Anion Carriers in Fatty Acid-Mediated Physiological Uncoupling

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Physiological aspects of uncoupling of oxidation and phosphorylation are reviewed in the context of involvement of mitochondrial anion carriers. It is assumed that the carriers facilitate electrophoretic translation of fatty acid anion, $RCOO^-$, from the inner to the outer leaflet of the mitochondrial membrane, whereas back movement of the protonated fatty acid, RCOOH, from the outer to the inner leaflet represents flip-flop of RCOOH via the phospholipid bilayer of the membrane. The $RCOO^-$ transport seems to be catalyzed by the ATP/ADP and aspartate/ glutamate antiporters, dicarboxylate carrier, and uncoupling proteins (UCP1, UCP2, $UCP3_L$, $UCP3_S$, and plant UCP). The fatty acid uncoupling is shown to be involved in the thermoregulatory heat production in animals and plants exposed to cold, as well as in performance of respiratory functions other than ATP synthesis, i.e., formation of useful substances, decomposition of unwanted substances, and antioxidant defense. Moreover, partial uncoupling might take part in optimization of the rate of ATP synthesis in aerobic cells.

KEY WORDS: Uncoupling; thermoregulation; mitochondria; ATP/ADP antiporter; aspartate/glutamate antiporter; uncoupling proteins 1, 2, 3; plant uncoupling protein.

THERMOREGULATORY UNCOUPLING

Definition

The expression "thermoregulatory uncoupling" is applied to define a specific type of biochemical thermoregulation when the respiration-produced electrochemical H^+ potential difference ($\Delta\bar{\mu}_{H^+}$) is dissipated to form heat. Such a mechanism is simpler and, hence, more operative than, say, cold-induced shivering, which requires (1) utilization of the respiratory $\Delta\bar{\mu}_{H^+}$ by the H^+-ATP-synthase to form intramitochondrial ATP from ADP and phosphate (P_i), (2) translocation of ATP by the ATP/ADP-antiporter to cytosol, (3) diffusion of ATP in cytosol to the actomyosin filaments, (4) ATP hydrolysis by actomyosin to cytosolic ADP and P_i, (5) diffusion of formed ADP and P_i to a mitochondrion, and (6) import of ADP and P_i to the mitochondrial matrix via the ATP/ADP antiporter and phosphate carrier. It is not surprising, therefore, that uncoupling substitutes for shivering in the course of cold adaptation of animals (Skulachev and Maslov, 1960).

Discovery

In 1958, I suggested that not only phosphorylating respiration but also uncoupled and noncoupled respirations can be of some physiological importance (Skulachev, 1958). Heat production in warm-blooded animals looked like the most obvious example when respiration is needed to produce heat rather than ATP. At that time, Beyer and his co-workers and Smith independently reported that cold acclimation of rats for several weeks resulted in some decrease in the P/O ratio of isolated liver mitochondria (Panagos *et al.*, 1958; Smith, 1958; Panagos and Beyer, 1960; see also Hannon, 1958). Unfortunately, the *in vivo* cold treatment was so long

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and the *in vitro* effect so small that the physiological importance of this observation remained obscure. It could well be a result of an *in vitro* artifact or a long-term *in vivo* epiphenomenon accompanying other cold-induced changes in the metabolic pattern.

In 1960, I published together with zoologist S.P. Maslov, a paper (Skulachev and Maslov, 1960; see also Skulachev, 1963) describing results of our experiments on pigeons exposed to severe cold treatment that caused a strong decrease in body temperature. Pigeons were shorn before the experiment (to exclude physical thermoregulation) and placed at $-15°C$ in a refrigerator equipped with a ventilator. Under such terrible conditions, the pigeon died in less than 1 hour if treated for the first time. However, the bird survived for several hours and maintained almost normal body temperature if put in the refrigerator for the second time on the next day after a 20–40 min first cold exposure (Fig. 1).

Measurement of respiration and phosphorylation in liver mitochondria isolated from control and cold-treated animals reveals that the cooling had no effect. On the other hand, breast muscle mitochondria proved to be almost completely uncoupled when isolated from a pigeon cooled for the second time. This uncoupling was accompanied by more than twofold increase in the *in vivo* measured oxygen consumption.

As to the first cold treatment, it also lowered the P/O ratio in the muscle mitochondria but the effect was much smaller than after the second exposure to cold. The *in vivo* O_2 consumption was unstable: initial stimulation changed to inhibition when the body temperature dropped drastically.

A series of repeated cold stresses results in partial restoration of the P/O ratio level and in some decrease of the *in vivo* O_2 consumption with the body temperature remaining the same as at the second cold exposure (Fig. 2). In the same experiments, it was found that the P/O ratio of mitochondria from pigeons before the second cooling was almost as high as that of the nontreated control. This meant that the P/O ratio decrease upon the second cold exposure developed on the minute time scale (Skulachev, 1962).

To explain these data, we suggested that uncoupling is a mechanism of urgent heat production in muscle. This mechanism fails to be actuated sufficiently rapidly on the first cold exposure, resulting in catastrophic drop of body temperature. On the second cold treatment, a pigeon, being better prepared for the cooling, succeeds in strong uncoupling before the body temperature becomes too low to carry out any physiological response. The series of repeated cooling seemed to mobilize thermoregulatory mechanisms other than uncoupling and stimulation of O_2 consumption.

Within the framework of the above logic, one might expect that injection of an artificial uncoupler into an animal before the first cooling would save it from sudden cold-induced death. Experiments on mice confirmed this expectation. It was found that injection of 2,4-*p*-dinitrophenol prolonged the survival time at $-15°C$ (Skulachev and Maslov, 1960). Further studies
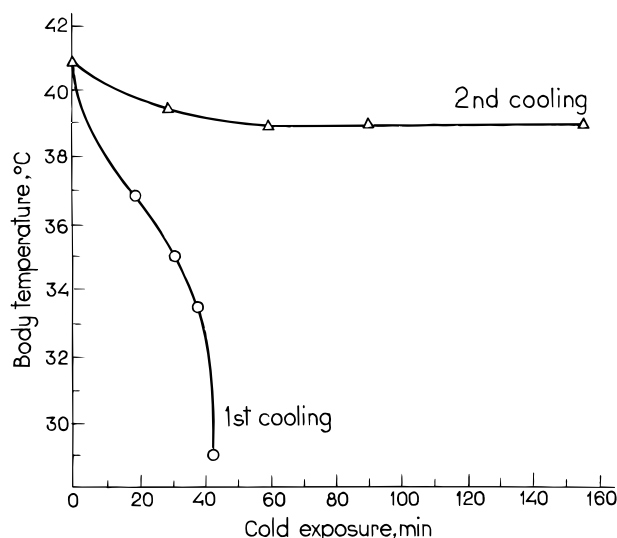

Fig. 1. Dynamics of the body temperatures of pigeons on the first and second cold exposures. ($-15°C$). Pigeons were shorn before the experiment (from Skulachev and Maslov, 1960).
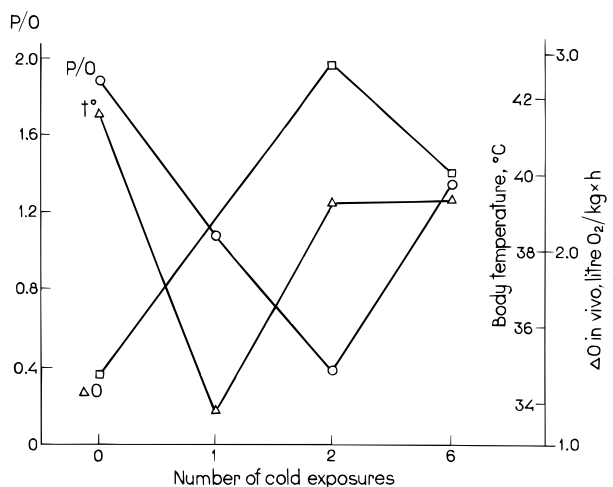

