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Regulation of UCP3 by nucleotides is different from regulation of UCP1

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Abstract UCP3 is an isoform of UCP1, expressed primarily in skeletal muscle. Functional properties of UCP3 are still largely unknown. Here, we report about the expression of UCP3 and of UCP1 in inclusion bodies of *Escherichia coli*. On solubilization and reconstitution into proteoliposomes, both UCP3 and UCP1 transport Cl^- at rates equal to the reconstituted native UCP1. Cl^- transport is inhibited by low concentrations of ATP, ADP, GTP and GDP. However, no H^+ transport activity is found possibly due to the lack of a cofactor presents in UCP from mitochondria. The specificity of inhibition by nucleoside tri- and diphosphate is different between UCP1 and UCP3. UCP1 is more sensitive to tri- than diphosphate whereas in UCP3, the gradient is reverse. These results show a new paradigm for the regulation of thermogenesis at various tissues by the ATP/ADP ratio. In brown adipose tissue, the thermogenesis is correlated with a low ATP/ADP whereas in skeletal muscle, non-shivering thermogenesis is active at a high ATP/ADP ratio, i.e. in the resting state.

Key words: Uncoupling protein; Chloride transport; ATP/ADP regulation

1. Introduction

The uncoupling protein from brown adipose tissue mitochondria has a well-defined uncoupling function for the thermogenesis of this tissue [1,2,3]. It short-circuits H^+ generated by the respiratory chain and thus converts most of the oxidative energy into heat instead of ATP. It requires fatty acids as cofactors for the H^+ transport function [4]. The transport is specifically inhibited by purine nucleotides [5]. Besides H^+, UCP1 also transports Cl^-, although at a lower rate [6,7]. This Cl^- transport is inhibited in the same way by purine nucleotides as H^+ transport. Although the physiological meaning of Cl^- transport is unclear, it clearly reflects a basic transport function of UCP without the necessity for the fatty acid cofactor such as in H^+ transport.

Whereas UCP1 is exclusively confined to brown adipose tissue, structurally similar proteins have been identified to occur in other organs of mammalians although the overall similarity of UCP2 and UCP3 to UCP1 is only about 55–57%, respectively [8–11]. There are a number of critical residues conserved which have been identified by mutagenesis to be important for the function of UCP. One exception is the absence of a His pair which was identified in UCP1 to be involved in H^+ transport [12]. This suggests that UCP2 and UCP3 may have functional properties similar to UCP1. However, the profile of these functions may differ and be adapted to the particular physiology of UCP in those organs. The elucidation of these different properties of the isoforms possesses a major challenge.

The approach to characterize UCP3 in isolated mitochondria from tissues and the isolation of UCP therefrom, which has been so successful for UCP1, turned out not to be possible for UCP3. Even after induction of UCP3 with triiodothyronine in skeletal muscle of rat, in the isolated mitochondria, only very low amounts of UCP3 could be determined (S.-G. Huang, unpublished work). Although there was a good expression of UCP2 and UCP3 reported in *Saccharomyces cerevisiae*, there was no report on the functional characterization of UCP2 and UCP3 in those mitochondria in contrast to the situation with UCP1 in *S. cerevisiae*. [12–14]. Apparently, the state of UCP3 in *S. cerevisiae* remained uncertain.

Here, we report on the expression of UCP1 and UCP3 in *Escherichia coli*. Similar as other mitochondrial carriers [15,16], UCP1 and UCP3 are deposited in high amounts in inclusion bodies. Partial renaturation therefrom permits us to characterize some functional properties of UCP. In particular the K^+ diffusion potential driven Cl^- transport could be determined and its inhibition by purine nucleotides shown. A striking difference was discovered to exist between the UCP isoforms. Whereas for UCP1, purine riboside triphosphates (GTP, ATP) are stronger inhibitors than the diphosphates (GDP, ADP), in UCP3, the opposite situation is found, diphosphates inhibit stronger than triphosphates. This has a major implication for the regulation of UCP3 versus UCP1 in the respective tissues and opens up a new understanding of the function of UCP3 versus UCP1.

2. Materials and methods

2.1. Materials

The fluorescence dyes *N*-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE) and pyranine (8-hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt) were purchased from Molecular Probes. Egg yolk phospholipid was isolated from fresh eggs and purified with Alumina B Super 1 from ICN Biomedicals as described previously [17]. The detergent *n*-decylpentaoxyethylene ($C_{10}E_5$) was obtained from Fluka and Amberlite XAD4 from Sigma.

