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Control of oxidative phosphorylation in rat muscle mitochondria: implications for mitochondrial myopathies

Thierry Letellier, Monique Malgat and Jean-Pierre Mazat

Université Bordeaux II, Bordeaux (France)

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The control of oxidative phosphorylation has been studied in normal skeletal muscle isolated from the hind legs of rats. The control coefficients of different steps of oxidative phosphorylation on the flux of O_2 consumption were determined by the inhibitor method and calculation was done according to the model of Gellerich et al. (FEBS Lett. 274 (1990) 167–170) using a non-linear regression fitting procedure. The respiration was recorded with pyruvate (+malate) and palmitoyl-L-carnitine (+malate) as respiratory substrates, which are the main substrates oxidized in the muscle. It appears that the control is broadly distributed among the different complexes of the respiratory chain, and of the ATP synthesis system. Our results also provide an explanation for the threshold effects often evidenced in the clinical manifestation of mitochondrial diseases.

Introduction

The metabolic control theory developed by Kacser and Burns [1] on the one hand and by Heinrich and Rapoport on the other [2] has been extensively applied to the study of the mitochondrial metabolism, for which it afforded a neat solution to the problem of the control of oxidative phosphorylation [3–13].

The most important elements defined in this framework are the control coefficients, which measure the amount of control that a particular step exerts on flux in a whole metabolic network. The main results of these studies are, according to the theory, (i) the control of oxidative phosphorylation can be shared amongst several steps; (ii) control distribution can change when the steady state is changed; (iii) control distribution is different in mitochondria isolated from different tissues.

The steps with high control coefficients are, by definition, those most sensitive to changes in the activity of the enzyme by which they are catalyzed. Such changes, however – even major ones – will have only little effect on the flux of the network if the control coefficient of the step is low. Thus, the measure of control coefficients is particularly relevant to the understanding of the clinical consequences of an enzymatic defect in a metabolic disease.

Inborn errors of oxidative phosphorylations are now broadly described [14,15] and are often associated with heteroplasmy of mitochondrial DNA [16–19]. One of the most severely affected tissues in these diseases is the skeletal muscle. For a better understanding of the effects of defects in oxidative phosphorylation in this tissue, we have studied, in this paper, the control distribution of this pathway in mitochondria isolated from normal rat skeletal muscle (hind legs), using pyruvate (+malate) and palmitoyl-L-carnitine (+malate), which are the main respiratory substrates in the muscle.

We have used the inhibitor method [3,8] for the determination of control coefficients and the method of Gellerich et al. [20] to determine the control coefficients by non linear-regression of the entire inhibition curves of the flux.

Materials and Methods

Chemicals

Rotenone, antimycin, oligomycin, carboxyatractyloside were from Sigma, Fresh solution of pyruvate, palmitoyl-L-carnitine, cyanide, mersalyl were used throughout this study. Ubiquinone I was a gift from Hofmann-Laroche.

Correspondence to: J.-P. Mazat, Université Bordeaux II, 146, rue Léo Saignat, 33076 Bordeaux-Cedex, France.

Isolation of mitochondria

Rat muscle mitochondria were isolated by differential centrifugation as described by Morgan-Hughes et al. [21]. The muscle of the two hind legs is collected in the isolation medium I (210 mM mannitol, 70 mM sucrose, 50 mM Tris-HCl (pH 7.4), 10 mM EDTA) and digested by trypsin (0.5 mg/g of muscle) for 30 min. The reaction is stopped by addition of trypsin inhibitor (soy bean 3:1 inhibitor to trypsin) and homogenised. The homogenate was centrifuged at $1000 \times g$ for 5 min. The supernatant was strained on gauze and recentrifuged at $7000 \times g$ for 10 min. The resulting pellet was resuspended in ice-cold isolation medium II (225 mM mannitol, 75 mM sucrose, 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA) and a new series of centrifugations ($1000 \times g$ and $7000 \times g$) were performed. The last pellet of mitochondria was resuspended into a minimum volume of isolation medium II in order to obtain a mitochondrial concentration between 20 and 40 mg protein/ml. Protein concentration was estimated by the biuret method using bovine serum albumin as standard.

