the presence of UCP1, since it cannot be transported in the neutral form back across the membrane and cannot carry H^+ in this way (Garlid *et al.*, 1996). Consequently, we come to the conclusion that its close competing analog, lauric acid can induce H^+ transport in the presence of UCP1 and does it just because lauric acid can flip-flop back in the neutral form (Garlid *et al.*, 1996). From the point of view of Klingenberg's local buffering hypothesis (Winkler and Klingenberg, 1994; Bienengraeber, *et al.*, 1998), lauric acid provides a pool of H^+, which is a part of the H^+ translocation pathway. H^+ ions are physically translocated by certain residues of UCP1 across the membrane according this hypothesis. It should be concluded that undecane sulfonate cannot provide local buffering whereas lauric acid can. To distinguish between the hypotheses one needs further arguments.

Ježek *et al.* (1997a, b) have found FA derivatives that have a pK_a in the physiological range, but they are unable to flip-flop. These FAs were called "inactive" FAs. Their inability to induce flip-flop, acidification was explained by their specific, most probably, U shape, a conformation in the membrane that does not

allow them to flip-flop. The inactive FAs are mostly dumbbell-shape molecules or bipolar molecules, such as phenylvaleric or 12-hydroxylauric acid. The support for the FA cycling mechanism comes from the observations that the inactive FAs did not induce H^+ transport in proteoliposomes containing UCP1, did not induce charge transfer, and neither inhibited Cl^- transport. We have concluded that being unable to flip-flop, the inactive FAs cannot undergo the cycling and, as a consequence, the H^+ translocation, mediated by UCP1, is prevented.

On the contrary, according the local buffering hypothesis, one should have to explain why these compounds cannot provide local buffering. There could be an explanation in the case when local buffering takes place in the second matrix segment, as predicted by Bienengraeber *et al.* (1998), and the site is, therefore, hidden for the FAs, being unable to flip-flop to the other side of the membrane. This is, however, not necessary in our experimental system, i.e., in the system with two orientions of the inserted UCP1. Here, at least half of the UCP1 molecules should be accessible toward the external inactive FAs. On the other hand, accepting that FAs interact with UCP1 solely in the matrix segment of the protein, one cannot exclude the possibility that FA release from the site proceeds exclusively toward the inner (matrix) leaflet of the lipid bilayer. The opposite is true. One should accept that a portion of FAs can be released toward the external (cytosolic) leaflet of the lipid bilayer. Since there must also be distribution because of the acid–base equilibria within the ensemble of FA molecules, some bound/unbound FA molecules must be dissociated (anions). One can easily recognize that this release mechanism is, in fact, identical to the anion uniport. In this sense, the local buffering hypothesis (in the matrix site) merges with our FA cycling hypothesis.

FATTY ACIDS AS MEDIATORS OF UNCOUPLING

It must be assumed that flip-flop acidification observed upon the FA addition to the liposomes (Fig. 1b) also proceeds transiently in mitochondria, when FAs are released from triglyceride storage and other sources (Ježek *et al.*, 1998). The flip-flop acidification causes a transient decrease in the protonmotive force that is observed experimentally upon the FA addition to all types of energized mitochondria. The FA cycling mediated by several proteins of the inner membrane represents the second, less transient, mechanism of uncoupling, which has an advantage in its multiple regulation which is different for each protein. With the regulation switched on, mitochondria are uncoupled until all nonesterified FAs are depleted from the membrane and binding sites on proteins, as well as from both cytosolic and matrix aqueous compartments. A major depleting mechanism is the carnitine cycle (Eaton *et al.*, 1996), that passes FAs to β-oxidation. It is inhibited by malonyl-CoA and has important organ-dependent regulatory links toward *de novo* lipogenesis and FA elongation. Uncoupling, based on FA-cycling, tunes the degree of the coupling of oxidative phosphorylation that results in slightly increased respiration rate. Thermodynamical analysis shows that in an open system, when oxidative phosphorylation is exchanging its products/precursors with the cell, such an acceleration is beneficial (Stucki, 1980). Bearing this in mind, one should judge the basic function of the newly discovered uncoupling proteins, UCP2 and UCP3, accordingly.