2.2. Bacterial expression and isolation of inclusion bodies containing UCP

The gene encoding hamster uncoupling protein 1 (haUCP1) was cloned from hamster brown adipose tissue (BAT) as described earlier

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Abbreviations: haUCP, hamster uncoupling protein; hUCP, human uncoupling protein; BAT, brown adipose tissue; MQAE, *N*-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide; IPTG, isopropyl β-D-thiogalactoside; PMSF, phenylmethanesulfonyl fluoride; TX-100, Triton X-100; ME, 2-mercaptoethanol; DTE, dithioerythritol

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and the human uncoupling protein 3 (hUCP3) gene was obtained from Merck. A colony of *E. coli* cells BL21 (DE3) which has been transfected with the plasmid (pET-24a) containing the UCP gene was inoculated into LB medium (10 g tryptone, 5 g yeast extract and 10 g NaCL in 1 l) containing ampicillin (150 μg/ml) and the culture was grown at 37°C. Induction of expression was carried out with isopropyl β-D-thiogalactoside (IPTG) (1 mM). 2 h following the addition of IPTG, cells were harvested and lysed by incubation for 30 min at 37°C in solution A (50 mM Tris-HCl and sucrose 25% pH 8.0) containing lysozyme (1 mg/300 ml culture). After addition of 18 mM EDTA, 1.5% Triton X-100 (TX-100) and 1 mM phenylmethanesulfonyl fluoride (PMSF), the suspension was sonicated (a total of 6–7 cycles of 30 s sonication on ice). Following centrifugation at 19 500 rpm for 30 min at 4°C, the pellet was re-suspended in 10 ml solution B (1 M urea, 1% TX-100, 0.1% 2-mercaptoethanol (ME) and 1 mM PMSF), sonicated and re-centrifuged. The resulting pellet was re-suspended in solution B once more, sonicated and re-centrifuged as above. This is followed by re-suspending the pellet in 20 ml of solution C (20 mM Tris-HCl, 1 mM EDTA, 10 mM ME and 0.5% TX-100, pH 8.0), sonicating and re-centrifuging. The resulting inclusion bodies were washed with solution D (50 mM Tris-HCl, 1 mM EDTA and 10 mM ME, pH 8.0) and the pellet was stored at −20°C.

2.3. Reconstitution of UCP from inclusion bodies into phospholipid vesicles and measurements of H^+ and Cl^- transport

Inclusion bodies (≈1 mg) were solubilized in 100 μl of 2% (w/v) sarcosyl in buffer A (50 mM NH_4HCO_3, 2 mM dithioerythritol (DTE) and 2 mM PMSF, pH 8.0) followed by centrifugation at 12 500 rpm for 30 min. The supernatant was then diluted by buffer A to a final sarcosyl concentration of 0.5%. The solubilized protein was treated with a suspension of 4 mg egg yolk phosphatidylcholine (PC) in 0.97% TX-100, 0.5 mM EDTA, 1 mM DTE, 1 mM PMSF and 50 mM Mops, pH 6.7, to a final volume of 660 μl. The final ratio of TX-100/protein = 32 and TX-100/PC = 2.4. The suspension was shaken with 60 mg Amberlite XAD4 overnight at 4°C.

The solubilized UCP was reconstituted into liposomes using the protocol described previously [13]. H^+ transport was assayed using pyranine as pH sensitive fluorescence probe at $\lambda_{ex} = 467$ nm and $\lambda_{em} = 510$ nm in a standard medium containing 280 mM sucrose, 0.5 mM HEPES, 0.2 mM EDTA, 1 μM pyranine, pH 6.8, and 125 μM laurate. Valinomycin (2.5 μM) was added to initiate the H^+ uptake driven by the K^+ efflux. Cl^- transport was measured by fluorescence of MQAE-loaded proteoliposome at $\lambda_{ex} = 355$ nm and $\lambda_{em} = 460$ nm in a medium containing 4 mM sodium phosphate, pH 6.8, and 155 mM KCl. Cl^- influx was initiated by the addition of valinomycin (2 μM) and the rate was determined according to the Eq. 1:

$$J_{cl} = ((F_i - F_s)(K_{Cl} + 1)/[Cl]_s)^{-1}(dF(0)/dt) \quad (1)$$

3. Results

3.1. Expression of UCP in inclusion bodies

The level of expression of UCP1 and UCP3 in the inclusion bodies collected from *E. coli* cells is illustrated in the protein-stained gel in Fig. 1. The gel contains native UCP1 isolated from hamster mitochondria parallel to the inclusion bodies. 2 h following induction with IPTG, a prominent protein band (apparent molecular mass 33–34 kDa) appears in the inclusion bodies fraction containing either UCP1 or UCP3. At least

