

# Thermoregulatory uncoupling in heart muscle mitochondria: involvement of the ATP/ADP antiporter and uncoupling protein

Ruben A. Simonyan, Vladimir P. Skulachev\*

*Department of Bioenergetics, A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119899, Russia*

Received 19 August 1998

**Abstract** Possible involvement of the ATP/ADP antiporter and uncoupling protein (UCP) in thermoregulatory uncoupling of oxidative phosphorylation in heart muscle has been studied. To this end, effects of carboxyatractylate (cAtr) and GDP, specific inhibitors of the antiporter and UCP, on the membrane potential of the oligomycin-treated mitochondria from cold-exposed (6°C, 48 h) and control rats have been measured. It is found that cAtr increases the membrane potential level in both cold-exposed and non-exposed groups, the effect being strongly enhanced by cooling. As for GDP, it is effective only in mitochondria from the cold-exposed rats. In these mitochondria, the coupling effect of GDP is smaller than that of cAtr. CDP, which does not interact with UCP, is without any influence on membrane potential. The cold exposure is found to increase the uncoupling efficiency of added natural (palmitate) or artificial (SF6847) uncouplers, the increase being cAtr- and GDP-sensitive in the case of palmitate. The fatty acid-free bovine serum albumin enhances  $\Delta\Psi$  in both cold-exposed and control groups, the effect being much larger in the former case. It is concluded that in heart muscle mitochondria the ATP/ADP antiporter is responsible for the ‘mild uncoupling’ under normal conditions and for major portion of the thermoregulatory uncoupling in the cold whereas the rest of thermoregulatory uncoupling is served by UCP (presumably by UCP2 since the UCP2 mRNA level is shown to strongly increase in rat heart muscle under the cold exposure conditions used).

© 1998 Federation of European Biochemical Societies.

**Key words:** Thermoregulatory uncoupling; Heart mitochondria; ATP/ADP antiporter; Uncoupling protein

## 1. Introduction

In 1960 our group found that a short-term cold exposure of pigeons adapted to cold stress resulted in considerable lowering of the P/O ratio in breast muscle mitochondria [1,2]. This phenomenon, called ‘thermoregulatory uncoupling’, was then reproduced in mice [3]. Later the same effect was described in the brown fat mitochondria [4] and, under natural conditions, in the skeletal muscle mitochondria of fur seal [5]. In the last decade, the above observations were extended to other animals and even to some plants (for review, see [6]). The study on the mechanism of thermoregulatory uncoupling carried out in our group revealed that it is mediated by free fatty acids [7]. It was assumed that the fatty acid-induced uncoupling mechanism is composed of (i) influx of the proto-

nated fatty acids to mitochondria through phospholipid regions of the inner mitochondrial membrane and (ii) efflux of the deprotonated fatty acid anions mediated by some anion carriers [6,8,9]. Carriers in question were shown to be the brown fat uncoupling protein [9,10], the ATP/ADP antiporter [11], the aspartate/glutamate antiporter [12,13] and the dicarboxylate carrier [14].

Quite recently, several types of mRNA homologous to the brown fat uncoupling protein mRNA were identified in many animal tissues as well as in plants (for reviews, see [6,10]). Boss et al. [15] reported that 48 h exposure of rats to 6°C resulted in 4.3-fold increase in the mRNA level of one of the uncoupling proteins, namely UCP2, in the heart muscle. On the other hand, Fleury et al. [16] failed to observe such an effect in mice exposed to 4°C for 10 days.

Observation of increased expression of UCP, a protein specialized in uncoupling, if it really occurs in a cold-dependent fashion, might be the final step of verification of the thermoregulatory uncoupling concept since in this case uncoupling cannot be explained by any in vitro artifacts that accompany isolation of mitochondria. Interrelations of UCP and other mitochondrial anion carriers in the thermoregulatory uncoupling is one more problem to be solved.

In this paper, we describe the effect of the 48 h cold exposure of rats on sensitivity of the heart muscle mitochondrial membrane potential to carboxyatractylate (cAtr) and GDP, specific inhibitors of the ATP/ADP antiporter and UCP, respectively. The results obtained indicate that cold exposure causes in heart a fatty acid-induced uncoupling mediated by the ATP/ADP antiporter and, to lesser degree, by UCP.

## 2. Materials and methods

The white rats (about 130 g) were exposed to 6°C for 48 h. Heart muscle mitochondria were isolated as described elsewhere [17].

