Minireview

A second mechanism of respiratory control

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Abstract According to the chemosmotic hypothesis, ATP is synthesized in mitochondria, bacteria and chloroplasts via the proton motive force Δp, the energy-rich intermediate of electron transport and photosynthetic phosphorylation. The general applicability of the chemosmotic hypothesis, however, was disputed until present. In particular the relationship between the rate of respiration and Δp in mitochondria was found variable, depending on the experimental conditions. Recently, a new mechanism of respiratory control was found, based on binding of ATP or ADP to subunit IV of cytochrome c oxidase, which is independent of Δp and could explain many previous results contradicting the chemosmotic hypothesis.
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Key words: Respiratory control; Chemosmotic hypothesis; Cytochrome c oxidase; ATP or ADP binding site; Allosteric inhibition; Oxidative phosphorylation

1. The first mechanism of respiratory control

Respiratory control was originally defined as stimulation of respiration, i.e. oxygen consumption of isolated mitochondria by ADP (active, state 3 respiration), followed by its decrease (controlled, state 4 respiration) due to conversion of ADP into ATP [1,2]. A molecular explanation for this phenomenon was presented by the chemosmotic hypothesis [3,4] which postulates the proton motive force Δp across the inner mitochondrial membrane as the energy-rich intermediate of oxidative phosphorylation ($\Delta p = \Delta \Psi$-$Z \times \Delta \mathrm{pH}$, $Z = 2.303 \times RT/F$; $\Delta p \times F = \Delta \mu_{H^+}$. Three proton pumps of the respiratory chain (NADH dehydrogenase, cytochrome c reductase and cytochrome c oxidase) generate Δp which is used by the proton gradient driven ATP-synthase for the synthesis of ATP from ADP and phosphate. Respiratory control is explained by the following events: uptake of ADP into mitochondria stimulates the ATP-synthase accompanied by a decrease of Δp, which in consequence stimulates the activity of the three proton pumps and thus mitochondrial respiration [5]. The chemosmotic hypothesis was strongly supported by the effect of 'uncoupler' of oxidative phosphorylation which increases the H^+ conductance of biological membranes and dissipates Δp. After the first description of 2,4-dinitrophenol as uncoupler of phosphorylation from oxidation by Loomis and Lipman in 1948 [6], numerous other compounds were identified to un-

couple oxidative phosphorylation, all representing hydrophobic weak acids [7].

2. Results in conflict with the chemosmotic interpretation of respiratory control

The chemosmotic hypothesis became established after Peter Mitchell received the Nobel price in chemistry in 1978. The hypothesis was strongly supported by results obtained with bacteria [8,9] and chloroplasts [10], but various observations, in particular from studies with mitochondria, could not be explained by that theory ([11], for review see [12,13]). In 1959, Ernster and colleagues [14] described the lack of respiratory control in skeletal muscle mitochondria from a patient with hypermetabolism (the first described case of a mitochondrial myopathy), although these mitochondria were able to synthesize ATP from added ADP. A second case of hypermetabolism which like the first one could not be explained by a thyroid disorder was published by DiMauro et al. [15]. The results demonstrate that the rate of respiration in mitochondria is not necessarily controlled by Δp. Weinbach and Garbus [7,16] suggested that the key to the uncoupling phenomenon (e.g. stimulation of respiration by uncoupler of oxidative phosphorylation) may be provided by the binding of uncouplers to mitochondrial proteins. Padan and Rottenberg [17] obtained an inverse relationship (with a linear graph) between $\Delta \mu_{H^+}$ and the rate of respiration in rat liver mitochondria under partly uncoupled conditions (presence of valinomycin and KCl), but measured almost no change of $\Delta \mu_{H^+}$ between the active state 3 and the controlled state 4, while respiration increased 3.5-fold. They concluded that their results are not compatible with the chemosmotic model of oxidative phosphorylation. From studies on the effect of pyridine homologues on the respiratory control and H^+/O ratio in mitochondria, Ho and Wang [18] concluded that the basal rate of electron transport is governed not directly by the proton gradient but by molecular processes in the energy transducing membrane. Wilson and Forman [19] concluded from measurements of respiratory rates at different Δp in rat liver mitochondria that large differences in slope are inconsistent with the membrane potential being the primary determinant of the mitochondrial respiratory rate. Similar conclusions were obtained in studies by Zoratti et al. [20] with rat liver mitochondria and by Mandolino et al. [21] with plant mitochondria. Zoratti and Petronelli [22] concluded that beef heart submitochondrial particles and rat liver or plant mitochondria behave differently. The former apparently exhibit only one J_{ATP} (rate of ATP synthesis) versus $\Delta \mu_{H^+}$ relationship while the latter show several. The common denominator of all of these studies is the lack of relationship between the magnitude of Δp