Fig. 2. Body temperature, *in vivo* oxygen consumption, and *in vitro* P/O ratio in breast muscle mitochondria oxidizing pyruvate and malate. Pigeons were exposed to $-15°C$ for 30 min (from Skulachev and Maslov, 1960).

revealed thermoregulatory uncoupling in mice on repeated cold exposure (Skulachev *et al.*, 1963). Later, a piece of evidence supporting the thermoregulatory uncoupling was obtained in my group by Zorov and Mokhova (1973) who studied respiration of intact diaphragm muscle of the cold-exposed rat and by Grav and Blix (1979) measuring respiratory control of the skeletal muscle mitochondria isolated from fur seals acclimated to cold under natural conditions. The cold-induced respiratory control decrease was abolished by adding serum albumin. Our group observed the same effect earlier (Skulachev, 1963). When I reported this observation at the 5th International Congress of Biochemistry in 1961, Efraim Racker mentioned in the discussion (Racker, 1963) that the albumin effect may be an indication of the involvement of fatty acids as thermoregulatory uncouplers (uncoupling by fatty acids was already described by Pressman and Lardy, 1956 and Scholefield, 1956). Further studies confirmed this suggestion. We found that repeated short-term cold exposure resulted in an increase in concentration of nonesterified fatty acids both in skeletal muscles and in isolated mitochondria. Fatty acids extracted from mitochondria of cold-treated animals caused uncoupling when added to control mitochondria (Levachev *et al.*, 1965).

Brown Fat and UCP1

In the 1970s, the focus of interest in the thermoregulatory uncoupling problem was shifted from muscles to brown fat. This occurred when it became clear that thermoregulatory heat production is the main physiological function of brown fat (Smith and Horwitz, 1969).

Attempts to study the energetics of brown fat mitochondria failed until serum albumin was added to the homogenization, washing and incubation media (Guillory and Racker, 1967; Joel *et al.*, 1967; Hohorst and Rafael, 1968). Energy coupling was further increased when the incubation mixture was supplemented with ATP or GTP (Hohorst and Rafael, 1968). In the same group, it was later found that brown fat mitochondria are two–four times more sensitive to the uncoupling action of fatty acids than mitochondria from liver, white fat, and heart and skeletal muscles (Rafael *et al.*, 1969).

Further studies revealed that in brown fat mitochondria, there is a usual level of respiratory chain enzymes, but the level of H^+-ATP-synthase is strongly lowered. On the other hand, they contain so-called uncoupling protein (or thermogenin) (Ricquier and Kader, 1976) which is absent in mitochondria from other tissues (for reviews, see Klingenberg, 1990; Klaus *et al.*, 1991). Thermogenin can amount to 10–15% of the total brown fat mitochondrial protein (Nicholls, 1979; Nicholls and Locke, 1984).

It is uncoupling protein (UCP) that is responsible for the high sensitivity of brown fat mitochondria to fatty acids. It was shown that in both brown fat mitochondria (Nicholls, 1976; Nicholls and Locke, 1984) and UCP-containing proteoliposomes (Bouillaud *et al.*, 1983; Klingenberg and Winkler, 1985; Strieleman *et al.*, 1985), 10^{-6}–$10^{-5}M$ fatty acid induces H^+ conductance, which is abolished by 0.1 mM ATP, ADP, GTP, or GDP.

Cold adaptation was found to stimulate transcription of UCP mRNA (Ricquier *et al.*, 1983) and its translation (Nicholls and Locke, 1984).

Several studies carried out on whole animal, brown fat *in situ*, brown adipocytes *in vitro*, and isolated brown fat mitochondria, revealed the following chain of events involved in the thermogenic response of this tissue to cold.

(1) Cold receptors in the skin send signals to the thermoregulatory center in the hypothalamus.

(2) From the hypothalamus, the signals are transmitted to brown fat via sympathetic neurons.

(3) Noradrenaline is released from neuron terminals into intercellular spaces of the brown adipose tissue.

(4) Noradrenaline binds to β-adrenergic receptors, which are localized on the outer surface of the brown fat cell plasma membrane.

(5) β-Adrenergic receptors, combining with noradrenaline, activate adenylate cyclase in the cytosol of the brown fat cell.

(6) Adenylate cyclase produces cAMP from ATP.

(7) cAMP switches on the protein kinase cascade resulting in the activation of triglyceride lipase.

(8) Lipase releases fatty acids and glycerol when triglycerides in intracellular neutral fat droplets are hydrolyzed.

(9) Fatty acids perform two functions: they are the respiratory substrates and the secondary cytosolic messengers of the hormonal signal. As substrates, fatty acids undergo the usual catabolic treatment. First, they are activated to form fatty acyl-CoA in the outer mitochondrial membrane. Then fatty acyls are transported into the mitochondrial matrix via the carnitine system,

and finally they yield reducing equivalents and CO_2 by means of the β-oxidation enzymes.

(10) Reducing equivalents supplied to the respiratory chain are transferred to O_2, the process being coupled to $\Delta\bar{\mu}_H^+$ generation. As a result, H^+ ions are exported from the matrix space.

(11) H^+ ions return to the matrix moving downhill. This is mediated by the fatty acid-uncoupling protein system. This final event results in heat production.

In fact, all 11 steps described above have been proved by direct experiments. In this context, of particular significance, are observations showing that the uncoupling effect of cold exposure can be reproduced (i) by the *in vivo* injection of noradrenaline as well as by noradrenaline addition to adipocytes or to homogenate but not to isolated mitochondria, (ii) by the addition of a penetrating cAMP analog (dibutyryl-cAMP) to cells or homogenate but not to mitochondria, and (iii) by the addition of palmitate to cells, homogenate, mitochondria or UCP proteoliposomes.

Significantly, not only cooling but also the waking up of an animal after hibernation is accompanied by uncoupling in brown fat tissue (for reviews, see Lindberg, 1970; Smith and Horwitz, 1969; Nicholls and Locke, 1984; Bukowiecki, 1984; Nedergard and Cannon, 1984; Bouillaud *et al.*, 1983; Klingenberg and Winkler, 1985).

The ATP/ADP Antiporter

The brown fat studies, in spite of obvious progress in solution of the problem of the thermoregulatory uncoupling mechanism, failed to answer the question of what happens in other tissues when additional oxygen is consumed in response to cold. Even in mammals, brown fat cannot be responsible for the major portion of this additional cold-induced respiration. In birds, where the thermoregulatory uncoupling was discovered, there is no brown fat at all. Nevertheless, the thermoregulatory uncoupling in pigeon muscles was found to be fatty acid-dependent like that in brown fat (see above). This forced us to search for protein(s) that are similar, but not identical, to the brown fat UCP and can catalyze uncoupling by fatty acids in muscles and, perhaps, in some other tissues involved in the cold-induced stimulation of respiration.

At least structurally, uncoupling protein belongs to the family of mitochondrial anion carriers. Like these carriers, uncoupling protein is about a 33-kDa molecular mass, composed of three domains, each

domain containing two putative transmembrane α-helices (Klingenberg, 1990).

Especially impressive similarity was revealed between uncoupling protein and the ATP/ADP antiporter. These proteins were found to have homology in primary, secondary, and tertiary structures. They form dimers and possess a purine nucleotide-binding site (one per dimer). Moreover, they contain no special signal sequence at the N-terminus of the polypeptide chain, a feature distinguishing the ATP/ADP antiporter and uncoupling protein from the great majority of other mitochondrial proteins (for reviews, see Skulachev, 1988; Klingenberg, 1990; Klaus *et al.*, 1991; Ježek and Garlid, 1998).