Membrane potential was measured with safranin O [18]. The 550–523 nm light absorption difference was measured by an Aminco DW 2000 spectrophotometer. The ratio of dye (nmol) to mitochondrial protein (mg) was equal to 20:1. Oxygen consumption was monitored by a Clark oxygen electrode and a Rank Brothers polarograph.

The incubation mixture for the  $\Delta\Psi$  measurement contained 250 mM sucrose, 10 mM MOPS, pH 7.4, 1 mM EGTA,  $2 \times 10^{-6}$  M rotenone, oligomycin ( $3 \mu\text{g ml}^{-1}$ ), 3 mM potassium phosphate,  $8 \times 10^{-6}$  M safranin O and mitochondria (1 mg protein  $\text{ml}^{-1}$ ).

Mitochondrial protein was measured with the biuret method.

Oligomycin, rotenone and SF6847 were dissolved in double-distilled ethanol.

EGTA and rotenone were from Serva. GDP, CDP, oligomycin, MOPS and fatty acid-free bovine serum albumin were from Sigma.

## 3. Results

In Fig. 1, typical results of the  $\Delta\Psi$  kinetics in the succinate-

\*Corresponding author. Fax: +7 (95) 939 03 38 or +7 (95) 939 31 81. E-mail: skulach@head.genebee.msu.su

**Abbreviations:**  $\Delta\Psi$ , transmembrane electric potential difference; cAtr, carboxyatractylate; SF6847, 3,5-di(*tert*-butyl)-4-hydroxybenzylidene-malononitrile; TMPD, *N,N'*-tetramethyl-*p*-phenylene diamine; UCP, uncoupling protein

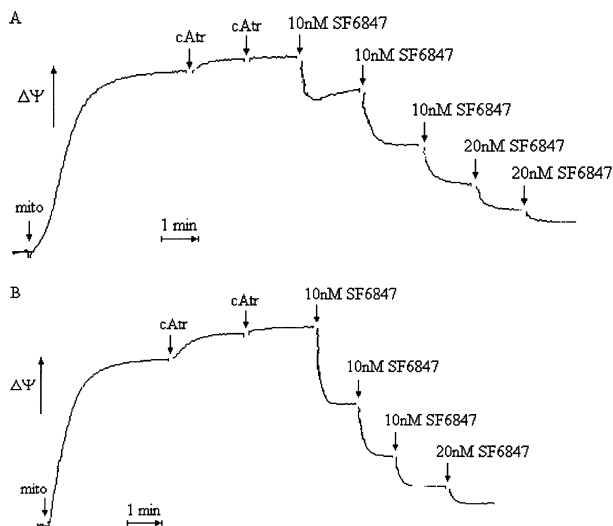


Fig. 1. The  $\Delta\Psi$  kinetics in the succinate-oxidizing heart muscle mitochondria from the control (A) and cold-exposed (B) rats. The incubation mixture was supplemented with 5 mM succinate. Additions: mitochondria (mito, 1 mg protein  $\text{ml}^{-1}$ ); cAtr,  $2 \times 10^{-6}$  M carboxyatractylate.

oxidizing heart muscle mitochondria from control (A) and cold-exposed (B) rats are shown. It is seen that in both cases cAtr causes some increases in the  $\Delta\Psi$  level, the effect being higher in the cold-exposed animals. Subsequent addition of an artificial protonophorous uncoupler, SF6847, entails a  $\Delta\Psi$  discharge. Note that the first addition of SF6847 (final concentration, 10 nM) induces two-fold stronger  $\Delta\Psi$  decrease in mitochondria from the cold-exposed rats compared with the non-exposed rats.

In Fig. 2, it is shown that bovine serum albumin increases the  $\Delta\Psi$  level both in mitochondria from the cold-exposed and

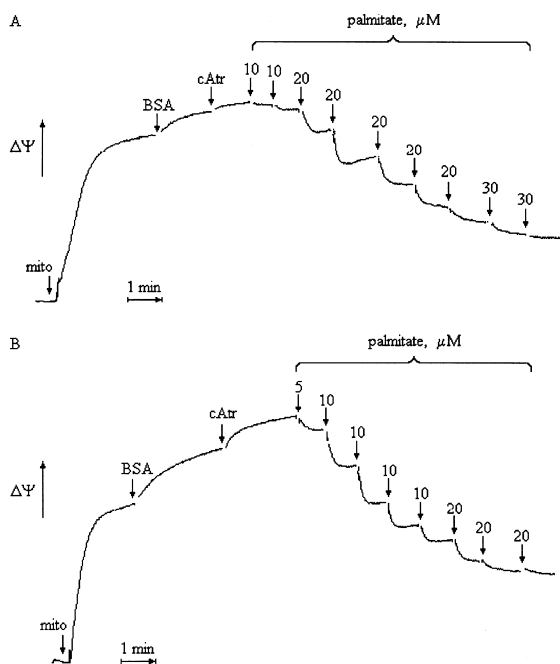


Fig. 2. Effect of bovine serum albumin (BSA), cAtr and palmitate on the  $\Delta\Psi$  level in mitochondria from the control and cold-exposed groups. For conditions, see Fig. 1. Additions: BSA (0.2 mg  $\text{ml}^{-1}$ );  $2 \times 10^{-6}$  M cAtr.