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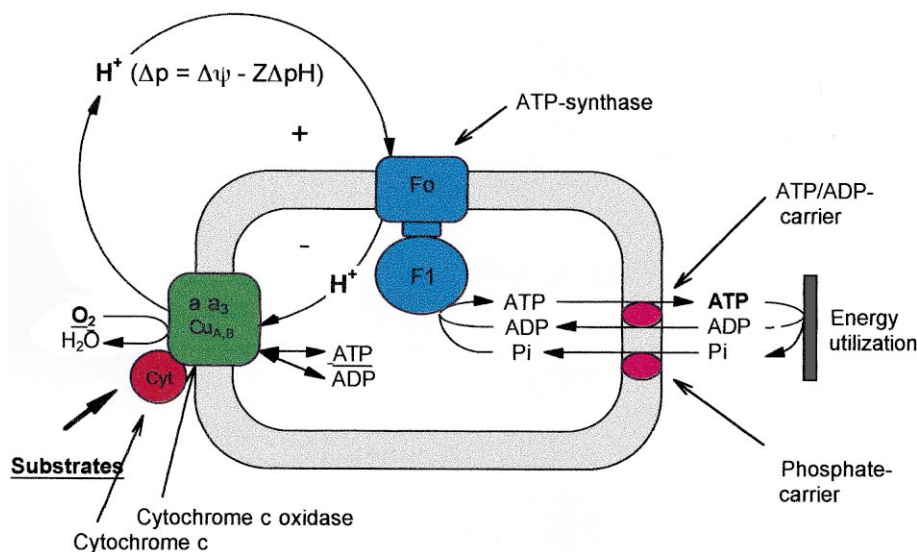E-mail: Kadenbach@chemie.uni-marburg.de

Fig. 1. Two mechanisms of the control of respiration and ATP synthesis in mitochondria according to the utilization of energy (ATP). The first mechanism of respiratory control is based on the proton motive force Δp across the inner mitochondrial membrane (grey). Activation of the ATP-synthase (blue) by ADP, taken up via the ATP/ADP carrier (margenta), decreases Δp which in consequence stimulates the three proton pumps of the respiratory chain (complexes I, III and IV). For simplicity, only complex IV (cytochrome *c* oxidase) and its substrate (cytochrome *c*) are shown in green and red, respectively. The second mechanism of respiratory control is based on the intramitochondrial ATP/ADP ratio. High ATP/ADP ratios inhibit cytochrome *c* oxidase activity allosterically. Uptake of ADP decreases the intramitochondrial ATP/ADP ratio accompanied by exchange of bound ATP by ADP at the matrix domain of subunit IV of cytochrome *c* oxidase, with subsequent stimulation of respiration.

and the rate of respiration or ATP synthesis in intact mitochondria, but this relationship was found under certain conditions, e.g. aged or uncoupled mitochondria or mitochondrial particles.

In recent studies, Starkov et al. [23,24] investigated the recoupling of the uncoupler-stimulated respiration of mitochondria by 6-ketocholestanol, male sex hormones and progesterone. The effect was specific to animal mitochondria and abolished by a very low concentration of fatty acids. The authors assumed that the action of low concentrations of the SF6847-like uncouplers on coupling membranes involves cytochrome *c* oxidase and perhaps some other membrane protein(s) as well.