In 1987, we showed that carboxyatractylate (CAtr), the most potent and specific inhibitor of the ATP/ADP antiporter, recouples rat skeletal muscle mitochondria partially uncoupled by a low concentration of palmitate (Andreyev *et al.*, 1987; see also Andreyev *et al.*, 1988, 1989). It was found that CAtr, added after $1.5 \times 10^{-5}M$ palmitate, increased $\Delta\Psi$ and lowered the respiration rate. The recoupling effect was found to be inherent in other ATP/ADP-antiporter ligands, namely ADP, atractylate, and bongkrekic acid, but in all these cases it was not as pronounced as with CAtr (Andreyev *et al.*, 1988, 1989). On the other hand, CAtr proved ineffective in inside-out submitochondrial particles, whereas bongkrekic acid showed the recoupling activity both in mitochondria and in the particles (Dedukhova *et al.*, 1991). In all cases, the antiporter inhibitors did not recouple when *p*-trifluoromethoxycarbonylcyanide phenylhydrazone (FCCP) or other artificial protonophores were used instead of fatty acids (Andreyev *et al.*, 1989). Our data were then confirmed by others on mitochondria (Schönfeld, 1990; Valcarce and Cuezva, 1991; Vianello *et al.*, 1994; Macri *et al.*, 1994; Schönfeld *et al.*, 1996), digitonin-permeabilized Ehrlich ascite tumor cells (Schönfeld *et al.*, 1996), and ATP/ADP-antiporter proteoliposomes (Tikhonova *et al.*, 1994; Brustovetsky and Klingenberg, 1994).

Recently, Polcic *et al.* (1997) reported that point mutation in the yeast ATP/ADP antiporter (R96H) not only inhibits the antiport, but also strongly lowers the uncoupling efficiency of fatty acids in mitochondria from this mutant. Similarly, Ježek *et al.* (1998) showed that deletions in the ATP/ADP antiporter genes abolish the CAtr-sensitive uncoupling by fatty acids in yeast mitochondria. (For further information concerning the ATP/ADP antiporter-mediated uncoupling by fatty acids, see Wojtczak and Wieckowski, 1999).

The Aspartate/Glutamate Antiporter and Dicarboxylate Carrier

Schönfeld (1990) reported that the degree of CAtr-induced recoupling of fatty acid-uncoupled mitochondria is the highest in heart muscle, the lowest in liver, with the kidney occupying an intermediate position. This corresponds to the relative concentrations of the ATP/ADP antiporter in the mitochondria of these tissues. Later, it was found that in liver mitochondria some other anion carriers can partially substitute for the ATP/ADP antiporter in mediating the fatty acid-induced uncoupling. Recently, in our group Bodrova et al. (1996) and Samartsev et al. (1997a,b) reported that glutamate, aspartate, and diethyl pyrocarbonate (an inhibitor of the glutamate/aspartate antiporter) can recouple, to some degree, mitochondria uncoupled by fatty acids. It was also shown (Samartsev et al., 1997b) that pH-dependent reciprocal changes are inherent in the contribution of the ATP/ADP antiporter and the aspartate/glutamate antiporter to the fatty acid-induced uncoupling in rat liver mitochondria. As one can see from Fig. 3, the recoupling effect of CAtr increases and that of glutamate (or aspartate) decreases with increase in pH within the range 7.0–7.8. The recoupling caused by the combined action of CAtr and glutamate (aspartate) was constant at those pH values, being as high as 80%. The residual 20% might be due to involvement of the dicarboxylate and, perhaps, phosphate carriers. According to Wieskowski and Wojtczak (1997), malonate caused small, but reproducible, recoupling in liver mitochondria uncoupled by myristate. In addition, myristate inhibited transport of malonate into mitochondria. They concluded that it is the dicarboxylate carrier that is responsible for these effects.

Strabergerova and Ježek (1996) reported that micromolar concentrations of N_3-laurate and N_3-palmitate induce photoinhibition of phosphate transport into mitochondria and combine with the phosphate carrier. It remains unclear whether this carrier not only interacts with fatty acids but also transports them.

UCP2, UCP3 and Plant UCP

Fleury et al. (1997) reported that there is a gene in the human genome that codes a protein designated UCP2; it has 59% amino acid identity to the human brown fat uncoupling protein (now UCP1). Both UCP1 and UCP2 exhibit the three mitochondrial carrier pro-
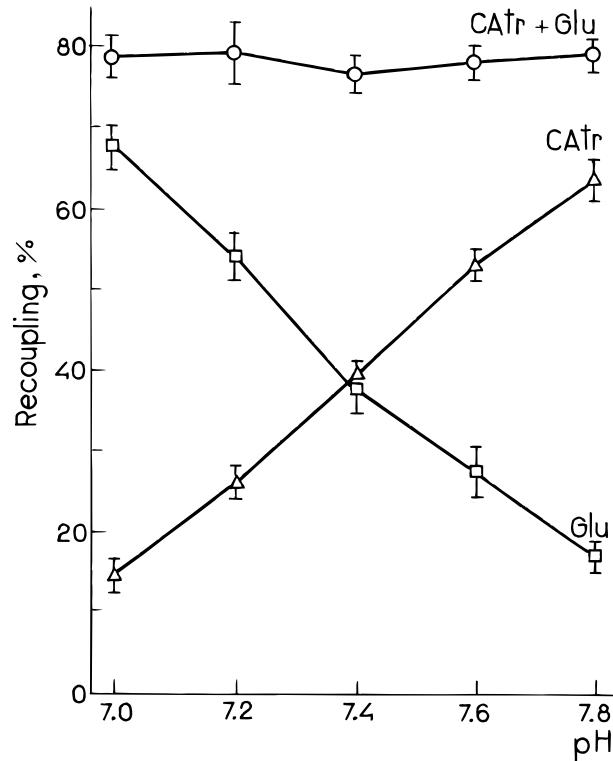

Fig. 3. Recoupling effect of CAtr, glutamate, and their combination at different pH values. Incubation mixture: 0.25 M sucrose, 5 mM potassium succinate, 2×10^{-6} M rotenone, 3 mM MgCl$_2$, 05 mM EGTA, 5 mM MOPS-KCl, oligomycin (2 μM/ml), rat liver mitochondria (1 mg protein/ml). Additions: 1×10^{-6} M CAtr and 2 mM glutamate (from Samartsev et al., 1997b).

tein motifs and the nucleotide binding site. A gene very similar to that of human UCP2 (95% identity) was also found in mouse. UCP2 maps to regions of human chromosome 11 and mouse chromosome 7 that have been linked to diabetes and obesity. In comparison with UCP1, UCP2 was found to cause stronger *in vivo* lowering of mitochondrial membrane potential when expressed in yeast and stronger inhibition of the growth of yeast. Mitochondria isolated from yeast expressing UCP2 showed a higher state 4-respiration rate and lower stimulation by FCCP or ADP. In contrast to UCP1, the UCP2 gene is widely expressed in adult human tissues (skeletal muscle, lung, heart, placenta, kidney, spleen, thymus, leucocytes, macrophages, bone marrow, and stomach). In mice, UCP2 gene expression was found in brown fat, white fat, and at a high level in heart and kidney. A low expression level was observed in brain.

In the same 1997, Giacobino and co-workers identified two more representatives of the UCP family

that are expressed specifically in brown fat and skeletal muscle tissues (Boss *et al.*, 1997a,b). The novel proteins were called $UCP3_L$ and $UCP3_S$, where L and S are for long and short, respectively. $UCP3_S$ is sequence identical to $UCP3_L$, but contains only 275 amino acids instead of the 312 found in $UCP3_L$ (amino acids at positions 276–312 are absent). $UCP3_L$ has 57 and 73% identity to UCP1 (307 amino acids) and UCP2 (309 amino acids), respectively. The identity to the most closely related mitochondrial anion carrier (the α-ketoglutarate/malate antiporter) is 32%. $UCP3_S$ contains no purine nucleotide-binding site responsible for the inhibition of fatty acid-mediated uncoupling in UCP1. UCP3 was described independently by Vidal-Puig *et al.* (1997).