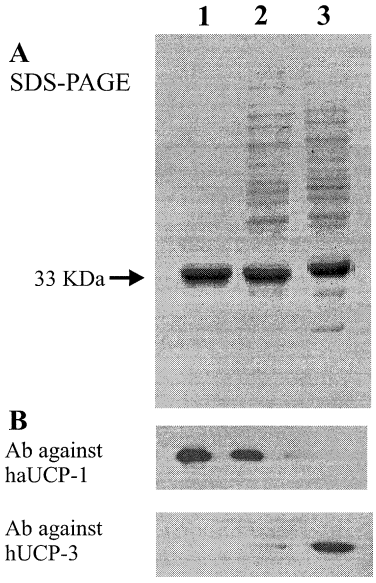

Fig. 1. Analysis of expression of UCPs in *E. coli*. (A) Coomassie-stained SDS-PAGE, 12.5% polyacrylamide of (lane 1) native haUCP1 (6 μg), (lane 2) inclusion bodies of haUCP1 (6 μg), (lane 3) inclusion bodies of hUCP3 (6 μg). (B) Immunoblot of UCPs expressed in *E. coli* using antisera against haUCP1 and hUCP3.

60% of total proteins can be attributed to UCP1 or UCP3. The identification of UCP1 and UCP3 is documented by the immunoblot (Fig. 1B) using whole protein antisera against haUCP1 and peptide antisera against hUCP3. The high specificity of the antisera is evident because there is virtually no cross-reaction between UCP1 and UCP3.

3.2. Transport measurements

The conditions for studying H^+ and Cl^- transport measurements mediated by the UCPs expressed in *E. coli* followed in principle the same as those applied for monitoring the transport function of native haUCP1 in reconstituted vesicles. H^+ uptake requires a K^+ diffusion potential, negative inside, generated by the valinomycin-catalyzed efflux of K^+ and a high buffering capacity inside as provided by 100 mM potassium phosphate inside the vesicles. Cl^- uptake is initiated by the valinomycin following the K^+ diffusion potential, positive inside in the presence of external 155 mM KCl.

Fig. 2 shows the recordings of the Cl^- influx into proteoliposomes reconstituted with either haUCP1 or hUCP3 and the influence of GTP and GDP on the transport inhibition. The extent and the rate of Cl^- uptake are similar with UCP1 and UCP3. In each instance, the Cl^- transport activity was inhibited maximally (85–90%) by the addition of an excess GTP or GDP which is a criterion for the involvement of UCP in the Cl^- transport. In the reconstituted vesicles with

Table 1
The K_i values for ATP, GTP, ADP and GDP inhibition of Cl^- transport in proteoliposomes, reconstituted with native haUCP1 and *E. coli* expressed haUCP1 and hUCP3 at pH 6.8 and 10°C

UCP	K_i (μM)		K_i^{ATP}/K_i^{ADP}	K_i (μM)		K_i^{GTP}/K_i^{GDP}
	ATP	ADP		GTP	GDP	
Native haUCP1	0.51	1.03	0.49	0.71	2.1	0.34
E. coli haUCP1	0.61	1.53	0.40	0.41	0.83	0.49
E .coli hUCP3	0.23	0.078	2.95	0.34	0.13	2.62

E. coli expressing UCP1 and UCP3, no marked H^+ transport activity could be measured with or without 125 μM laurate. The vesicles were similar to those of Cl^- transport measurements except that they were loaded with potassium phosphate to generate a K^+ diffusion potential positive outside to drive the H^+ uptake. The absence of H^+ transport does not exclude that native UCP3 can catalyze H^+ transport, because H^+ transport is well-established for the reconstituted native haUCP1. Possible reasons are discussed below. The rates of H^+ and Cl^- uptake are summarized in Fig. 3.