3. The control of respiration in intact cells

The control of cell respiration in vivo according to the utilization of ATP is not clear [25–27]. In fact, neither the mitochondrial membrane potential $\Delta\Psi_m$[26], nor the cytosolic ATP/ADP ratio, calculated from ^{31}P-NMR data [25], could be shown to vary according to the rate of cell respiration. Also, using ^{31}P-NMR techniques, no correlation between the concentrations of ADP or Pi and the rate of respiration could be found in vivo in heart [28] and kidney [29]. Nevertheless, Chance et al. [30] considered ADP as the principal control parameter of the oxidative metabolism in skeletal muscle and this was further supported by recent results suggesting that the apparent kinetic order of the transduction function of the signal cytosolic ADP concentration is at least second order. A sigmoidal relationship was found between the cytosolic ADP concentration and the rate of oxidative phosphorylation, measured by ^{31}P-NMR in the human arm in vivo [31]. For other investigators, however, it is still unclear what ultimately limits myocardial performance under awake exercizing conditions [27].

4. The second mechanism of respiratory control

A second mechanism of respiratory control was recently described, which is based on the intramitochondrial ATP/ADP ratio. It was suggested to control the rate of cell respiration according to the utilization of ATP in eucaryotes [32], including yeast, but not in bacteria [33]. The second mechanism of respiratory control (see Fig. 1) is independent of Δp [34] and due to binding of ATP or ADP to the matrix domain of subunit IV of eucaryotic cytochrome *c* oxidase, since a monoclonal antibody against subunit IV completely abolished the inhibition of activity at high ATP/ADP ratios. The corresponding subunit IV does not occur in bacterial cytochrome *c* oxidase [35]. The binding site for ATP at subunit IV has previously been identified by photoaffinity labelling [36–38]. This binding site represents one of seven high affinity bindings sites for ATP or ADP and three additional only for ADP, in bovine heart cytochrome *c* oxidase [39–41]. High intramitochondrial ATP/ADP ratios convert the Michaelis-Menten type kinetics of the enzyme activity into sigmoidal kinetics with complete inhibition up to 1–6 μM cytochrome *c*. With the reconstituted enzyme, half maximal inhibition of activity was obtained at an ATP/ADP ratio of 28 [34] and the sigmoidal relationship between the intraliposomal ADP concentration (at decreasing ATP/ADP ratios) and the rate of respiration was found to be very similar to that described by Jeneson et al. [31] between the cytosolic ADP concentration and the rate of oxidative phosphorylation, measured by ^{31}P-NMR in the human arm in vivo.

The allosteric inhibition of cytochrome *c* oxidase by ATP is sensitive to various compounds (conditions) which uncouple the second mechanism of the respiratory control. No ATP inhibition is obtained, i.e. the same activity is measured in the presence of ATP and of ADP (or at ATP/ADP ratios <20) under the following conditions. (1) Measurement of

activity in the presence of laurylmaltoside [32], a detergent frequently used for activity measurements. (2) Measurement of activity in the presence of TMPD (N,N,N',N'-tetramethyl-p-phenylenediamine), an electron carrier between ascorbate and cytochrome c. (3) A low concentration of 3,5-diiodo-L-thyronine binds specifically to the matrix oriented subunit Va and completely abolishes the ATP inhibition [42]. (4) Submicromolar concentrations of palmitate (much lower than those required for uncoupling) and micromolar concentrations of other fatty acids (Hammerschmidt and Kadenbach, 1999, in preparation). (5) Ethanol concentrations above 40 mM (0.2%) (Hammerschmidt and Kadenbach, unpublished results). (6) Dephosphorylation of cytochrome c oxidase reduces the ATP inhibition (probably at subunit IV [43]). The ATP inhibition of reconstituted bovine heart cytochrome c oxidase can be enhanced by cAMP-dependent phosphorylation with protein kinase A (Bender and Kadenbach, in preparation). (7) Insufficient cardiolipin of the isolated or reconstituted enzyme [32]. From this list follows that in isolated mitochondria the second mechanism of respiratory control is easily abolished.