Boss *et al.* (1997b) confirmed that UCP2 mRNA was expressed in various tissues: heart > brown fat > white adipose tissue > skeletal muscle. As for UCP3, its mRNA was most highly expressed in rat brown fat, at high level in Musculus tensor fascia latae (fast-twitch glycolytic muscle), M. tibialis anterior (fast-twitch oxidative-glycolytic), M. gastrocnemicus (mixed), and less in M. soleus (slow-twitch oxidative). This suggests that UCP3 is more expressed in glycolytic than in oxidative muscles. UCP3 mRNA was also detected, although at a much lower level, in rat heart and kidney. In skeletal muscles, the amount of UCP3 mRNA is much higher than that of UCP2 mRNA (Boss *et al.*, 1997b).

Recently, Fleury *et al.* (1997) postulated the existence of a UCP4, which is predominantly expressed in neural tissues. A corresponding gene was found in the X chromosome.

In plants, mRNA for one more uncoupling protein, StUCP, was found by Mueller-Roeber's laboratory in cooperation with Ricquier's group (Laloi *et al.*, 1997). This finding is in line with an earlier observation of Vercesi *et al.* (1995) of uncoupling in potato tuber mitochondria, which is sensitive to purine nucleotides, like that mediated by UCP1 (see also Saviani and Martins, 1998). They isolated a 32-kDa protein from these mitochondria and reconstituted proteoliposomes showed an increased H^+ conductance lowered by ATP and GTP by 50 and 35%, respectively. This protein was called "plant uncoupling mitochondrial protein" (PUMP). This name is somewhat ironic because of H^+ pumps present in the same organelles. As for the name stUCP (st from the Latin name for potato, *Solanum tuberosum*) suggested by Mueller-Roeber's group, it would be adequate only if it would be shown that the protein in question is specific for potato. If this is not the case, the name pUCP (p for plant) seems more appropriate.

Quite recently, Vercesi's and Jezek's groups found a gene of 81% identity to the potato UCP gene in *Arabidopsis* (Maya *et al.*, 1998) and detected immunologically plant UCP in climatic fruits [more than 10 fruits of various species were investigated (Zackova *et al.*, 1998)].

Several pieces of evidence have already been reported indicating the involvement of new members of the UCP family in thermoregulatory uncoupling.

Boss *et al.* (1997a) have shown that expression of UCP2 mRNA increased 2.5-fold in soleus muscle and brown fat and 4.3-fold in heart after 48-h cold exposure of rats to 6°C. The same cold treatment caused threefold increase in UCP1 mRNA level in brown fat. However, Fleury *et al.* (1997) failed to observe any effect of cold on UCP2 mRNA in mice exposed for a longer period (10 days) to 4°C. This is hardly surprising if it is taken into account that long-term cold acclimation mobilizes thermoregulatory mechanisms other than uncoupling, as was shown by our group as early as 1960 with pigeons (see above, Fig. 2) and later with mice (Skulachev *et al.*, 1963). Direct confirmation of this statement was quite recently reported by Lin *et al.* (1998). It was found (Fig. 4) that the UCP3 mRNA level in skeletal muscle of rats exposed to 5°C increases threefold between 6 and 24 h of cold exposure and then decreases to 50% of the control value after 6 days in the cold. As to UCP2 mRNA, it almost doubles on day 3 and returns to normal after 6 days. These dynamics of UCP2 and UCP3 explain why Boss *et al.* (1997a) failed to observe any significant changes in muscle UCP3 mRNA after 2 days of cold exposure.

Figure 4 also shows the mRNA level of glucose transporter isoform 4. This transporter is localized in the membrane of special intracellular vesicles, which fuse with the outer cell membrane in an insulin-dependent fashion. Expression of the glucose transporter gene is known to be stimulated by muscle contraction (Ren *et al.*, 1994). It is seen that the glucose transporter mRNA level is augmented already at 3 h and then gradually lowers during further cold treatment. Lin and co-authors (1998) suggested that the glucose transporter response is due to switching on of shivering thermogenesis, the first line of thermoregulatory defense in muscle, whereas expression of UCP3 is related to thermoregulatory uncoupling, i.e., the next line of defense. In regard to UCP2, its amount in skeletal muscle is much less than that of UCP3. The
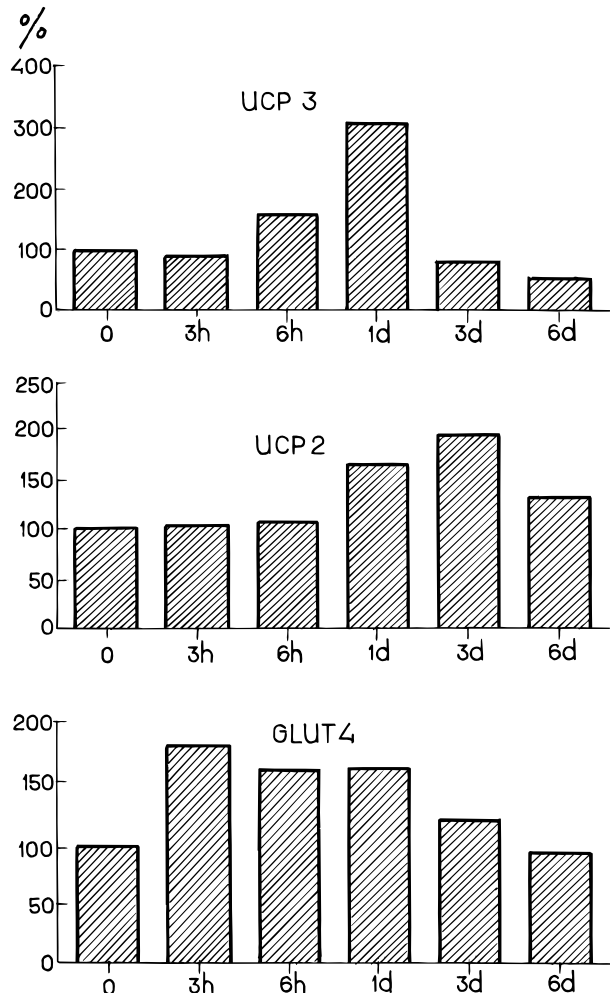
Fig. 4. Effect of cold exposure (5°C) on UCP3, UCP2 and glucose transporter-4 (GLU4) mRNA levels in rat skeletal muscle (from Lin *et al.*, 1998)

relative contribution of UCP2 to the total uncoupling activity of mitochondrial proteins should be modest. Nevertheless, UCP2 tries, to some degree, to support uncoupling when the UCP3 level decreases (Fig. 5). In this context, it is noteworthy that UCP2 differs from UCP3 in a regulatory aspect. UCP2, like UCP1, contains the nucleotide-binding sequence responsible for inhibition of the uncoupling activity by GDP, whereas one of two forms of UCP3, namely UCP3$_S$, does not. Thus, UCP3$_S$ seems to provide more potent uncoupling mechanism than UCP2 or UCP3$_L$ possessing the nucleotide-binding sequence (Boss *et al.*, 1997a).

An impressive picture of regulation of uncoupling proteins in brown fat was revealed by Villarroya and

his colleagues (Carmona *et al.*, 1998). Brown fat is unique tissue where all three UCPs can be expressed. It was found, however, that the UCP2 gene is the only one which is expressed in brown fat in prenatal development of mice. UCP3 mRNA appears suddenly at birth and reaches the adult level in a few hours. UCP2 mRNA also increases after birth but more slowly than UCP3 and UCP1 mRNA. Five-hour exposure of adult animals to 4°C was shown to increase mRNA levels for UCP1 and UCP2, but not for UCP3, this fact indicating that thermoregulatory responses of UCPs in muscles and brown fat are different.

According to Boyer *et al.* (1998), hibernation results in increases in the mRNA levels for UCP2 in white fat (1.6-fold) and UCP3 in skeletal muscle (3-fold).

