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Reversible inhibition of electron transfer in the ubiquinol

Cytochrome *c* reductase segment of the mitochondrial respiratory chain in hibernating ground squirrels

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Electron transfer through the ubiquinol:cytochrome c_1-segment of liver mitochondria isolated from hibernating ground squirrels *Citellus undulatus* is repressed by 70–80% as compared to mitochondria from the active animals. The inhibition site is likely to be localized between ubiquinone and the cytochrome bc_1 complex. Partial release of the inhibition can be observed upon swelling of the isolated mitochondria in a hypoosmotic medium, the effect being prevented by phospholipase A_2 inhibitors. Possible role of phospholipase A_2 in regulation of ubiquinol oxidation by complex bc_1 is discussed.

Oxidative phosphorylation; Electron flow regulation; Hibernation; Phospholipase A_2; Ubiquinol: cytochrome c_1 reductase; *Citellus undulatus*

1. INTRODUCTION

As there has been achieved significant progress in the studies of the structure and mechanism of the mitochondrial respiratory chain, more and more attention becomes attached to a problem of regulation of this system.

A discovery of cytochrome *c* oxidase tissue specificity has opened a clear perspective for the studies of this enzyme regulation by various effectors via the 'minor' subunits [1]. As to the other energy-transducing segments of the respiratory chain, little is known about their regulation.

A promising model for the studies of the respiratory chain regulation might be hibernating animals, since profound changes in their metabolism should most certainly involve appropriate control over energy production.

For many years, the data concerning mitochondrial respiration and its regulation in hibernators had been rather controversial (e.g. see References in [2]). However, it has been shown recently by 3 groups on 3 different ground squirrel species that respiration of mitochondria isolated from the livers of the animals during hibernation period is depressed severely [3–5], the inhibition being localized somewhere in the middle part of the respiratory chain [4,5], which corroborates the early finding of Roberts and Chaffee [6].

In this work we confirm inhibition of electron transfer through the respiratory chain of liver mitochondria in hibernating ground squirrels and show that: (i) inhibition of the uncoupled respiration is associated specifically with the ubiquinol : cytochrome *c* reductase segment; (ii) the inhibition can be partly reversed in isolated mitochondria by mild hypoosmotic treatment; (iii) the reversal of the inhibition is likely to be mediated by phospholipase A_2 activity.

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Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; BrPhBr, bromphenacylbromide

2. MATERIALS AND METHODS

The experiments have been carried out on ground squirrels *Citellus undulatus* during their active (body temperature: 37°C) and hibernating (body temperature: 5°C) periods. Handling of the animals and isolation of mitochondria from liver were as described [4].

Mitochondrial respiratory activity was measured with a Clark-type electrode in a 2 ml cell thermostatted at 27°C. Optical measurements were carried out in a Hitachi 557 or an Aminco DW2a spectrophotometer.

The standard (isoosmotic) basic incubation medium contained 250 mM sucrose, 3 mM $MgCl_2$, 3 mM KH_2PO_4 and 10 mM Tris-HCl, pH 7.4. The 'hypoosmotic' medium had the same composition except that sucrose concentration was 60 mM. Protein was determined by the method of Lowry et al. [7].

3. RESULTS

When assayed in a standard medium, the uncoupled respiration of liver mitochondria isolated from hibernating ground squirrels is inhibited by 70–80% as com-

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pared to mitochondria from the active animals. This effect has been observed with each of the substrates tested (including β-oxybutyrate, glutamate, α-ketoglutarate, palmitoyl-L-carnitine, succinate and duroquinol) except for ascorbate + TMPD, where no significant inhibition was found (data not included [4]). These results are in agreement with [5] and suggest the inhibition being associated with the middle part of the respiratory chain.

This conclusion is directly confirmed by a spectrophotometric assay of the mitochondrial succinate:ferricyanide reductase activity which proved to be ~5-fold lower in mitochondria from the hibernating animals (see typical recordings in Fig. 1).

Accordingly, a 85% inhibition of the antimycin-sensitive Q_2H_2:cytochrome *c* reductase activity in the hibernator mitochondria has been observed (data not shown). These results are in variance with the data of Gehnrich and Aprille on *C. tridecemlineatus* [5] who found only a 32% decrease of the succinate:cytochrome *c* reductase activity in hibernators despite the 64% inhibition of the respiration with succinate.

In subsequent experiments cytochrome reduction in mitochondria from hibernating and active animals has been studied spectrophotometrically (Fig. 2).