Respiration studies

Mitochondrial oxygen consumption was monitored at 30°C in a 1 ml thermostatically controlled chamber equipped with a Clark oxygen electrode, in the buffer: 75 mM mannitol, 25 mM sucrose, 100 mM KCl, 10 mM Tris-phosphate, 10 mM Tris-HCl (pH 7.4), 50 μM EDTA, plus respiratory substrate (10 mM pyruvate or 10 μM palmitoyl L-carnitine 10 mM malate). The mitochondrial concentration used for this study was 1 mg/ml. State 3 (according to Chance) was obtained by addition of 2 mM ADP.

For each inhibition curve, the inhibitor was preincubated with mitochondria before the stimulation by ADP.

Enzymatic determination

All the enzymatic determinations were done on mitochondria broken down by osmotic shock, by dilution in a low osmolarity buffer.

Determination of isolated complex I activity (NADH ubiquinone reductase). The oxidation of NADH by complex I was recorded using the ubiquinone analogue ubiquinone$_1$ (UQ_1) as electron acceptor [22]. The basic assay medium (35 mM KH_2PO_4, 5 mM $MgCl_2$, 2 mM KCN (pH 7.2)) was supplemented with defatted BSA (2.5 mg/ml), antimycin (5 μg/ml), 65 μM UQ_1 and 0.13 mM NADH in a final volume of 1 ml. The enzyme activity was measured at 30°C and the reaction started with 50 μg mitochondrial protein. The decrease in absorption due to NADH oxidation was measured at 340 nm both in the absence and in the presence of rotenone 5 μg/ml.

Determination of isolated complex III activity (ubiquinol cytochrome c reductase). The oxidation of ubiquinol (UQ_1H_2) by complex III was determined using cytochrome *c* (III) as electron acceptor [22]. The assay was carried out in basic medium supplemented with 2.5 mg/ml defatted BSA, 15 μM cytochrome *c*(III) and rotenone (5 μg/ml) in a final volume of 1 ml at 30°C. The reaction was started with 10 μg of mitochondrial protein and the enzyme activity was measured at 550 nm.

Determination of isolated complex IV activity (cytochrome c oxidase). Two methods were used for the determination of this step: first, the activity was measured using the method described by Wharton and Tzagoloff [23]. Cytochrome oxidase activity was determined spectrophotometrically using cytochrome *c*(II) as substrate. The oxidation of the cytochrome *c* was monitored at 550 nm at 30°C.

In the second method, cytochrome oxidase activity was isolated from the rest of the respiratory chain by means of antimycin, with ascorbate-TMPD as electron donor system. The rate was monitored by the oximetric method described above.

Determination of control coefficients

The control coefficients of the various steps involved in the oxidative phosphorylations were determined with specific inhibitors of these steps, according to the definition:

$$C_i = \left. \frac{\frac{\partial \ln J}{\partial I}(I=0)}{\frac{\partial \ln v_i}{\partial I}(I=0)} \right|_{\text{steady state}}$$

i.e., the ratio of the initial slope (I) of the inhibition curve of the whole flux J to the initial slope (I) of the inhibition curve of the isolated step v_i in the same conditions as in the pathway. In practical terms, the control coefficient is calculated from the inverted ratio of the intercept of the two slopes with the x-axis. In some cases, nevertheless, it is impossible to determine the activity of the isolated step in the same conditions as in the pathway. This is the case for the activity of ATP synthase, for the adenine nucleotide translocator and for the P_i carrier, which are dependent on the level of the $\Delta\mu_{H^+}$ generated by the respiratory chain. In these cases, the method presented by Gellerich et al. [20] was used. This method is based on non-linear regression to fit the experimental data to a model assuming non-competitive inhibition. The equation of the model involves the dissociation constant of the inhibitor, the concentration of the titrated enzyme and the control coefficient. The non-linear fitting was done using the programme 'Simfit' [24]. This method was

also used in cases where the inhibition curve of the isolated step can be approached experimentally, to determine more accurately the initial slope of the inhibition curve of the whole flux. In these latter cases, the parameters determined with the inhibition curve of the flux are also used to draw the theoretical inhibition curve of the isolated step.

Results

Control of complex I activity

We used the specific inhibitor rotenone to inhibit complex I activity.