Laloi *et al.* (1997) found that plant UCP mRNA level strongly increases when plants are exposed to cold and decreases when the cooling ceases (intact potato plants and potato tubers were studied). A similar effect was observed by Maia *et al.* (1998) in all tissues of *Arabidopsis*.

There is a principal difficulty in any study when one first treats an organism in some way, then isolates mitochondria, and tries to follow *in vitro* certain *in vivo* changes in energy coupling. The problem is how to discriminate between, say, (1) *in vivo* uncoupling and (2) increased sensitivity of mitochondria to an *in vitro* damage. Such a dilemma does not exist if we deal with a cold-induced increase in the mRNA levels of UCPs. This is why discovery of the cold-induced muscle UCPs can be regarded as the final proof of the thermoregulatory uncoupling first described on isolated mitochondria (see above). Respectively, similar findings in plants confirmed original publications of Voynikov's group concerning uncoupling and rise of the fatty acid level in winter cereals exposed for 1 hr to 4°C (Voynikov *et al.*, 1981; Pobezhimova, 1997).

Certainly, for UCPs other than UCP1, a lot of work should be done to finalize conclusions concerning their uncoupling activity. Among the most important problems to be solved, is direct measurement of UCP2 and UCP3 protein concentrations in mitochondria of various tissues; isolation of these proteins and their reconstitution in proteoliposomes; elucidation of the role of fatty acids for uncoupling by purified UCP2 and UCP3 [according to a quite recent report of K. Garlid, fatty acids are absolutely necessary for uncoupling by these proteins in proteoliposomes, purine nucleotides being inhibitory (Garlid *et al.*, 1999)].
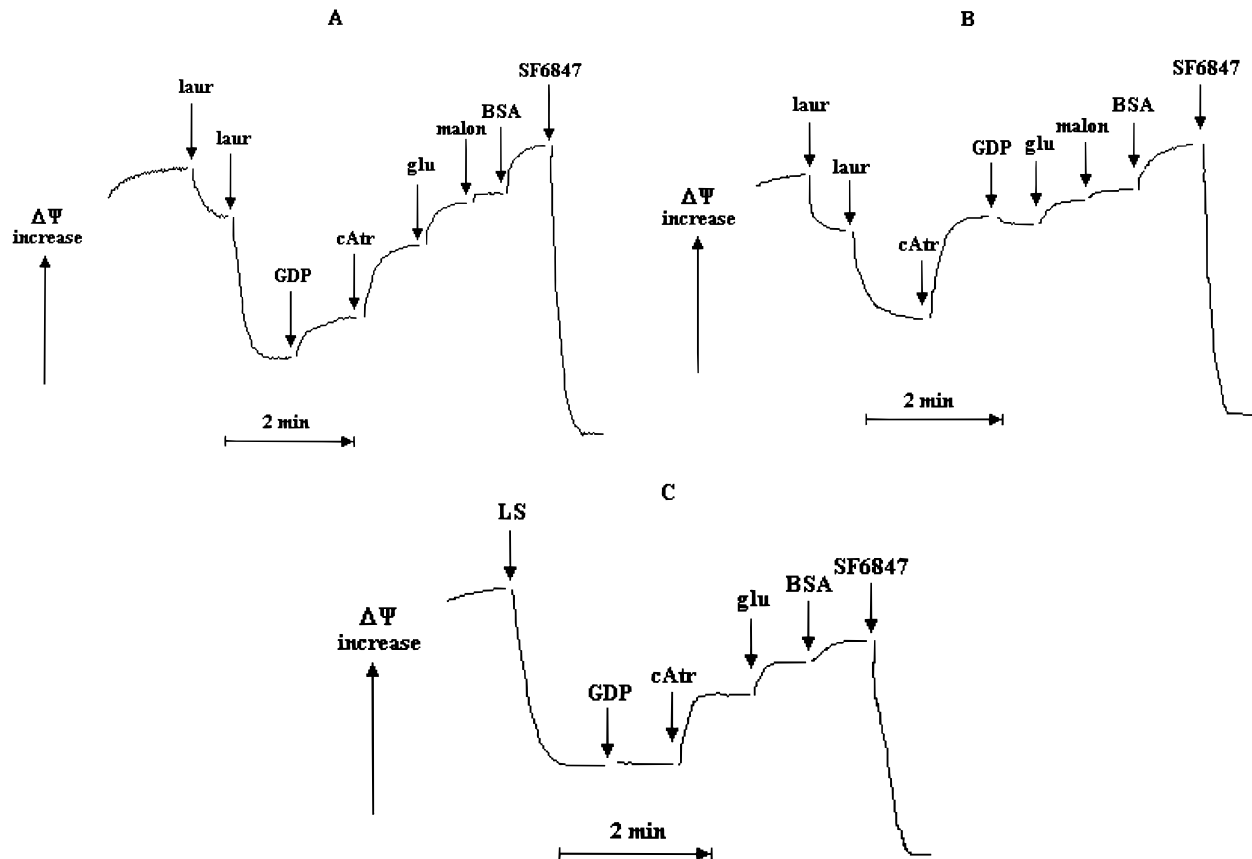
Fig. 5. Comparison of the laurate and lauryl sulfate uncouplings in rat skeletal muscle mitochondria. Incubation mixture: 250 mM sucrose, 10 mM MOPS (pH 7.4), 2 mM EGTA, 0.12 mM TMPD, 5 mM ascorbate, 2 μM rotenone, oligomycin (3 mg/ml), 8 μM safranine O, 3 mM potassium phosphate, BSA (2 mg/ml), rat skeletal muscle mitochondria (1 mg protein/ml). Additions: laur, 20 μM laurate; LS, 200 μM lauryl sulfate; 200 μM GDP; 2 μM CAtr; glu, 7 mM glutamate; malon, 2 mM malonate; BSA, bovine serum albumin (2 mg/ml); 60 nM SF6847.

Nevertheless, the total amount of already available information seems to allow one to conclude that UCPs other than UCP1 take part in thermoregulation.

Recently, Dr. R.A. Simonyan in my group performed experiments on heart mitochondria from rats treated exactly as recommended by Boss *et al.* (1997a), i.e., 48 h at 6°C. According to Boss *et al.*, this treatment resulted in more than fourfold increase in the heart muscle UCP2 mRNA level. It was found that such mitochondria are (1) partially uncoupled, (2) more sensitive to uncoupling action low concentrations of added fatty acids, and (3) acquire the ability to respond by recoupling to added GDP. All these observations are consistent with the assumption that the UCP2 level is increased by cold and that this UCP uncouples in a fatty acid-dependent fashion (Simonyan and Skulachev, 1998).

PHYSIOLOGICAL UNCOUPLING UNRELATED TO THERMOREGULATION

Heat production is not the only physiological function facilitated by uncoupling. In some other cases, restriction of respiration by ADP availability creates problems that may be solved if respiration is uncoupled. The cases in question include production of useful substances, removal of unwanted substances, and even ATP formation at the maximal rate (Skulachev, 1962, 1998). For instance, conversion of such a highly reduced substrate as lipids to more oxidized carbohydrates requires many reducing equivalents to be consumed by the respiratory chain. Coupled ATP synthesis can, in principle, limit the overall process, and this limitation can be overcome by means of uncoupling.

Removal of lactic acid, which accumulates in muscle due to hard work, is carried out via its oxidation during rest. Large amounts of oxygen are consumed for this purpose under conditions when muscle stops working. Again, just as in the previous case, some uncoupling looks favorable to avoid deceleration of the lactate oxidation because of lack of ADP.

Partial uncoupling can stimulate the rate of ATP synthesis if the resulting increase in the overall rate of electron flow and of coupled ATP synthesis appears to be higher than the decrease in ATP synthesis rate due to lowering of the thermodynamic efficiency of respiratory phosphorylation (Skulachev, 1962; Stucki, 1980 a,b).

