CYCLIC RESPONSE OF ENERGY LINKED FUNCTIONS OF LIVER MITOCHONDRIA TO UNCOUPLING THIOPHENE DERIVATIVES

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Received 15 December 1969

1. Introduction

Various theories on the molecular mechanism of uncoupling by lipid soluble weak acids have been developed, depending on the model of energy conservation presumed [1–3]. Independent of whether an "energized state" or a high-energy intermediate are dissipated, or whether a proton gradient is discharged by means of an uncoupling agent, the latter is assumed to act as proton conductor across a lipophilic space [2–5]. Recently, we have described the derivatives of 2-anilido-thiophen as a new group of potent uncouplers, representing lipid soluble compounds, carrying an acid NH-group, which is essential for the uncoupling action [6]. Two of these derivatives are of particular interest: 2-(2′,6′-dimethylanilino)-3,4-dinitro-5-chlorothiophene (DDCT), and 2-bromo-3,4-dinitro-5-(4′-chloranilino)-thiophene (BDCT). Generally, uncoupling appears as a catalytical process, changing energy linked equilibria to a new steady state, as for instance the respiratory rate, the redox-states of electron carriers, or the ion exchange reactions. However, by addition of the above thiophene-derivatives to energized liver mitochondria, a transitory uncoupling of energy linked reactions can be observed, comparable to the phosphorylating cycles produced by small amounts of ADP + P_i, when added to respiring mitochondria. By means of their special properties, the new compounds are considered to serve as useful tools for kinetic studies of initial stages of uncoupling and for the resolution of the reactions involved. The present report is to give examples for the cyclic response of some energy dependent mitochondrial parameters on addition of DDCT or BDCT.

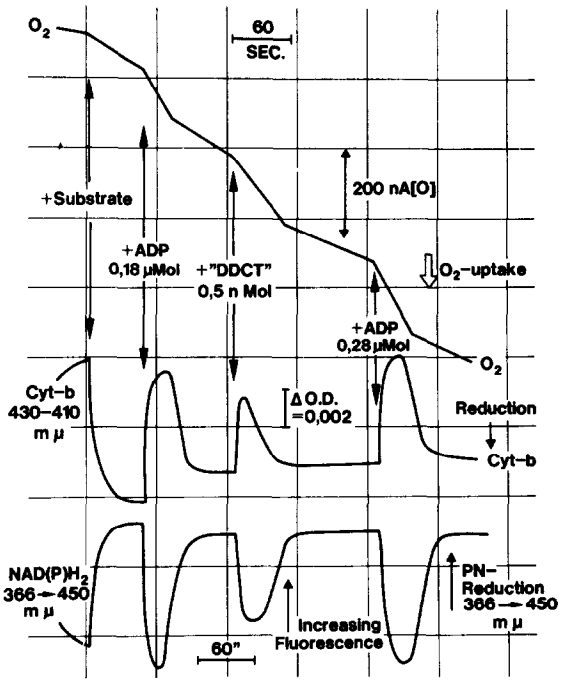

Fig. 1. Cyclic response of the respiratory chain to DDCT. 2.45 ml buffer, containing 0.25 M sucrose, 3 mM phosphate, 10 mM TRA, 2 mM Mg^{2+}, 10 mM KCl, pH 7.3, t = 25°C; substrate 5 mM β-hydroxybutyrate. Other additions indicated in the figure.

Table 1
Recovery of state 4 after transitory uncoupling by DDCT.

Substrate	state 4_1 →	state 3u →	state 4_2
Succinate (n = 6)	32.6 ± 1.7	130.5 ± 2.6	35.8 ± 1.4
β-Hydroxy-butyrate (n = 7)	16.8 ± 0.5	45.4 ± 3.3	18.4 ± 1.7

Mitochondrial protein 4.0 mg, total vol. 2.32 ml, t = 25°C, addition of 0.5 nmol DDCT for state 3u. For other conditions see fig. 1. Figures give oxygen uptake as nA(O)/min/mg prot.