The model of Gellerich et al. [20] does not give a good fit for the inhibition curves of respiration by rotenone. Thus, the initial slopes were simply calculated by linear regression on the first points. The corresponding control coefficients are calculated to be

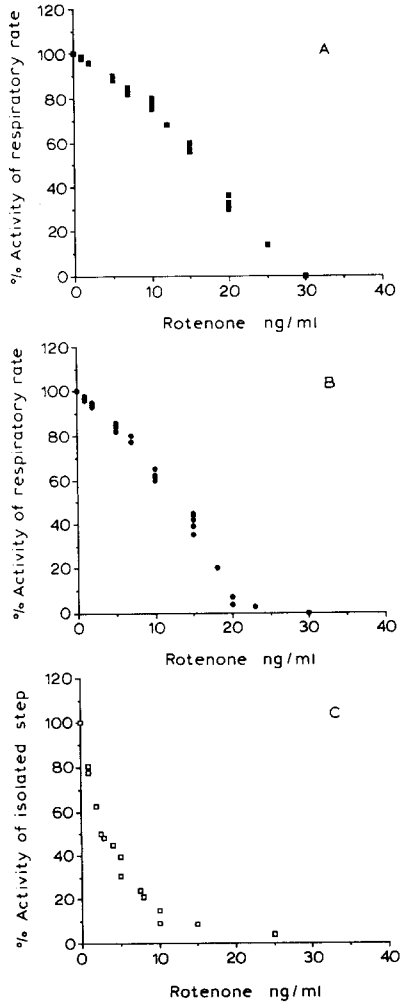

Fig. 1. Inhibition curves by rotenone. Inhibition of O_2 consumption flux with pyruvate (+malate) (A) or palmitoyl-L-carnitine (+malate) (B) as respiratory substrate. Inhibition of complex I activity (C). The conditions for measuring complex I activity are described in Materials and methods.

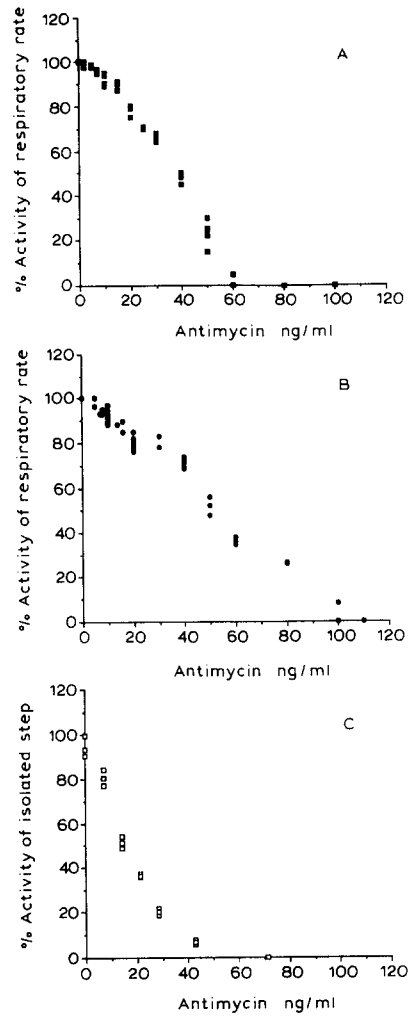

Fig. 2. Inhibition curves by antimycin. Inhibition of O_2 consumption flux with pyruvate (+malate) (A) or palmitoyl-L-carnitine (+malate) (B) as respiratory substrate. Inhibition of complex III activity (C). The conditions for measuring complex III activity are described in Materials and Methods.

0.11 and 0.14 for respiration with pyruvate and palamitoylcarnitine, respectively.

Control of complex III activity

We used the specific inhibitor antimycin to inhibit complex III activity.

Once again, the model does not give a good fit of the inhibition curves of respiration by antimycin. The initial slopes were simply calculated by linear regression on the first points as above. The calculated control coefficients are thus 0.22 and 0.23 for respiration with pyruvate and palmitoylcarnitine, respectively.

Control of complex IV (cytochrome c oxidase) activity

In the study of the control of the cytochrome c oxidase activity, the inhibitor used was KCN.

The inhibition curves for this inhibitor of oxygen consumption with pyruvate (+malate) and palmitoyl-

L-carnitine (+malate) as respiratory substrates are shown in Fig. 3A and B, respectively. The initial slopes of the inhibition curves of the whole respiratory flux can be estimated from the first points. We obtained 40 μM and 32.5 μM, respectively, as intercepts of the initial slope with the x-axis.