Addition of succinate to mitochondria from the non-hibernating ground squirrels at the high protein concentration used results in a very rapid anaerobiosis accompanied by an almost complete reduction of site 2 cytochromes; increment of absorbance at 550 and 562 nm induced by dithionite addition to anaerobic mitochondria is largely due to cytochrome b_5 contribution. In case of mitochondria from hibernating animals, there is a significant delay in the onset of anaerobiosis consistent with the inhibition of electron transfer. Notably, difference spectra of the aerobic steady-state show little reduction of either *b* or *c* cytochromes pointing to the inhibition being localized on the reducing side of the bc_1-site hemoproteins.

Altogether our data may indicate impairment of ubiquinol oxidation by complex bc_1.

As discovered recently, mild hypoosmotic treatment can induce a number of profound changes in respiration and oxidative phosphorylation of liver mitochondria [8,9]. Following these observations we found the repressed respiration of the hibernator mitochondria to be activated 2–3-fold upon an incubation for less than 5 min in a hypotonic medium (Table I). The same effect is observed with respect to the succinate:ferricyanide reductase activity (Fig. 1). No activation is observed for the cytochrome oxidase activity in the hibernating animals (Table I) and none of the activities is stimulated in mitochondria from non-hibernators (not shown). Thus hypotonic treatment appears to entail partial release of the block in the bc_1-site of the respiratory chain imposed in hibernators.

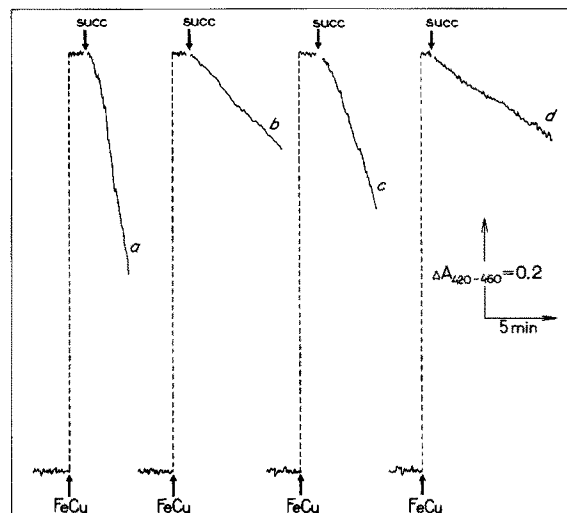

Fig. 1. Reversible inhibition of the succinate:ferricyanide reductase activity of liver mitochondria in hibernating ground squirrels. Mitochondria from the active (a) and hibernating animals (b–d) are suspended at 0.86 and 0.78 mg protein/ml, respectively. (a, b) The standard medium supplemented with 3 μM rotenone, 1 μM CCCP and 2 mM KCN. (c) The hypoosmotic medium with the same additions. (d) As (c), but in the presence of 20 μM BrPhBr. In each case, mitochondria were preincubated in the cell for ~5 min and, where indicated, 1 mM ferricyanide and 10 mM succinate have been added. The succinate:ferricyanide reductase activity was more than 90% sensitive to myxothiazol. The BrPhBr effect was not observed without the preincubation.

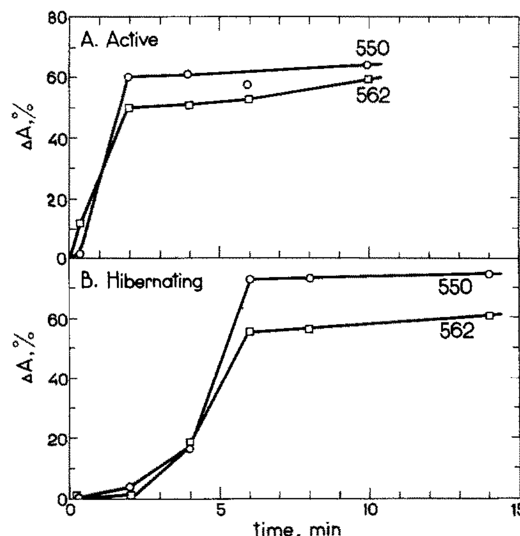

Fig. 2. Effect of hibernation on the *b* and $(c+c_1)$ cytochrome reduction by succinate in liver mitochondria. The sample and reference spectrophotometric cells contained mitochondria from the active (A) or hibernating animals (B) (3.3 mg protein/ml) in the aerobic standard medium supplemented with 3 μM rotenone and 1 μM CCCP. At the zero time, 10 mM succinate was added to the sample and difference spectra have been recorded immediately and then each two minutes in the range 540–580 nm at a rate of 2 nm/s. Finally dithionite was added to the sample. For each spectrum, the extent of cytochromes *b* and $(c+c_1)$ reduction was evaluated measuring ΔA at 562 and 551 nm, respectively, relative to the baseline connecting the points of the spectrum at 540 and 575 nm. The corresponding ΔA values for the dithionite-induced spectrum have been taken in each case as 100.