In principle, any of the integral membrane proteins of the MACP gene family could attract the anionic carboxylic headgroup of FA and permit its passage to the other side of the membrane, since the majority of them contain conserved positively charged residues in the hydrophobic core of the membrane. Among them, indeed the ADP/ATP carrier (Skulachev, 1991; Brustovetski and Klingenberg, 1994, Schönfeld *et al.*, 1996, Polčič *et al.*, 1997) and glutamate/aspartate carrier (Samartsev *et al.*, 1997) were reported to significantly contribute to FA-induced uncoupling in mitochondria. CAT-sensitive uncoupling, induced by azido-FA, was turned back to a coupled state upon UV irradiation (Schönfeld *et al.*, 1996). Thus, azido-FA, covalently attached to the ADP/ATP carrier, was unable to induce coupling, which again supports the existence of the FA cycling mechanism. FA-induced uncoupling in yeast mitochondria of the mutant strain DNY1 was not sensitive to CAT (Ježek *et al.*, 1998). DNY1 has the deleted AAC2 gene, coding the functional AAC. Similar results were independently reported for the JL-1-3 strain, having deleted all AAC genes (AAC1, AAC2, and AAC3), or the *op1* mutant, in which R96H substitution is present in the product of AAC2 gene (Polčič *et al.*, 1997). Note, that products of AAC1 and AAC3 isogenes do not participate in the ADP/ATP transport. The inability of AAC, with the R96H substitution, to transport FA anions suggests that the Arg 96 could be the residue attracting the anionic headgroup. One must interpret with caution, since the substitution of the corresponding Arg 83 on UCP1 did not abolish the

FA cycling (Modrianský *et al.*, 1997). The other carriers, such as dicarboxylate carrier (Wieckowski and Wojtczak, 1997) and phosphate carrier (Engstová *et al.*, 1998) were found to, at least, interact with FAs. We have suggested that the recently discovered uncoupling proteins, ubiquitous UCP2 and skeletal muscle-specific UCP3, also provide FA cycling (Ježek and Garlid, 1998; Ježek *et al.*, 1998).

EXISTENCE OF FATTY ACID-BINDING SITES

Attempts to indicate the existence of FA-binding sites on the integral membrane proteins using radiolabeled palmitate failed (Cunningham *et al.*, 1986). A hopeful indication was later provided by the photoaffinity labeling of whole BAT (Růžička *et al.*, 1996) and rat heart mitochondria (Schönfeld *et al.*, 1996; Engstová *et al.*, 1998), when [$^3H_4$] azido-FA labeled the most hydrophobic proteins. Among them, the strongest bands on the autoradiograms were represented by UCP1, ADP/ATP carrier, and phosphate carrier. The isolated UCP1 can bind the photoaffinity label in a quite low stoichiometry, less then 0.5 per dimer (Růžička *et al.*, 1996). The best indication of the FA-binding site existence in UCP1 was provided by EPR studies using 5-Doxyl-stearic acid (Ježek and Freisleben, 1994; Ježek *et al.*, 1995), which exhibits a specific EPR signal reflecting the immobilized probe. This specific signal was prevented by lauric acid and alkyl sulfonates and modulated by nucleotide addition. The latter effect on the spin-label signal probably reflects conformational changes involved in the allosteric nucleotide-mediated gating.