Hard Muscle Work

Some observations were published showing that partial uncoupling occurs in liver and muscle mitochondria isolated from animals performing stenuous muscle work (Davies *et al.*, 1982; Klug *et al.*, 1984). In liver, fatty acids were found to mediate uncoupling (Klug *et al.*, 1984). Such an effect cannot be UCP-linked since UCP1 and UCP3 are absent in liver and UCP2 is present in fetal liver cells only (Hondy *et al.*, 1998) As to the adult liver, UCP2 was found in Kupffer cells but absent in hepatocytes (Larrouy *et al.*, 1997). This means that fatty acids can uncouple in liver via carriers others than UCPs, i.e., the ATP/ADP or aspartate/glutamate antiporters or dicarboxylate carrier. In muscle the work-induced partial uncoupling, if it occurs, seems also unrelated to UCPs. In fact, Giacobino and co-workers (Boss *et al.*, 1998a) observed that endurance training resulted in a *decrease* in the skeletal muscle UCP3 and UCP2 mRNA levels (by factors 4 and 2, respectively). They explained this effect assuming that lowering of the UCP levels diminishes respiration energy dissipation and, hence, should be favorable for muscle work.

Lactate Oxidation

One more complication of energy coupling control in muscle mitochondria may be due to the oxygen debt problem, i.e., necessity to oxidize excess lactate accumulated during hard muscular work. As was already mentioned, here some uncoupling may be required.

In yeast lactate is oxidized via cytochrome b_2 (housed in the mitochondrial intermembrane space) in such a way that the electron flow by-passes two of three respiratory chain energy coupling sires (Appleby and Morton, 1959; Kotel'nikova and Zvyagil'skaya, 1973). In *Mycobacterium phlei*, lactate oxidase is non-coupled (Brodie, 1959). We observed that efficiency of oxidative phosphorylation in muscle mitochondria is lower with lactate than with other respiratory substrates (including pyruvate) (Skulachev, 1962). The mechanistic reasons for such an effect still remain obscure. This was not a result of pyruvate cycling recently postulated by Jezek and Borecky (1998). They measured/State-4 respiration and $\Delta\Psi$ in brown fat mitochondria oxidizing α-glycerophosphate in the presence of rotenone. It was found that addition of pyruvate caused some uncoupling, which was abolished by the pyruvate, H^+-symporter inhibitor α-cyano-4-hydroxycinnamate. They speculated that pyruvate anion enters mitochondria together with H^+ via the symporter and goes back without H^+ via UCP1, which is known to facilitate translocation of many monanions including pyruvate (Jezek and Garlid, 1990). In muscle, the same mechanism might be involved in partial uncoupling of the lactate oxidation assuming that UCP3 and UCP2, like UCP1, are competent in uniport of the lactate dehydrogenase-formed pyruvate. However, we failed to observe any pyruvate-induced $\Delta\Psi$ decrease in skeletal muscle mitochondria oxidizing TMPD and ascorbate (R.A. Simonyan and V.P. Skulachev, unpublished). Apparently there are also some other reasons for low-energy coupling of lactate oxidation.

Fasting

According to Giacobino and co-workers, 1- to 2-day fasting stimulates expression of the UCP2 and UCP3 genes in muscles by factors 2.5–3.5 and 5.5, respectively (Boss *et al.*, 1997a, 1998b; Samec *et al.*, 1998). Refeeding resulted in a decrease in the UCP2 and UCP3 mRNA contents below the control level, which remain low until day 10. Fasting lowers the total metabolic rate. Because heat production due to aerobic–catabolic processes decreases, the maintenance of normal thermogenesis might probably result in stimulation of UCP3 and UCP2 syntheses (Boss *et al.*, 1998b). In this context, a fasting-induced increase in circulation of fatty acid is noteworthy (Guezennec *et al.*, 1988; Ohno *et al.*, 1990).

Interestingly, 50% food restriction caused an effect opposite to fasting, i.e., strong lowering of the UCP3 mRNA level (Boss *et al.*, 1998b), an effect that might be accounted for by the necessity of more effective food consumption.

Obesity

Inherited obesity was reported to be accompanied by an elevated expression of UCP2 (Gimeno *et al.*, 1997; Simoneau *et al.*, 1998) [see, however, (Emilsson, 1998)]. A positive correlation between the level of circulating fatty acids and the UCP3 mRNA was revealed when human obesity was studied (Boss *et al.*, 1998c). Fat diet was shown to stimulate UCP3 and UCP2 mRNA syntheses in skeletal muscle and white fat (Matsuda *et al.*, 1997; Surwit *et al.*, 1998).

Zhou *et al.* (1997) found that overexpression of the adipocyte-produced protein hormone leptin gives rise to enhancement of UCP2 mRNA in pancreatic islets (Six-fold) and in white fat (more than tenfold).

Augmentation (1.8–2.7-fold) of skeletal muscle UCP2, $UCP3_L$, and $UCP3_S$ mRNA levels in type 2 diabetes was observed by Bao *et al.* (1998). In the same paper, a good correspondence of (1) percentage of body fat and (2) UCP2 and $UCP3_S$ mRNA levels was found. $UCP3_L$ mRNA tended to increase as a function of obesity.

Thus, UCP2-and UCP3-mediated uncoupling seems to be involved in oxidation of excess food, especially fat, just as was previously shown for UCP1 (for reviews, see Boss *et al.*, 1998d; Skulachev, 1998).

Antioxidant Defense

A very steep dependence of $O_2^{-\cdot}$ formation by mitochondria upon the $\Delta\mu_H^-$ level has been revealed (Liu and Huang, 1996; Liu, 1997; Korshunov *et al.*, 1997). In the Q cycle, such an effect can be explained by the $\Delta\bar{\mu}_H^+$-linked inhibition of $CoQ^{-\cdot}$ oxidation (Skulachev, 1996). Apparently, a similar mechanism is inherent in the energy-coupled NADPH-CoQ reductase (Korshunov *et al.*, 1998; Skulachev, 1998). The threshold $\Delta\bar{\mu}_H^+$ value above which strong stimulation of $O_2^{-\cdot}$ formation occurs was shown to be slightly higher than that in state 3 (Korshunov *et al.*, 1997).

In our group it was postulated that there is a special mechanism called "mild uncoupling," which prevents an increase in $\Delta\bar{\mu}_H^+$ and, hence, in the $O_2^{-\cdot}$ generation, when ADP is exhausted (Skulachev, 1994,

1996). Fatty acids seem to be good candidates for the role of mild uncouplers. In rat heart mitochondria, very low concentrations of fatty acids were found to abolish the $O_2^{-\cdot}$ production at the state 3 → state 4 transition. The effect of fatty acids was almost completely abolished by CAtr and partially by GDP (Korshunov *et al.*, 1998).

Such relationships, on the face of it, suggest that it is the ATP/ADP antiporter that is mainly responsible for mild uncoupling whereas UCP2, also present in heart muscle, plays only a minor role. However, a quite recent observation of Simonyan in this group (see below, Fig. 5) seems to indicate that $UCP3_L$, in contrast to UCP1 is CAtr-sensitive. As for UCP2, it remains unclear whether it is inhibited by CAtr.

Recently, Casteilla and co-workers reported that GDP increases $\Delta\Psi$ and H_2O_2 production by mitochondria from brown fat, spleen, thymus, and nonparenchymal liver cells expressing UCP2. GDP was completely ineffective in parenchymal hepatocytes deprived of UCP2 (Negre-Salvayre *et al.*, 1997).

At the hormonal level, mild uncoupling was assumed to be controlled by thyroid hormones (Skulachev, 1994, 1996, 1998) that were recently found to induce synthesis of mRNA for UCP2 (Lanni *et al.*, 1997; Masaki *et al.*, 1997) and UCP3 (Gong *et al.*, 1997; Larkin *et al.*, 1997).