Table I

Effect of the medium osmolarity and of phospholipase A_2 inhibitors on the respiratory activity of liver mitochondria from hibernating ground squirrels

Substrate	Rate of oxygen consumption (nmol·min^{-1}·(mg protein)$^{-1}$)				
Medium composition:	Iso	Hypo	Hypo + BrPhBr	Hypo + EGTA + A23187	Hypo + Nupercain
β-Oxybutyrate (4 mM)	23 ± 4	54 ± 4	22 ± 3	24 ± 1	21 ± 3
Succinate (4 mM)	40 ± 5	99 ± 9	34 ± 5	25 ± 5	31 ± 4
Palmitoyl-L-carnitine (100 μM)	29 ± 4	55 ± 4	24 ± 3	26 ± 3	19 ± 3
Duroquinol (200 μM)	42 ± 6	115 ± 9	44 ± 4	31 ± 5	26 ± 4
Ascorbate (4 mM) + TMPD (400 μM)	120 ± 11	108 ± 10	110 ± 9	113 ± 9	74 ± 9

The respiration rate was measured as described in section 2 in the standard (Iso) or hypoosmotic (Hypo) medium in the presence of the uncoupler (1 μM CCCP). Other additions where indicated: BrPhBr, 20 μM; EGTA, 1 mM; A23187, 0.1 μg/mg protein; nupercain, 500 μM. Mitochondria were preincubated with the inhibitors for at least 5 min

That hypoosmolarity can activate phospholipase A_2 in rat liver mitochondria was reported in [10]. We have found the release of electron transfer in hibernator mitochondria observed in the hypotonic medium to be fully prevented by any of the three phospholipase A_2 inhibitors tested (Table I, Fig. 1). No significant effect of these compounds on the corresponding activities of the control mitochondria (non-hibernating animals) was found. An approx. 1.5-fold decrease of the cytochrome oxidase activity in the presence of nupercain (Table I) is in line with the moderate inhibitory action of local anaesthetics on this enzyme [11]. Electron microscopy showed that swelling of the hibernator mitochondria in the hypotonic medium was not affected by the phospholipase inhibitors.

4. DISCUSSION

Our data indicate that electron transfer capacity of the ubiquinol/bc_1-span of the liver mitochondria respiratory chain may subject to a strong physiological control. The ~80% depression of the succinate:ferricyanide and QH_2:cytochrome *c* reductase activities in hibernating *C. undulatus* mitochondria is a much more significant effect than the 32% inhibition reported earlier for *C. tridecemlineatus* [5] and in case of our experiments can fully account for the decrease of the respiratory activity of the mitochondria from hibernators.

Somewhat unusually, the inhibition of electron transfer through the middle part of the respiratory chain in hibernating animals does not result in a crossover between *b* and c_1 cytochrome as is typical of virtually all bc_1-site inhibitors [12], but rather is localized on the reducing side of the cytochromes. Hence the inhibition is not likely to be associated specifically with either QH_2-oxidizing or Q-reducing centres *o* and *i* of the Q-cycle [12,13].

Perhaps the most interesting novel observation of this work consists in the easy re-activation of electron transfer in hibernating animals upon swelling of isolated mitochondria. It is likely that the role of swelling is confined to phospholipase A_2 activation. What might be then the role of phospholipase?

One possibility is that there occurs accumulation of some inhibitory effector in the membranes of hibernator liver mitochondria which can be removed by phospholipase A_2 directly (e.g. hydrolysis) or indirectly via some regulatory cascade switched on by phospholipid hydrolysis. The putative lipid-soluble effector could serve as an allosteric inhibitor of complex bc_1 or simply as a ubiquinone antagonist.

Alternatively, the role of phospholipase A_2 might consist of providing appropriate membrane fluidity required for diffusion of ubiquinone and Q-reactive enzymes within the membrane. In the absence of such fluidity, electron flow in mitochondria from hibernating animals might be confined to the ~20–30% of bc_1 molecules which form direct complexes with the dehydrogenases [14] without involvement of the CoQ pool.

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