We were able to measure the inhibition of the isolated step using two methods. The step was first isolated from the respiratory chain by means of antimycin, with TMPD + ascorbate as an electron donor system. The cytochrome c oxidase activity was measured by the rate of oxygen consumption (Fig. 3C) and the initial slope found to intercept the x-axis at 7.5 μM KCN. The activity of cytochrome c oxidase can also be recorded spectrophotometrically by following the oxidation of reduced cytochrome c at 550 nm. This curve is very similar to the previous one (Fig. 3C) and once again, the initial slope is found to intercept the x-axis at 7 μM KCN (not shown). The calculated control coefficients are thus 0.19 and 0.23, respectively. Both curves have been also fitted with the model and give control coefficients of 0.17 and 0.22, respectively.

The theoretical inhibition curves have been drawn in Fig. 3A and B using the parameters listed in Table I. Using these parameters, we have also drawn the theoretical inhibition curves of the isolated step. It can be seen in Fig. 3C that these curves fit the experimental points well.

Control coefficient of the ATP synthase system

The system of ATP synthesis involves three main steps: the ATP synthase itself and two carriers, the P_i carrier and the adenine nucleotide translocator. All these steps require the existence of a $\Delta\mu_{H^+}$. This means that, in practical terms, it is impossible to study the isolated step, and particularly its inhibition, by a specific inhibitor. The method of Gellerich et al. [20] is

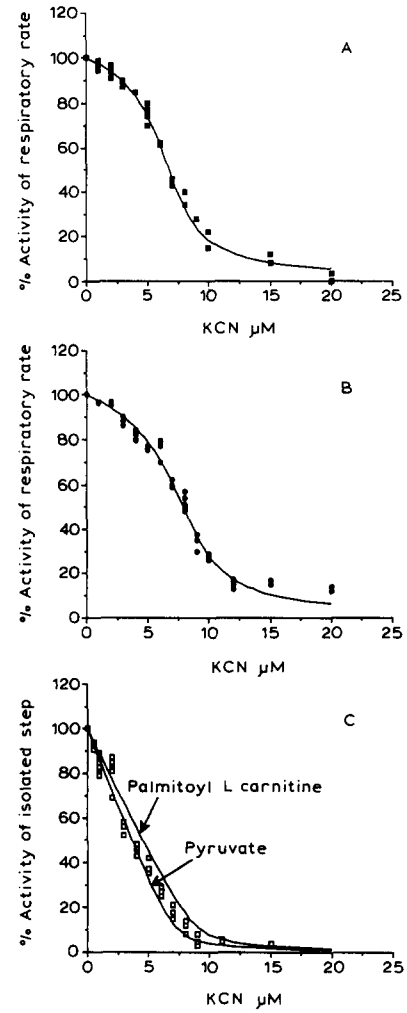

Fig. 3. Inhibition curves by KCN. Inhibition of O_2 consumption fluxes with pyruvate (+malate) as respiratory substrate (A) or palmitoyl-L-carnitine (+malate) as respiratory substrate (B). Inhibition of complex IV activity (C). The conditions for measuring complex IV activity are described in Materials and Methods. The theoretical curves are drawn according to the values listed in Table I.

TABLE I

Control coefficients and model parameters

The values of the parameters have been calculated according to the model of Gellerich et al. [20] using the non-linear fitting procedure of Holzhütter and Colosimo [24], except in the case of complexes I and III, for which a direct determination of the control coefficients was done. All the K_d and E_o values are expressed in μM.

	Pyruvate-malate			Palmitoyl-L-carnitine-malate		
	K_I	E_o	C_i^{resp}	K_I	E_o	C_i^{resp}
Complex I	–	–	0.11	–	–	0.14
Complex III	–	–	0.22	–	–	0.23
Complex IV	0.122	7.1	0.17	0.165	8.77	0.22
Translocase	0.010	1.18	0.09	0.024	1.40	0.10
P_i carrier	0.022	7.87	0.08	0.029	5.89	0.17
ATPase	0.56 E-3	0.031	0.07	0.80 E-3	0.049	0.11
			$\Sigma = 0.73$			$\Sigma = 0.97$

the only one we can use in these cases. The experimental points and the theoretical inhibition curves drawn with the model are shown in Figs. 4 to 6. The values of the parameters used are indicated in Table I.