In hepatocytes, where there are no proteins of the UCP family, thyroid hormones were found to also decrease energy coupling. In our group, it was recently shown using intact hepatocytes that energy coupling decreases in the series hypothyroid > euthyroid > hyperthyroid (Bobyleva *et al.*, 1998). This effect may be due to thyroid hormone-induced increase in the content of the ATP/ADP antiporter in liver mitochondria (Luciakova and Nelson, 1992; Lunardi *et al.*, 1992; Bobyleva *et al.*, 1997; Schönfeld *et al.*, 1997).

In adult liver, the ATP/ADP antiporter isoform 2 is present. However, isoform 1 (which in adults was found in muscles) is expressed in hepatocytes around birth and disappears in the second week of postnatal life (Grado *et al.*, 1998; Florian and Schönfeld, 1998). Possible relationships between the isoform dynamics and their ability to uncouple remain obscure.

MECHANISM OF UNCOUPLING BY FATTY ACIDS

UCPs and Mitochondrial Anion Carriers Transport Fatty Acid Anions

Noncharged (protonated) forms of fatty acids (RCOOH) are shown to cross the phospholipid mem-

brane on the millisecond time scale (Kamp *et al.*, 1992) whereas their anionic forms ($RCOO^-$) require minutes to do this (Kamp and Hamilton, 1992). This is why fatty acids do not show protonophorous activity in phospholipid bilayers (for review, see Skulachev, 1998). On the other hand, they acquire such activity if some proteins are inlaid in the bilayer. The proteins in question are mitochondrial anion carriers, namely the ATP/ADP antiporter, the aspartate/glutamate antiporter, and the dicarboxylate carrier, as well as UCPs that belong to the anion carrier family not only for structural reasons, but also because of their ability to translocate Cl^-, pyruvate, and other monoanions (for reviews, see Skulachev, 1988, 1991).

The above reasoning forced me, in 1988, to postulate that the role of UCP and the ATP/ADP antiporter in fatty acid-mediated uncoupling consists in that these proteins facilitate translocation of fatty acid anions thus completing the protonophorous fatty acid cycle (Skulachev, 1988, 1991). In particular, it was assumed that fatty acids interact with the anion translocation machinery (intramembrane cationic amino acid residues) of the ATP/ADP antiporter without the ATP(ADP)-specific hydrophilic gate being involved. In regard to UCPs, they were considered as ATP/ADP antiporter derivatives, which (1) translocate fatty acid anions, and (2) bind nucleotides on one membrane side but cannot release them on the opposite membrane side. In other words, in UCPs the main (nucleotide transporting) function of the antiporter is lost, whereas the additional (fatty acid transporting) function is retained. During the last decade, several pieces of evidence were reported supporting this hypothesis.

(1) Not only the ATP/ADP antiporter but also two other mitochondrial anion carriers (the aspartate/glutamate antiporter and the dicarboxylate carrier) were found to be involved in fatty acid uncoupling (Samartsev *et al.*, 1997a; Wieskowski and Wojtczak, 1997).

(2) Point mutation of the yeast mitochondrial ATP/ADP antiporter (an intramembrane cationic amino acid residue, R96, is replaced by H) strongly inhibits the uncoupling activity of fatty acids (Polcic *et al.*, 1997).

(3) In permeabilized ascite cells, N_3-laurate was found to uncouple in the dark but not after illumination. Moreover, illumination of the N_3-laurate-treated cells prevented uncoupling by myristate and simultaneously inhibited the ATP/ADP antiport (Schönfeld *et al.*, 1996).

(4) It was shown that only those fatty acid derivatives that easily penetrate a phospholipid bilayer in their protonated forms can uncouple at low concentrations (Wojtczak *et al.*, 1998).

(5) Relationships as in (4) are inherent in UCP1 (Jezek, *et al.*, 1997a,b; Garlid *et al.*, 1998).

(6) An azido derivative of a fatty acid specifically inactivates UCP1 when combined stoichiometrically with the UCP1 dimer. Stearate and alkyl sulfonates prevent this UCP1 labeling (Ruzicka *et al.*, 1996).

(7) Undecanesulfonate anion is shown to be transported by UCP1 with K_m very similar to the K_m from laurate when laurate-mediated H^+ transport was measured. Undecanesulfonate is a competitive inhibitor of the laurate-induced H^+ transport and both undecanesulfonate and laurate are competitive inhibitors of Cl^- transport via UCP1. On the other hand, undecanesulfonate, in contrast to its close analog laurate, cannot facilitate H^+ transport by UCP1 since it is a very strong acid (Garlid *et al.*, 1998).

Mitochondrial Anion Carriers, But Not UCPs, Protonate Lauryl Sulfate

In our group, uncoupling effects of laurate and lauryl sulfate were recently compared (Samartsev *et al.*, 1999). It was found that in rat liver and skeletal muscle mitochondria, lauryl sulfate (other name, dodecyl sulfate) uncouples in a CAtr- and glutamate (aspartate)-sensitive fashion. Lauryl sulfate, when added at the same concentration as laurate (tens of micromoles per liter), did not affect mitochondrial energy coupling (see also Wojtczak *et al.*, 1998), but when the concentration of lauryl sulfate was increased by factors 5–10, an uncoupling took place, which was almost completely reversed by combined addition of CAtr and glutamate or aspartate (Samartsev *et al.*, 1999). The uncoupling by lauryl sulfate, like that by laurate, was accompanied by a CAtr-sensitive increase in the H^+ conductance of mitochondria (Brustovetsky *et al.*, 1990).

In liver mitochondria, other qualitative characteristics of uncoupling by laurate and lauryl sulfate proved to be identical, pointing to similarity of the molecular mechanisms of their effects. On the other hand, in skeletal muscle mitochondria one feature of the laurate action was found to be absent in the case of lauryl sulfate—recoupling by GDP. As one can see in Fig. 5, addition of GDP to mitochondria uncoupled by laurate caused an increase in $\Delta\Psi$. GDP was absolutely ineffective when lauryl sulfate was employed as an uncoupler. The GDP recoupling with laurate was not observed in the presence of CAtr.

It was suggested (Skulachev, 1998) that cationic groups (e.g., lysine or arginine residues) of mitochondrial anion carriers are involved in binding of transported anions (A^{n-}) on one membrane side, whereas protonation of A^{n-} facilitates release of A^{n-} to the water phase on the other membrane side. Apparently, the mechanism responsible for A^{n-} protonation is also used for protonation of lauryl sulfate translocated by anion carriers from the inner to the outer surface of the mitochondrial membrane. If this is the case, the protonated lauryl sulfate, which is released from the anion carrier protein not far from the outer surface of the mitochondrial membrane, has two options: to go (1) to the nearest (outer) water phase or (2) to the opposite membrane side. The majority of the protonated lauryl sulfate molecules uses option (1) as more probable, whereas the minority uses option (2). This is why lauryl sulfate appears to be a much less efficient uncoupler than laurate. The uncoupling efficiency of lauryl sulfate would be even lower if its concentration near the outer membrane were equal to that near the inner one. However, this is not the case. Lauryl sulfate accumulates in the outer membrane leaflet due to electrophoretic efflux of its anionic form, mediated by the anion carriers. These relationships are illustrated by Fig. 6.

The postulated protonation mechanism seems to be essential for transport of substances containing more than one anionic group, such as ATP^{4-}, ADP^{3-}, dicarboxylic amino acids, etc. On the other hand, it may be not necessary for uncoupling proteins specialized in translocation of the fatty acid monoanions, since decomposition of a complex of a monoanion with a cationic group of a UCP should be much easier than decomposition of a complex of, say, ATP^{4-} with four cationic groups of the ATP/ADP antiporter. As was already mentioned, the brown fat UCP1 still binds purine nucleotides, transports them to some depth in the membrane, but cannot release the nucleotides on the opposite membrane side (probably because the protonation mechanism was lost when the ATP/ADP antiporter evolved to UCP). UCP1 seems to catalyze the fatty acid circuit, being involved in the fatty acid anion translocation. As for the fatty acid anion protonation, it may occur spontaneously on the membrane surface due to the strong alkaline shift of pK_a of fatty acids in the membrane–water interface (Sankaram *et al.*, 1990). Such an assumption explains why fatty acids enhance, while lauryl sulfate and other alkyl sulfates and sulfonates do not, the H^+ conductance in UCP1 proteoliposomes. In fact, alkyl sulfate and

sulfonate anions are transported by UCP1 and interfere with fatty acid-linked H^+ conductance. Dicarboxylates and other di- and polyanions are not transported by UCP1 (Garlid, 1990; Jezek and Garlid, 1990). On the contrary, the ATP/ADP antiporter was shown in our group to mediate uncoupling by dicarboxylic fatty acids in a CAtr-sensitive manner (Bondarenko *et al.*, 1994; see also Hermesh *et al.*, 1998).