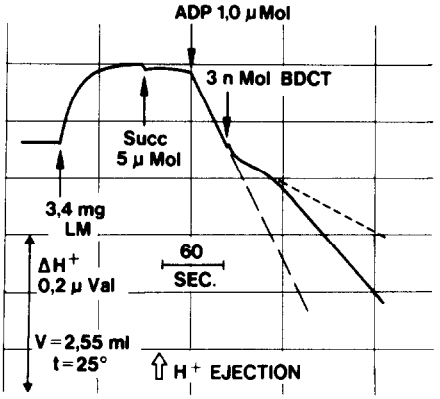

Fig. 2. Proton uptake during oxidative phosphorylation. Incubation mixture: 0.125 M sucrose, 50 mM NaCl, 2 mM phosphate, pH = 7.4, other conditions as specified in the figure.

2. Methods

Preparation of liver mitochondria and measurement of oxygen uptake were carried out as described previously [7, 8]. Incubations were performed as specified in the legends to the figures. The redox-states of cytochromes were monitored in a Phoenix-dual-wavelength spectrophotometer. Fluorescence of pyridine nucleotides was measured according to the method of Estabrook [9]. Proton liberation and uptake during ATP-hydrolysis or ADP-phosphorylation were monitored with a glass electrode in conjunction with a Knick-pH-meter, equipped with a compensation circuit and a recorder. The uncouplers DDCT and BDCT were synthesized together with an extensive line of other thiophene derivatives by K.H.B.

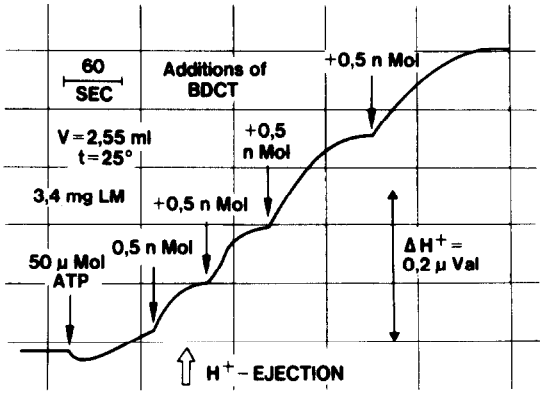

Fig. 3. ATPase activation by BDCT. Incubation mixture: 0.125 M sucrose, 50 mM NaCl, 1 mM TRA, pH 7.4, t = 25°C.

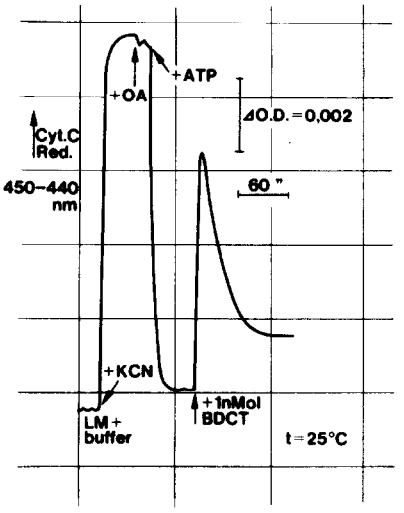

Fig. 4. Redox cycles of cytochrome *c* in anaerobic mitochondria. Conditions: 0.25 M sucrose, 10 mM TRA, pH 7.2 mM, 0.2 mM EDTA, 1 mM KCN, 3 mM oxaloacetate, 2 mM ATP, 4.8 mg mitochondrial protein, total vol. 2.1 ml, t = 25°C. Addition of 1.0 nmole BDCT.

3. Results

Fig. 1 shows a synoptic registration of mitochondrial respiration, the reduction of cytochrome *b*, and the reduction of pyridine nucleotides. After addition of substrate, respiration is initiated and is accelerated from state 4 to state 3 by addition of ADP. After completion of a normal phosphorylating cycle, DDCT is

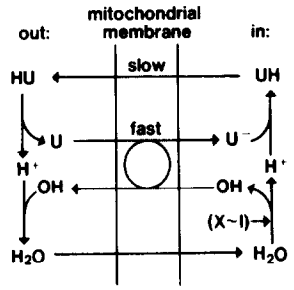

Fig. 5. Proposed mechanism of uncoupling, according to Van Dam [3], as modified for DDCT and BDCT. u = uncoupler.