Discussion

Comparative value of the control coefficients

Table I summarizes the control coefficient values determined in this work. Two main overall features are apparent from this table. Firstly, and most strikingly, control is broadly distributed among all the steps of the oxidative phosphorylation. No control coefficient much exceeds 0.2 (0.23 at the most), and none is much less than 0.1 (0.07 at the least). This means that the control of the oxidative phosphorylation in muscle is not confined to one or a few particular step(s). Secondly, the control coefficient values are systematically lower when the respiratory substrate is pyruvate (+malate) rather

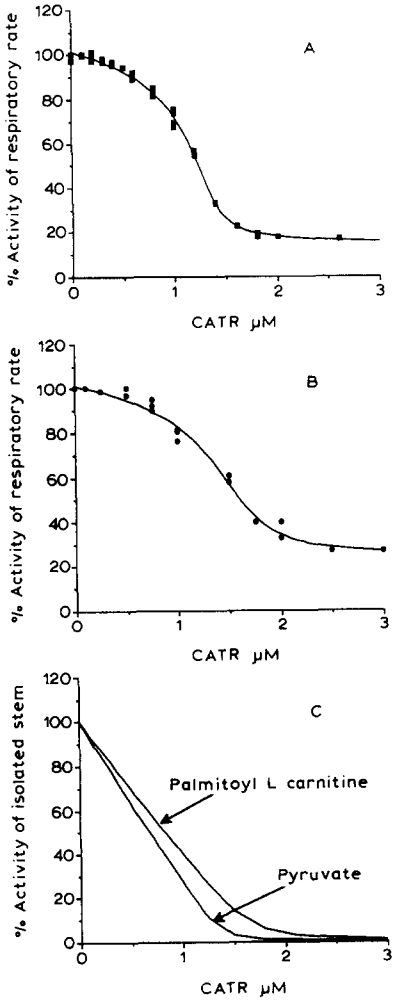

Fig. 4. Inhibition curves by carboxyatractylate. Inhibition of O_2 consumption flux with pyruvate (+malate) (A) or palmitoyl-L-carnitine (+malate) (B) as respiratory substrate. Theoretical inhibition curves of adenine nucleotide translocator activity (C). The theoretical curves are drawn according to the values listed in Table I.

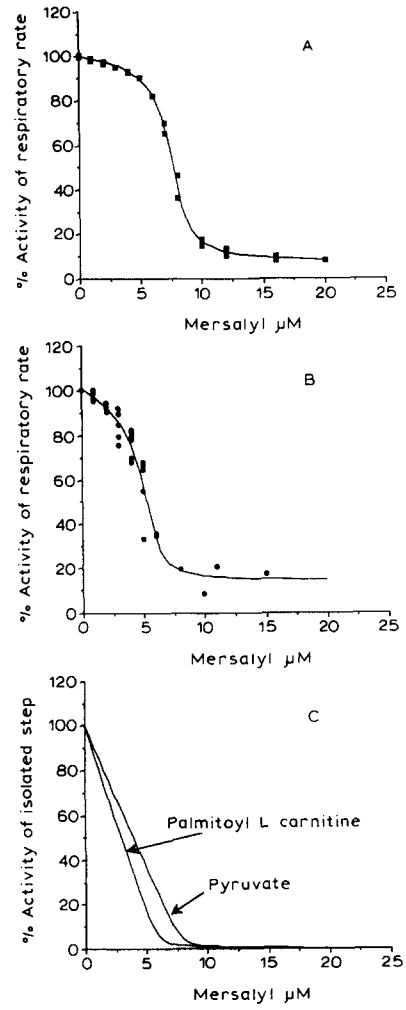

Fig. 5. Inhibition curves by mersalyl. Inhibition of O_2 consumption flux with pyruvate (+malate) (A) or palmitoyl-L-carnitine (+malate) (B) as respiratory substrate. Theoretical inhibition curves of phosphate carrier activity (C). The theoretical curves are drawn according to the values listed in Table I.

than palmitoylcarnitine (+malate). The explanation of this difference can be found in the difference in the steady-state. The rate of respiration with pyruvate is around 300 natom O/min per mg protein, whereas the same variable is only around 80 natom O/min per mg protein when palmitoyl-carnitine is used at a concentration of 10 μM. Thus, the total control coefficient is less than one with pyruvate as a respiratory substrate. In this case, the missing control can be found at the level of pyruvate transport or of pyruvate dehydrogenase.