Returning to Fig. 5, it is noteworthy that the GDP recoupling in the sample with laurate is seen only if GDP is added before CAtr. If the CAtr addition is the first one, (1) the recoupling effect of CAtr is larger than in the sample already treated with GDP, and (2) GDP added after CAtr is ineffective. The simplest explanation of such relationship consists in that CAtr inhibits the fatty acid uncoupling mediated by UCP3 like that by the ATP/ADP antiporter and in contrast to that by UCP1. UCP3 is the major representative of the UCP family in skeletal muscle (Boss *et al.*, 1998c). It seems probable that UCP3 is responsible for uncoupling by laurate (not by lauryl sulfate) sensitive to GDP and CAtr, whereas the ATP/ADP and aspartate/glutamate antiporters are involved in both laurate and lauryl sulfate uncoupling.

"Stationary" Hypothesis of Fatty Acid Uncoupling

A hypothesis was put forward assuming that fatty acids operate in a "stationary" fashion, either by changing the protein conformation or, when anchored within the protein hydrophobic core, by facilitating H^+ trafficking (Winkler and Klingenberg, 1994). This scheme cannot explain why UCPs and other proteins involved in fatty acid uncoupling are *anion* carriers. Moreover, it contradicts facts summarized in items (1–7), p.

As an argument in favor of their scheme, Klingenberg and co-workers (Bienengraeber *et al.*, 1998) mentioned results of their experiments on the double mutant UCP1 (H145Q and H147N) expressed in yeast. The mutant protein was found to still catalyze Cl^- transport in the GDP-sensitive manner, but H^+ transport was strongly decreased. H145 and H147 are localized in the hydrophilic link between α-helical columns III and IV, on the matrix surface of the protein.

However, according to Garlid *et al.* (1998), this finding is fully consistent with the fatty acid cycle model. They suggested that the histidines in question take part in organization of the fatty acid anion-binding site on the water–membrane interface near the matrix
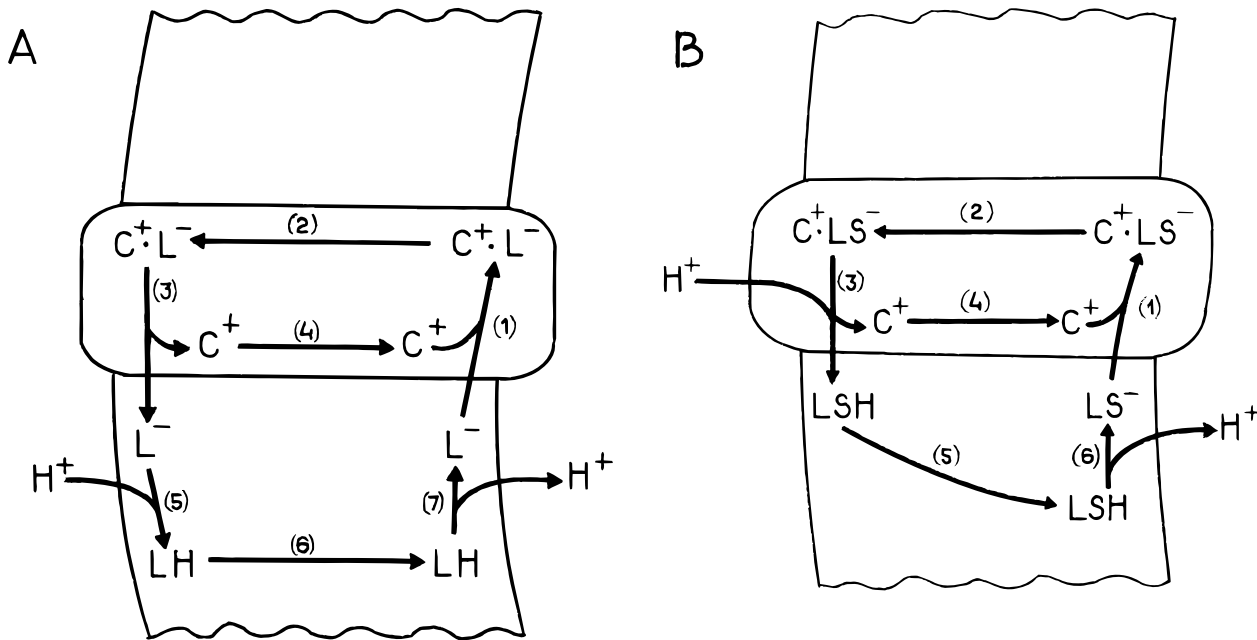
Fig. 6. Mechanisms of uncoupling by laurate (A) and lauryl sulfate (B) mediated by UCPs and the ATP/ADP or aspartate/glutamate antiporter, respectively. (A) Scheme for laurate and a UCP. (1) Binding of laurate anion (L^-) to a cationic amino acid residue of a UCP in the inner membrane leaflet; (2) translocation of the electroneutral complex C^+L^- to the outer membrane leaflet; (3) dissociation of the complex; (4) electrophoretic translocation of C^+ to its initial position; (5) protonation of the laurate anion by an extramitochondrial proton; (6) flip-flop of the protonated laurate from the outer to the inner membrane leaflet; (7) dissociation of the protonated laurate resulting in release of H^+ to the mitochondrial matrix. (B) Similar scheme for lauryl sulfate (LS^-) and the ATP/ADP or aspartate/glutamate antiporter. In contrast to a UCP, decomposition of the C^+LS^- complex is facilitated by protonation of bound LS^- (step 3). Released LSH goes mainly to the outer membrane surface (not shown since this process does not take part in the uncoupling). However, some amount of the LSH formed inside the membrane moves to the inner membrane surface (step 5). Please note, that, according to the scheme, UCPs do not possess the protonation mechanism and, hence, cannot organize protonophorous uncoupling by lauryl sulfate, although they can transport the lauryl sulfate anion. On the other hand, the antiporters can facilitate uncoupling by both laurate and lauryl sulfate.

surface of the UCP protein. Garlid *et al.* (1998) predicted that the histidines will be required for transport of long-chain alkyl sulfonates, but not for short-chain sulfonates, because the latter do not use the surface binding site.

Gonzalez-Borraso *et al.* (1998) suggested that low concentrations of fatty acids are operating in a "stationary" regime in UCP, whereas fatty acid cycling by anion carriers requires three orders of magnitude higher fatty acid level. The data of Gonzalez-Barroso's paper were carefully analyzed by Garlid and co-workers (1998). It was concluded that the 3 order of magnitude difference in the fatty acid sensitivities between UCPs and anion carriers is a result of a mistake in calculation. Apparently, UCP1 is really more sensitive to fatty acids than the ATP/ADP and aspartate/glutamate antiporters but the difference is no more than several times (Rafael *et al.*, 1969). This difference may explain recent observations by Matthias *et al.* (1998) on UCP1 knock-out mice. They concluded that UCP1 is responsible for endogenous GDP-sensitive uncoupling in brown fat mitochondria (caused apparently by low level of endogenous fatty acids), but is not necessary for uncoupling by added fatty acids (that can operate via UCP2, UCP3, the ATP/ADP and aspartate/glutamate antiporters and the dicarboxylate carrier still present in the knock-out mice).

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