non-exposed rats. In the former case, the coupling effect appears to be larger. Palmitate added after albumin and cAtr lowers  $\Delta\Psi$ . Again, as with SF6847, low concentrations of the uncoupler are more efficient in the cold-exposed group.

In the presence of cAtr, GDP reduced the palmitate uncoupling whereas in the control group GDP was inefficient (not shown in figures).

Fig. 3 shows the malonate titration of  $\Delta\Psi$ . One can see that the cold exposure makes  $\Delta\Psi$  more sensitive to malonate. cAtr lowers the  $\Delta\Psi$  sensitivity, the effect being more pronounced in the control animals. The qualitative difference between mitochondria from control and cold-exposed rats consists in the fact that in the latter case GDP causes some decrease in the malonate sensitivity of  $\Delta\Psi$ , whereas in the former case GDP is without effect. CDP fails to replace GDP. It is also obvious that cAtr is more efficient than GDP but cannot substitute for GDP (Fig. 3B).

In Fig. 4, ascorbate (+TMPD) was used instead of succinate as oxidation substrate. It is seen that in the control group (Fig. 4A), addition of  $1 \times 10^{-5}$  M TMPD appears to be sufficient to observe the maximal  $\Delta\Psi$  level. As for cAtr, it increases  $\Delta\Psi$  only slightly. In the cold-exposed group (Fig. 4B), much larger TMPD concentrations are required, and cAtr causes pronounced  $\Delta\Psi$  increase. Effect of subsequent GDP addition is very small in contrast to that in Fig. 3 where GDP was added before mitochondria and the respiration rate was strongly lowered by malonate.

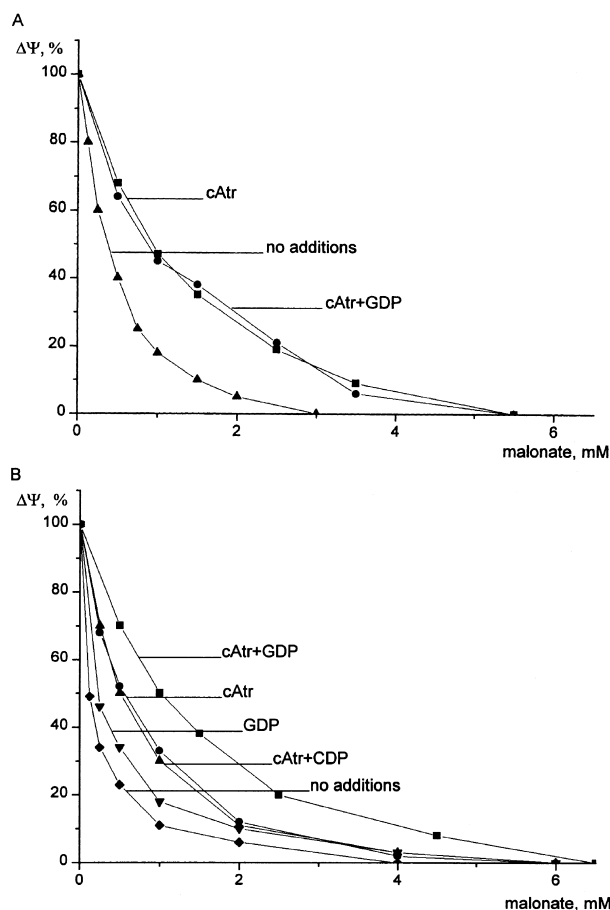


Fig. 3. The malonate titration of  $\Delta\Psi$  in the heart muscle mitochondria from the control (A) and cold-exposed (B) rats. For conditions, see Fig. 1. Additions:  $2 \times 10^{-6}$  M cAtr and 0.5 mM GDP or CDP.