5. Metabolic control by cytochrome c oxidase

The second mechanism of respiratory control could only be effective if cytochrome c oxidase represents the rate limiting step of mitochondrial respiration. Although cytochrome c oxidase catalyzes the only irreversible reaction of the respiratory chain, namely the reduction of dioxygen to water, its activity is not assumed to represent the rate limiting step of cell respiration. From application of the metabolic control analysis [44–46], a 5–7-fold excess of cytochrome c oxidase capacity over the amount required to support the endogenous respiration of isolated mitochondria was found [47–49]. A limitation in the metabolic control analysis applied to isolated mitochondria, as compared to the in vivo situation, however, is the significant alteration of the environment due to the possible loss of essential metabolites and to the absence of the cytosolic substrates and coenzymes. Recent metabolic control analysis of respiration in intact cultured cells revealed a tight control of cell respiration by cytochrome c oxidase, exceeding its capacity above the endogenous respiratory activity only by 7–25% [50,51]. In addition, in saponin-permeabilized muscle fibers, a higher control strength for cytochrome c oxidase was found than in isolated mitochondria, in particular at lower oxygen pressure [52]. Similar conclusions were already made by Erecinska and Wilson [53], who proposed the near equilibrium hypothesis, assuming in mitochondria an equilibrium between the reactions of NADH oxidation and cytochrome c reduction, but an irreversible and rate limiting reaction of cytochrome c oxidase. We propose that the different control strength of cytochrome c oxidase, measured in isolated mitochondria and intact cells, is mainly due to abolition (uncoupling) of the second mechanism of respiratory control during isolation of mitochondria (e.g. presence of free fatty acids).

6. Why is a second mechanism of respiratory control evolved in eucaryotes?

The first mechanism of respiratory control is based on the mitochondrial proton motive force Δp [5], which is generally assumed to represent the energy-rich intermediate of oxidative phosphorylation. The results presented above suggest, however, that in eucaryotes Δp does not represent the only parameter controlling the rate of respiration. The physiological significance of the second mechanism of respiratory control by the intramitochondrial ATP/ADP ratio, via binding of the nucleotides to and possibly phosphorylation of subunit IV of cytochrome c oxidase ([43], Bender and Kadenbach, in preparation), could be to maintain a constant and relatively low mitochondrial membrane potential $\Delta\Psi$, the major component of Δp. Control of respiration by Δp alone would require a decrease of $\Delta\Psi$ when the rate of ATP synthesis increases, which is scarcely the case in vivo [26]. Therefore we propose that the homeostasis of mitochondrial $\Delta\Psi$ in vivo is achieved by the second mechanism of respiratory control.

Furthermore, the control of respiration by Δp alone would allow $\Delta\Psi$ to rise to levels as high as 160–220 mV, as indeed measured with isolated mitochondria [54]. High $\Delta\Psi$ values, however, are disadvantageous to eucaryotic cells in three respects. (1) They support the formation of reactive oxygen species like the superoxide radical anion [55–57]. Increased production of oxygen radicals would increase deleterious mutations of DNA, in particular of mitochondrial DNA. (2) The unspecific and energetically unproductive proton leak of biological membranes increases exponentially with the membrane potential [58]. Therefore a large part of the oxidative energy would be released as heat at high values of $\Delta\Psi$. (3) Above 120 mV, free fatty acids act as uncoupler of oxidative phosphorylation [59]. Therefore it appears reasonable that in vivo, a rather low $\Delta\Psi$ of about 100–130 mV is maintained, as measured in perfused rat hearts and this value was only little affected by varying the rate of respiration by a factor of four [26].

7. Conclusion

The second mechanism of respiratory control could explain the inconsistencies with the chemosmotic hypothesis of many investigations, in particular the discrepancies between the rate of respiration and the magnitude of Δp. It presents an obvious explanation for the homeostasis of ATP/ADP ratios and of the mitochondrial Δp in eucaryotic cells, despite large variations of the rates of respiration and ATP synthesis.

Finally, the second mechanism of respiratory control presents one answer to the long standing question on the physiological function of supernumerary subunits of respiratory chain complexes [60] and proves the postulated functions of nuclear coded subunits of cytochrome c oxidase, representing receptors for nucleotides and hormones, which regulate via conformational changes the catalytic activity of the enzyme, i.e. the rate of respiration and the efficiency of energy transduction [61–63]. The latter has been demonstrated for subunit VIaH (heart-type), which decreases in mammalian skeletal muscle at rest (high intramitochondrial ATP/ADP ratios) the H^+/e^- stoichiometry from 1.0 to 0.5, thus participating in thermogenesis [64].

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