3.3. Inhibition by nucleoside di- versus triphosphates

Most interestingly, as shown in Fig. 2, the specificity of inhibition of the Cl^- transport by GTP and GDP is different between UCP1 and UCP3. Whereas in UCP1 the Cl^- transport activity is more sensitive to nucleoside tri- than diphosphate, the effect is reversed in UCP3. This effect is shown in more detail in Fig. 4 with the concentration-dependence for the inhibition of haUCP1 and hUCP3 by GDP and GTP. With UCP1, the Cl^- uptake rate is inhibited at a lower concentration of GTP than GDP. With UCP3, the concentration-dependence of the GTP inhibition is about the same as with UCP1, however, the sensitivity to GDP is drastically increased. The same sensitivity relations are also observed with ATP and ADP. Table 1 gives a summary of K_i values evaluated from concentration-dependence of the inhibition of Cl^- transport by ATP, ADP, GTP and GDP. It also gives the ratio of the inhibition constants for nucleoside tri- and diphosphate. These ratios ($K_i^{NTP}/K_i^{NDP} \approx 0.35$–$0.49$) are about the same for native UCP1 isolated from BAT mitochondria and for UCP1 re-natured from inclusion bodies, illustrating that the binding properties of the re-natured UCP reflect well the native one. For UCP3, this ratio increases 8-fold to about 2.6–2.9, reflecting the much stronger affinity for ADP and GDP than for ATP and GTP.

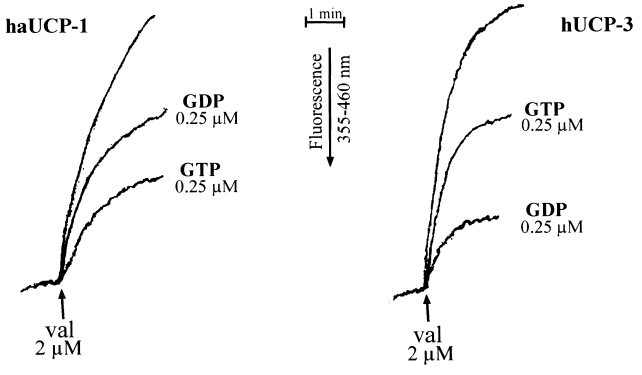

Fig. 2. Cl^- influx into phospholipid vesicles reconstituted with *E. coli* expressed haUCP1 and hUCP3. The recordings show also the different inhibition effect of the Cl^- transport activity by nucleoside tri- and diphosphate between haUCP1 and hUCP3. Cl^- influx was monitored by fluorescence of MQAE loaded into vesicle at $\lambda_{ex} = 355$ nm and $\lambda_{em} = 460$ nm at pH 6.8 and 10°C. A 50 μl portion of vesicles was suspended in 4 mM sodium phosphate buffer containing 155 mM KCl to a final volume of 385 μl. The Cl^- influx rate was monitored after addition of 2 μM valinomycin. Tributyltin acetate (40 μM) was added to equilibrate the internal and external chloride. The flux rate was calculated from the fluorescence data by using Eq. 1.

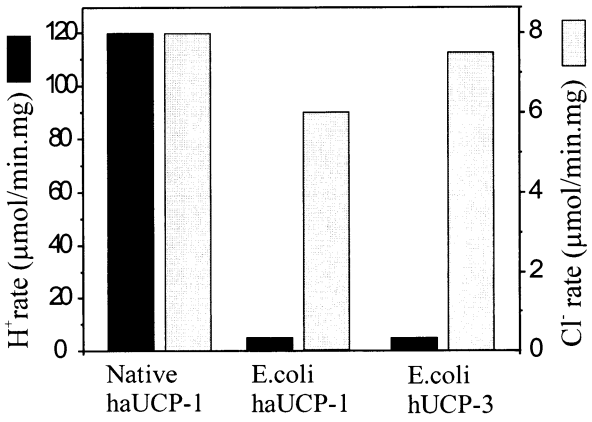

Fig. 3. H^+ and Cl^- transport into proteoliposomes reconstituted with purified native haUCP1 from BAT mitochondria and with *E. coli* expressed haUCP1 and hUCP3. H^+ influx was measured as the change in external pH monitored by pyranine fluorescence at $\lambda_{ex} = 467$ nm and $\lambda_{em} = 510$ nm at pH 6.8 and 10°C. A 50 μl portion of vesicles was added to 0.5 mM HEPES buffer, pH 7.5, containing 1 μM pyranine, 0.5 mM EDTA and 280 mM sucrose to a final volume of 330 μl. Valinomycin of final concentration 2.5 μM was added to generate a membrane potential in the presence of 125 μM laurate. H_2SO_4 was added in steps of 20 nmol H^+ to adjust the pH to 6.8. The uncoupling with 1 μM carbonylcyanide *m*-chlorophenyl hydrazone (CCCP) was used to determine the capacity of H^+ conductance across the vesicle. For Cl^- transport, the measurements are as described in the legend of Fig. 2.