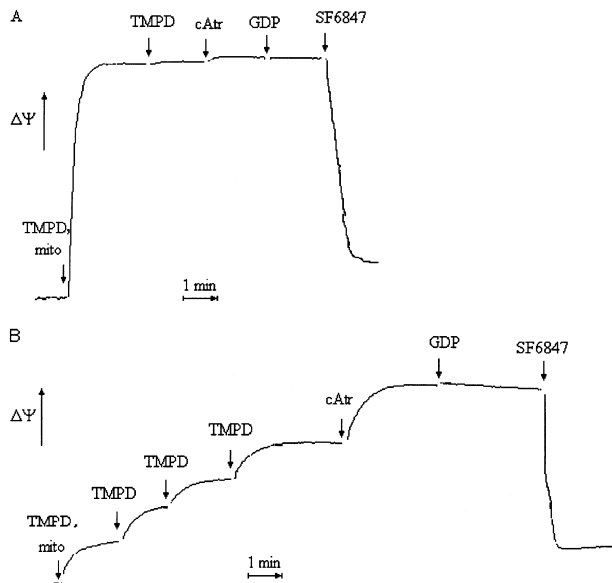


Fig. 4. The  $\Delta\Psi$  kinetics of the ascorbate-oxidizing mitochondria from the control (A) and cold-exposed (B) rats. The incubation mixture was supplemented with 5 mM ascorbate. Additions: mitochondria (mito);  $1 \times 10^{-5}$  M TMPD;  $2 \times 10^{-6}$  M cAtr; 0.5 mM GDP; 50 nM SF6847.

Measurements of the respiration rate in the presence of oligomycin showed that cAtr significantly inhibited this rate only if rats were exposed to cold (not shown in figures).

#### 4. Discussion

The data presented in this paper are consistent with assumption that the 48 h cold exposure of rats results in a decrease in the energy coupling of the heart muscle mitochondria.

(1) Recoupler cAtr inhibiting the ATP/ADP antiporter-mediated uncoupling by fatty acids causes stronger  $\Delta\Psi$  increase in the cold-exposed animals than in the control group as if the level of the ATP/ADP antiporter and/or endogenous fatty acids were increased by cooling (Figs. 1, 3 and 4).

(2) Low concentrations of both artificial (SF6847) and natural (palmitate) uncouplers when added to mitochondria from the cold-exposed group induce stronger  $\Delta\Psi$  lowering than the same concentrations added to mitochondria from the control animals (Figs. 1 and 2). Just this result could be expected assuming that level of endogenous uncoupler(s) is increased due to cold exposure.

(3) Decrease in the succinate oxidation rate by increasing malonate concentrations reduces  $\Delta\Psi$  stronger in the cold-treated rats (Fig. 3). Such an effect like the effect (2) can be easily accounted for suggesting that cooling results in an increase in the endogenous uncoupler(s) level.

(4) Larger [TMPD] and, hence, higher rate of the ascorbate oxidation are required to maintain maximal  $\Delta\Psi$  level in the cold-exposed group compared with the control one (Fig. 4).

(5) Cold exposure causes appearance of the recoupling action of a UCP inhibitor, GDP (Fig. 3). This observation is in line with data by Boss et al. [15] that 48 h exposure of rats to 6°C entails a manifold rise of the UCP2 level in heart muscle.

(6) Fatty acid-free bovine serum albumin exhibits more pronounced recoupling effect when added to mitochondria

from cold-exposed rats than from non-exposed animals (Fig. 2). This fact is consistent with assumption that endogenous fatty acids mediate the thermoregulatory uncoupling.

It should be emphasized that even in the cold-exposed animals, the ATP/ADP antiporter seems to dominate in mediating the uncoupling by both endogenous and exogenous fatty acids. This conclusion is supported by the fact that recoupling effects of cAtr are always stronger than those of GDP. In the control group, GDP was in fact without any measurable influence on  $\Delta\Psi$ . Even in the cold-treated rats cAtr increased  $\Delta\Psi$  stronger than GDP (Figs. 3 and 4). On the other hand, targets of these two recouplers were clearly different since cAtr failed to replace GDP and what was more, GDP proved to be more efficient when added after cAtr than before cAtr (Fig. 3). It is noteworthy that CDP, a nucleotide which cannot inhibit UCP, does not substitute for GDP in its effect on mitochondria from the cold-exposed animals (Fig. 3).

All the above relationships are inherent in the action of endogenous fatty acids or low concentration of added palmitate. High concentrations of palmitate uncouple in a cAtr- and GDP-insensitive manner. In this case, other mitochondrial anion carriers or other uncoupling mechanism(s) might be actuated [6,8]. It is noteworthy that the fatty acid-stimulated opening of the permeability transition pore is excluded in our experiments since EGTA was present in the incubation mixture.