added during the controlled state of respiration. In contrast to classical uncouplers, DDCT does not induce a new steady state of rapid respiration. However, after a period of stimulated oxygen uptake, referred to as state 3u, the system returns to the state 4 rate of inhibited respiration. This phenomenon can be repeated several times by successive additions of small amounts of DDCT or BDCT. In the latter case the recovery from the uncoupled state to the controlled state is even more pronounced. The effect is independent of the substrate oxidized by the mitochondria. As demonstrated in table 1, after a single addition of DDCT to liver mitochondria, oxidizing succinate or hydroxybutyrate, the transitory state 3u is established. Thereafter the original state 4 rate is fully recovered, indicating that the uncoupling effect of DDCT disappears after a certain period of time. In aerobic systems oligomycin does not prevent the occurrence of this cyclic response, suggesting that no phosphorylated intermediates are involved in the entire process of energy consumption by DDCT and BDCT.

The records of cytochrome *b* and pyridine nucleotide reduction illustrate, that simultaneously with an accelerated respiration a more oxidized state of the respiratory carriers is observed, also returning to the state 4 degree of reduction when the activity of the uncoupler has ceased. The redox cycles closely resemble those obtained with ADP or with Ca^{2+} during its active accumulation. From this an analogous process is suggested to occur with DDCT, presumably an energy dependent uptake of the uncoupler terminated by its accumulation in the inner membrane. It must be stressed that after completion of the described cycles the system of oxidative phosphorylation remains intact and normal ADP/O ratios and respiratory control are obtained. This is outlined in fig. 1 by addition of ADP after a full cycle of DDCT action. It has been found that a linear correlation exists between the $-\Delta O_2$ jump (duration of state 3u) and the added amount of BDCT, which is in agreement with the concept, that BDCT or DDCT are accumulated during the period of rapid respiration.

Based on the idea of an energy dependent uptake of the uncouplers, other energy linked reactions and equilibria were also expected to show a cyclic response. In fact, the OH^- production during aerobic phosphorylation of ADP by liver mitochondria proceeds at a diminished rate for a transitory interval after addition of BDCT, and then returns to normal. This is readily explained by the assumption that during the period of BDCT interaction, energy from oxidative phosphorylation is derived for translocation of the inhibitor, as shown in fig. 2. Additional evidence for energy dependent uptake of these uncouplers is obtained from the pulsed activation of ATPase. Fig. 3 shows a series of subsequent additions of BDCT to liver mitochondria, each producing a pulse of H^+ liberation due to splitting of ATP.

Moreover, in anaerobic systems, energized by excess ATP, cytochrome *c* is oxidized by reversed electron transfer to the pyridine nucleotide level [10]. BDCT initiates cycles of reduction/oxidation, thus resembling the response of aerobic mitochondria. During the initial phase of BDCT uptake high energy states X~I may be split, as indicated by the reduction of cytochrome *c*. After termination of uptake, reoxidation occurs at the expense of excess ATP present. The experiment is demonstrated in fig. 4.

4. Conclusions

The above findings are difficult to reconcile with chemi-osmotic energy coupling, which ascribes to uncouplers a catalytical role as proton conductors across a coupling membrane. However, the existence of stoichiometrically defined transitory states of limited duration, as reported here, easily meets the requirements of a model of energy dependent anion translocation, as described by Van Dam [3], Slater [11], and Harris [12]. In accordance with a slight

modification of Van Dam's mechanism proposed for uncoupling by weak acids, which is referred to in fig. 5, we assume that BDCT and DDCT enter the mitochondria as an anion, leading to a decay of an energy dependent separation of water molecules into H^+ and OH^-. Actually, a continuous operation of an "uncoupling cycle" does not occur due to the particular partition coefficients, *p*Ks and other structural properties of the above compounds, suggesting that the rate of passive outward diffusion of the undissociated acids is much slower than the rate of uptake in exchange for endogenous OH^-, or anions. Thus, accumulation results in undissociated forms of DDCT or BDCT in the hydrophobic membrane space. Moreover, on the basis of recent studies of Skulachev et al. [13, 14] the interference of the above proton conducting compounds with ion movement in an electric field created by the energy dependent H^+/OH^- separation in mitochondria may be explained likewise. More detailed kinetic data on the phenomenon of pulsed uncoupling and the exchange reactions under varying conditions will be presented elsewhere.

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