4. Discussion

Whereas in brown adipose tissue uncoupling of oxidative phosphorylation is consistent with the heat generation, the function of uncoupling in other organs is less well-defined. UCP1 is characterized as the key uncoupling protein in brown adipose tissue mitochondria, whereas the function of UCP2 and UCP3 as uncoupler are not yet confirmed [18]. Uncoupling by UCP2 and UCP3 has been claimed to be shown after expression in yeast cells using a mitochondrial membrane potential probe and the distribution of its fluorescence signal over the cell population determined in a single cell counter [9,19]. The observed shift in the fluorescence versus cell count pattern is ambivalent since it may also reflect a decrease of the mitochondrial content. UCP2 and UCP3 introduction seems to reduce the biogenesis of mitochondria since the growth rate of the transfected yeast cells is strongly decreased. More experimental evidence for the function of UCP2 and UCP3 was obviously necessary. The lack of reports on the functional characterization of UCP2 and UCP3 in mitochondria from yeast cells was indicative of a deleterious effect of UCP2 and UCP3 which is absent in UCP1.

Here, we report that UCP1 and UCP3 can be expressed in *E. coli* similar as other mitochondrial carriers [15,16]. These proteins are deposited in inclusion bodies from which UCP1 and UCP3 can be partially re-natured and reconstituted into proteoliposomes. The procedure for the reconstitution from inclusion bodies differs from the reconstitution of UCP1 isolated from mitochondria in that the solubilizing reagent lauryl sarcosyl intervenes and the replacement by the non-ionic detergent phospholipid mixture had to be distributed over several steps in order to assure renaturation. The two transport functions known from UCP1, H^+ transport and Cl^- (anion) transport were determined according to the procedures ap-

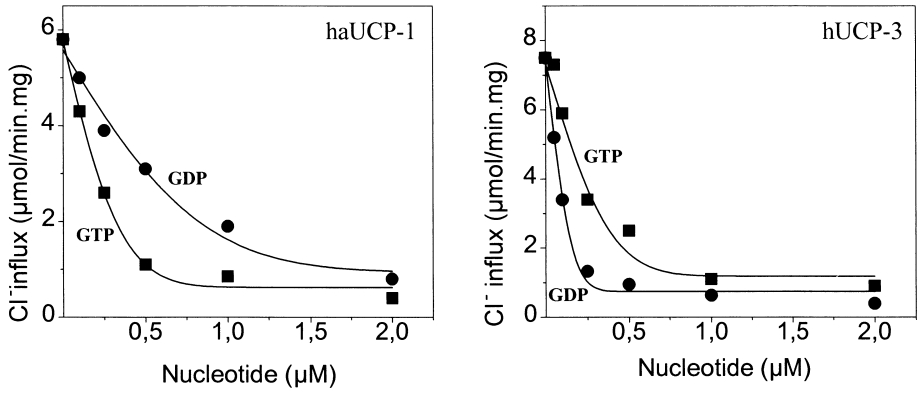

Fig. 4. Titration with GTP and GDP of the Cl^- influx into proteoliposomes reconstituted with haUCP1 and hUCP3. The figures contain the dose-responses for inhibition of the Cl^- influx by external addition of nucleotides. Cl^- transport measurements were performed as described in the legend to Fig. 2. The K_i values for GTP and GDP inhibition are contained in Table 1.

plied for UCP1 reconstituted from yeast cells [13]. Under identical conditions, no H^+ transport could be measured in the vesicles reconstituted with UCP1 and UCP3 derived from the inclusion bodies. However, the capability to transport Cl^- and its specific inhibition by purine nucleotide (ATP, ADP, GTP and GDP) could be reconstituted. Cl^- transport in vesicles reconstituted with the re-natured UCP1 and UCP3 from *E. coli* has about the same activity as the native UCP1.