The small but reproducible recoupling effects of cAtr and albumin on the control mitochondria in the absence of any added uncouplers is consistent with the assumption that normally the mitochondrial energy coupling is not maximal. This observation is in line with a recent finding by Bobyleva et al. [19] that mitochondrial membrane potential in the intact rat liver cells of the euthyroid rats appears to be somewhat lower than maximal level observed in the hypothyroid animals. Such a 'mild uncoupling' inherent in normal mitochondria may be essential to prevent the  $O_2^-$  production in the respiratory chain [17,20,21]. The above data indicate that it is the ATP/ADP antiporter, rather than UCP2, that is involved in the 'mild uncoupling' in heart muscle. When additional (thermoregulatory) uncoupling appears to be required due to lowering in the ambient temperature, the UCP2-mediated uncoupling is actuated. However, even after 48 h cold exposure, the contribution of the ATP/ADP antiporter to uncoupling seems to be still larger than that of UCP2.

**Acknowledgements:** The authors thank Dr. V.I. Dedukhova for measurement of respiration, Dr. B.V. Chernyak and Dr. E.N. Mokhova for useful discussion. This work was supported by RFBR Grants N 96-04-00022 and 96-15-98070.

#### References

- [1] Skulachev, V.P. and Maslov, S.P. (1960) *Biokhimiya* (Mosc.) 25, 1058–1064.
- [2] Skulachev, V.P. (1963) *Proc. 5th Int. Congr. Biochem.* 5, 365–374.
- [3] Skulachev, V.P. and Maslov, S.P. (1963) *Biokhimiya* (Mosc.) 28, 70–79.
- [4] Nicholls, D.G. (1976) *FEBS Lett.* 61, 103–110.
- [5] Grav, H.J. and Blix, A.S. (1979) *Science* 204, 87–89.
- [6] Skulachev, V.P. (1998) *Biochim. Biophys. Acta* 1363, 100–124.
- [7] Levachev, M.M., Mishukova, E.A., Sivkova, V.G. and Skulachev, V.P. (1965) *Biokhimiya* (Mosc.) 30, 864–874.
- [8] Skulachev, V.P. (1988) *Membrane Bioenergetics*, Springer, Berlin, p. 442.

- [9] Skulachev, V.P. (1991) *FEBS Lett.* 294, 18–162.
- [10] Jezek, P., Engstova, H., Zackova, M., Vercesi, A.E., Costa, A.D.T., Arruda, P. and Garlid, K. (1998) *Biochim. Biophys. Acta* 1365, 319–327.
- [11] Andreyev, A.Y., Bondareva, T.O., Dedukhova, V.I., Mokhova, E.N., Skulachev, V.P., Tsofina, L.M., Volkov, N.I. and Vygodi-na, T.V. (1989) *Eur. J. Biochem.* 182, 585–592.
- [12] Bodrova, M.E., Markova, O.V., Mokhova, E.N. and Samartsev, V.N. (1996) *Biochem. (Mosc.)* 60, 1027–1033.
- [13] Samartsev, V.N., Mokhova, E.N. and Skulachev, V.P. (1997) *FEBS Lett.* 412, 179–182.
- [14] Wieckowski, R. and Wojtczak, L. (1997) *Biochem. Biophys. Res. Commun.* 232, 414–417.
- [15] Boss, O., Samec, C., Dulloo, A., Saydoux, J., Muzzin, P. and Giacobino, J.-P. (1997) *FEBS Lett.* 412, 111–114.
- [16] Fleury, C., Neverova, M., Collins, S., Raimbault, S., Champigny, O., Levi-Neyrueis, C., Bouillaud, F., Seldin, M.F., Surwit, R.S., Ricquier, D. and Warden, C.H. (1997) *Nat. Genet.* 15, 269–272.
- [17] Korshunov, S.S., Skulachev, V.P. and Starkov, A.A. (1997) *FEBS Lett.* 416, 15–18.
- [18] Akerman, K.E.O. and Wikström, M.K.F. (1976) *FEBS Lett.* 68, 191–197.
- [19] Bobyleva, V., Pazzienza, T.L., Maseroli, R., Tomasi, A., Salvioli, S., Cossarizza, A., Franceschi, A. and Skulachev, V.P. (1998) *FEBS Lett.* 430, 409–413.
- [20] Skulachev, V.P. (1994) *Biochem. (Mosc.)* 59, 1910–1912.
- [21] Skulachev, V.P. (1996) *Q. Rev. Biophys.* 29, 169–202.