Method of Gellerich et al. and difficulties in the control measurements by the inhibitor method

We have already [6] pointed out the difficulties in determining control coefficients by the inhibitor method. For this reason, the method developed by Gellerich et al. [20] was of great help in calculating

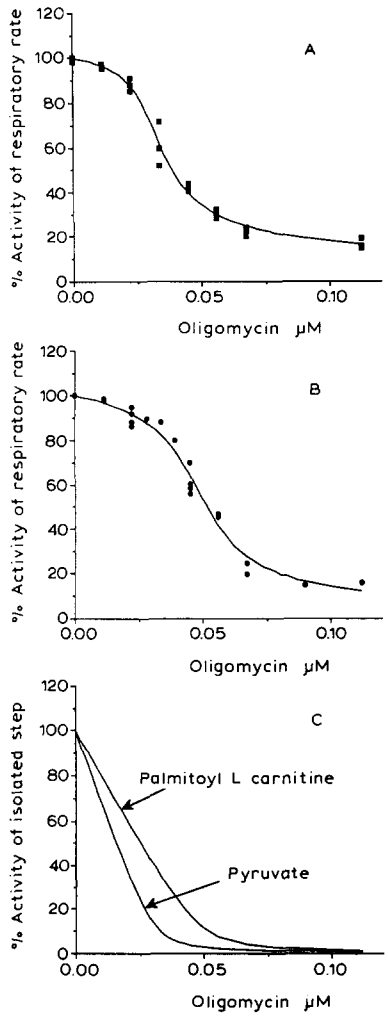

Fig. 6. Inhibition curves by oligomycin. Inhibition of O_2 consumption flux with pyruvate (+malate) (A) or palmitoyl-L-carnitine (+malate) (B) as respiratory substrate. Theoretical inhibition curves of ATP synthase activity (C). The theoretical curves are drawn according to the values listed in Table I.

these values and was used extensively in this study. The main advantage of this method is that it uses the whole inhibition curve to calculate the control coefficient. Our previous calculations of the control coefficients were done using linear regression on the first points. From a comparison of the two methods, it appears that the initial slope is usually overestimated by linear regression on the first points, leading to higher control coefficient values.

In some cases, we have applied the Gellerich et al. method and also calculated the control coefficient directly by determining the inhibition curve of the isolated step. This was the case in the determination of the control coefficient of the cytochrome oxidase (Fig. 3). The methods give nearly identical results. This is confirmed by drawing the theoretical curves according to the model, using the parameters determined by the fitting procedure. It must be pointed out that this procedure was applied to the inhibition curves of the whole respiratory flux, to determine a set of parameters, especially the concentration of the complex (here the cytochrome *c* oxidase) in terms of inhibitor binding sites, and the inhibitor dissociation constant for these sites. With these parameters it is possible to draw the theoretical curve of the inhibited isolated step (Fig. 3C) which fit the experimental results well.

The same assay was not so successful when applied to our results with the inhibition of complex I by rotenone and of complex III by antimycin, contrary to the work of Gellerich et al. with rat liver mitochondria [20]. These last cases reveal some limitations of the method, probably because the inhibition equation assumed in the model does not correspond to those applicable to rotenone and antimycin. Fortunately in these cases, it has been possible to determine separately the inhibition curve of the isolated step.

Implications for mitochondrial diseases

In mitochondrial myopathies, Wallace [18,19] has pointed out that the clinical expression of a mutation can present a very sharp threshold as a function of the relative amount of mutated mtDNA.

The threshold effect can be summarized by these two points:

(1) Only a small amount of a given activity is enough to sustain a quasi-normal respiration flux.

(2) A small decrease in this value however brings about an abrupt collapse in respiration.

This observation must be correlated with the sigmoidal aspects of the inhibition curves of the whole flux compared with the hyperbolic-type inhibition curve of the isolated steps. For instance, comparing Fig. 3A and B with Fig. 3C shows that when the activity of the (isolated) step decrease to below 10%, the respiration flux declines abruptly, giving rise to a threshold effect.

Our results show that very little activity at a step can nevertheless sustain an appreciable flux of respiration (and thus of ATP synthesis), which explains the threshold effect observed by Wallace when the activity of the step is further decreased.

These observations illustrate the value of metabolic control analysis in the understanding not only of the normal behaviour of metabolic pathways, but also of the phenotypic and clinical consequences of metabolic defects.

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