The reason for the defect of H^+ transport is not clear in view of the reconstitution of the Cl^- transport capacity. One might suggest that a cofactor responsible for H^+ transport by UCP1 is missing which is only present when UCP1 is inserted into mitochondria. Cl^- and H^+ transport in native UCP1 have been shown to be equally sensitive to purine nucleotide. They are both controlled from the same nucleotide binding site which is located separately from the translocation channel and they induce inhibition by a conformation change. Therefore, inhibition of Cl^- transport by nucleotides should be representative for the regulation of any potential H^+ transport activity of UCP in mitochondria.

The striking difference in the response of the two UCP isoforms to purine di- and triphosphate is highly significant for the regulation of UCP activities. In previous work of this laboratory, a very detailed analysis of the nucleoside triphosphate and diphosphate binding on UCP1 was presented [3,20–22]. At $pH < 7$, nucleoside triphosphate (GTP, ATP) had a higher binding affinity than the diphosphate (GDP, ATP). The binding was strongly pH-dependent [5,19]. The affinity decreases more strongly with nucleoside triphosphate than with diphosphate, thus reversing the affinity at $pH > 7.2$. The groups involved in controlling the pH of binding are identified as E190 and in addition H214 for only the nucleoside triphosphate binding [13,14]. Interestingly, these groups are also present in UCP2 and UCP3.

In the present study, the inhibition constants determined for ATP, ADP, GTP and GDP in UCP1 and UCP3 faithfully reflect the dissociation constants since there is only very little competition between Cl^- and nucleotide binding. Although the affinity for ATP is 2.6-fold higher for hUCP3 than haUCP1, it is nearly the same for GTP. However, the affinity for nucleoside diphosphate increases about 6.5-fold for GDP and 19.6-fold for ADP in hUCP3 over haUCP1. As a result, the affinity ratios GTP/GDP and ATP/ADP change from about 0.48 and 0.4 in UCP1 to 2.6 and 2.9 in UCP3, i.e. there is an 8-fold change in the inhibitory sensitivity towards the ATP/ADP ratio.

The different responses of UCP isoforms to the di- and triphosphates have important repercussions for the regulation of UCP by the intracellular phosphorylation potential as represented by the ATP/ADP ratio. In UCP1, a decrease of the ATP/ADP ratio, whereas in UCP3, an increase of the ATP/ADP ratio would increase uncoupling as illustrated in Fig. 5. This finding can be well-correlated with the role of uncoupling in various tissues. In brown adipose tissue, heat generation is the main function. Correspondingly, a low phosphorylation potential is in line with the overwhelming uncoupling in this tissue. In contrast, in skeletal muscle, the main function is energy transfer by ATP to muscle contraction. Thus, during high muscle activity, all oxidative energy should be converted to ATP and little diverted to uncoupling. Correspondingly, UCP3 should be inhibited when the ADP level rises parallel with a decrease of the ATP/ADP ratio. At this stage, heat is generated mainly by the muscle contraction cycle and will be sufficient to cover the heat demand of the skeletal muscle. In the resting state at a high ATP/ADP ratio and a low ADP level, UCP3 should be activated. In this state, the muscle activity cannot fulfil the heat demand and therefore, additional heat generation by uncoupling is necessary.

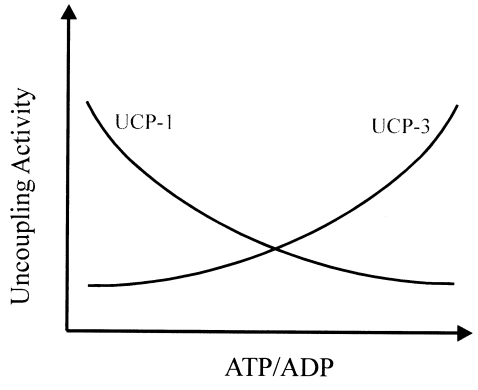

Fig. 5. Regulation of the uncoupling activity (H^+ transport) by energy charge (ATP/ADP ratio). In brown adipose tissue, energy dissipation (thermogenesis) is the main function mediated by the activity of UCP1 which is active at a low energy charge. In skeletal muscle, the regulation of energy dissipation is mediated by UCP3 which is active at a high energy charge.

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