FATTY ACID CYCLING IN PLANT MITOCHONDRIA

The experiments, referred above to as type I, were also performed with potato tuber mitochondria, leading to the discovery of the plant uncoupling mitochondrial protein (PUMP; Vercesi *et al.*, 1995). A 33-kDa crude protein has been isolated by the same way as traditionally used for UCP1 on hydroxylapatite and the first reconstitution confirmed its uncoupling function in the presence of FAs (Vercesi *et al.*, 1995). Later, the type I and II experiments, performed with mitochondria from potato (Ježek *et al.*, 1996), tomato (Vercesi *et al.*, 1999), and many other plant species and plant parts including seeds (Ježek, Vicente *et al.*, unpublished), have established that the uncoupling is induced by FAs, also in plant mitochondria and inhibited by purine nucleotide di and triphosphates. The latter, however, require much higher doses than for UCP1. As an exception, a high affinity of 8-azido-ATP to PUMP has been reported (Saviani *et al.*, 1997). In tomato, a state of mitochondrial uncoupling has been correlated with the amount of FA present. Reconstitution of PUMP isolated from potato tuber and tomato mitochondria has suggested that the FA cycling mechanism is likely to be involved and confirmed that PUMP is able to translocate hexane sulfonate and undecane sulfonate, but is unable to transport Cl^- and pyruvate (Ježek *et al.*, 1997c).

Laloi *et al.* (1997) have cloned a protein 44% homologous to UCP1 from the potato gene library and called stUCP. Using MALDI-mass spectroscopy, Ježek *et al.* (unpublished) have sequenced some peptides derived by trypsin cleavage from the isolated potato PUMP. The peptides were exactly identical to the corresponding portion of the *ST*UCP sequence and altogether covered approx. 35%, including the region of the second matrix segment. This provides profound evidence that PUMP is a product of the *ST*UCP gene. Moreover, direct proof has been obtained that the second matrix segment of PUMP does not contain any histidine. Hence, contrary to Bienengraeber *et al.* (1998), one can conclude that no H^+ dissociating groups with pK_a around 7 are present in the given region of PUMP (*ST*UCP), in spite of fact that this protein is able to facilitate FA-induced H^+ uniport. In conclusion, the FA-binding site could be located in the region of the second matrix segment and the histidine substitutions in the UCP1 sequence most probably lead to alternation of FA interaction with UCP1.

Meanwhile, the homologous sequence of PUMP (*AT*UCP) from the *Arabidopsis thaliana* gene library has been cloned (Maia *et al.*, 1998) and its expression in yeast has been achieved (Arruda, personal communication). In addition, antibodies raised against potato PUMP identified a 33-kDa PUMP band and its dimer in tomato mitochondria (Vercesi *et al.*, 1999), in mitochondrial fractions prepared from homogenates of many tropical and mild-climate fruits, such as banana, apple, peach, melons, orange, and other fruits (Ježek *et al.*, 1998), and in highly purified mitochondria from many other plant species and plant parts, namely in mitochondria from seeds (Ježek, Vicente *et al.*, unpublished).

In plants, uncoupling mediated by PUMP, might serve to optimize oxidative phosphorylation, as well as to switch-off rapidly ATP production, thus contributing to the termination of synthetic processes during the final stages of seed formation and senescence. PUMP can be responsible for the climacteric respiratory burst, a transient stage, existing in many fruits during their ripening. On the contrary, accelerated respiration and/or metabolism due to a mild uncoupling by PUMP in seeds should be beneficial to seed germination.

FUTURE PERSPECTIVES

In conclusion, many common questions need to be solved to evaluate similarities or different features in the functioning of uncoupling proteins in such distinct tissues as plant tissues, on the one hand, and mammalian tissues, such as heart, lymphocyte, white fat, or skeletal muscle, on the other hand. We must accept that the regulated uncoupling is a widespread phenomenon, existing "from banana to lymphocytes." Of course, there could be different regulatory mechanisms controlling PUMP in plants and still hypothetical uncoupling mediated by UCP2 in mammalian tissues (or by UCP3 in skeletal muscle). The detailed function of these proteins and their regulation awaits further investigation. The research on UCPs will bring a significant understanding of phenomena in such seemingly distinct fields as plant physiology, physiology of body weight regulation (pathophysiology of obesity), fever, adaptive thermogenesis, and other physiological disciplines.

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