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Review

What do mitochondrial diseases teach us about normal mitochondrial functions... that we already knew: threshold expression of mitochondrial defects

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Abstract

This paper shows how metabolic control analysis (MCA) can help to explain two important features of mitochondrial diseases: (i) the existence of a threshold in the expression of the complex deficiencies on the respiratory flux or on ATP synthesis, i.e. the fact that it is necessary to have a large complex deficiency in order to observe a substantial decrease in these fluxes; (ii) the tissue specificity, i.e. the fact that all tissues are not affected, even if the complex deficiency is present in all of them. We also show the limits of MCA, particularly when considering the in vivo situation. However, MCA offers a new way to consider mitochondrial diseases. The fact that fluxes only slightly change, when a complex is affected, is done at the expense of great changes in intermediate metabolite concentrations; intermediate metabolites situated upstream from the deficient complex are more reduced, leading to a greater generation of free radicals. This could bring an explanation for the diseases observed in conditions where the mitochondrial rate of ATP synthesis is only slightly affected.

Keywords: Inborn error of metabolism; Mitochondria; Oxidative phosphorylation; Mitochondrial diseases; Metabolic control analysis

1. Introduction

One of the first paradoxes, which early appeared in the study of mitochondrial diseases, is that several unrelated tissues can be affected, leading to an unexpected association of neuromuscular and/or non-neuromuscular symptoms (mitochondrial myopathy, peripheral neuropathy, encephalopathy and gastrointestinal diseases (MNGIE), neurogenic muscle weakness, ataxia, retinitis pigmentosa (NARP), diabetes and deafness...) [1–5]. This is well understandable when taking into account the simple fact that nearly all tissues contain mitochondria. However, such an argumentation should lead to the obvious conclusion that all tissues should be affected. This apparent contradiction emphasizes several well-known, but often forgotten properties of mitochondria.

First, mitochondria play different roles in cell physiology not only to supply energy to the cell but also to maintain the redox potential, to store an appreciable amount of calcium [6–8], to modulate calcium signals [9–11], to produce heat [12] and free radicals [13], to introduce cells in apoptosis [11,14,15], etc. A mutation can more or less affect one or the other of these functions and thus at the

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same time the tissues which depend on it. Second, the diversity of mitochondria themselves, already obvious in electronic microscopy observations [16]: liver mitochondria differ from heart mitochondria which are different from muscle and brain mitochondria. Mitochondria from some other tissues are still more specific (adipose tissues, spermatozoon) [16]. Thus it is difficult to admit, although not impossible, that all types of mitochondria in different tissues uniformly perform the same function of ATP production.

Third, the dual origin of the oxidative phosphorylation complex subunits, encoded by nuclear or mitochondrial DNA (mtDNA). This implies a complex regulation of nuclear vs. mitochondrial gene expression (gene dosage), which, although out of the scope of this paper, has to be kept in mind. This leads to the fourth point, the particularities of mitochondrial genetics, i.e. the nearly complete maternal inheritance of mtDNA and the important concept of *heteroplasmy* of mtDNA mutations (Fig. 1): most of the cells contain hundreds of mitochondria, each containing two to ten mitochondrial DNA; it means that each cell contains thousands of mtDNA, i.e. thousands of copies of the genes coding for the mtDNA encoded subunits of oxidative phosphorylation complexes (in comparison to only two copies of the nuclear DNA encoded subunits of the same complexes!). When there is a mtDNA mutation, not all the mtDNA molecules necessarily carry the mutation, so that a mixture of mutated and wild-type mtDNA coexists in the same cell, or even in the same mitochondria. The heteroplasmy is defined as the percentage of mutated mtDNA over the total mtDNA molecules. Due to the great number of mtDNA molecules in a cell, heteroplasmy of mtDNA mutations can vary, quasi-continuously, between 0 (no mutation, normal situation, also called homoplasmy) to 100%. Heteroplasmy is not necessarily the same in all cells, in all tissues and in all the members of the same family. The heteroplasmy, with its large natural variations, often in the same patient, has immediately formulated the question of the expression of mtDNA mutations in quantitative terms, thus logically leading to consider metabolic control analysis (MCA) as an appropriate tool. This led to the emergence of another concept, the concept of *threshold* in the expression of mitochondrial defects as a function of the intensity of the defect.

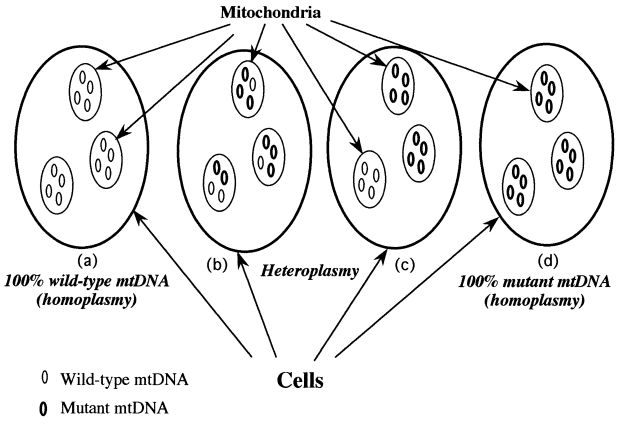

Fig. 1. Heteroplasmy of mutant mtDNA. Different cells (large circle) with different percentages of mutant mtDNA (small bold circles) in their mitochondria (intermediate circles). The nucleus is not represented. Note the difference in the two cases of heteroplasmy: in (b), all mitochondria contain wild-type and mutant mtDNA; in (c) mitochondria contain only one type of mtDNA, either wild-type or mutant.

1.1. The threshold in the expression of mtDNA mutations

It rapidly appeared that the expression of a mtDNA mutation is not proportional to its degree of heteroplasmy, i.e. that it is necessary to have a high degree of heteroplasmy (sometimes more than 90%) in order to observe the clinical signs of the pathology. Low levels of heteroplasmy in any tissue or more generally in a subject give no sign of disease. This is also true at the level of individual cells [17]. In 1986, D.C. Wallace already described this phenomenon for a mutation located in the mitochondrial 16S RNA coding gene and conferring chloramphenicol resistance [2,18]. Indeed, human hybrids with less than 10% of mutated mtDNA were killed in the presence of chloramphenicol whereas hybrids with 12% mutated mtDNA could grow in the presence of the drug.

In MERRF (myoclonic epilepsy with ragged red fibers) patients, the complete range of phenotypic and biochemical variation from normal to severely affected was found in individuals carrying between 27% and 2% wild-type mtDNA coexisting with mutated mtDNA [19]. In this case, a small percentage of

normal mtDNA appears to be enough to maintain a normal phenotype. The same observation was reported by Attardi's group on cell lines in the case of MELAS (mitochondrial encephalopathy, lactic acidosis and stroke-like episodes) 3243 mutation [20,21]; they evidenced a threshold in heteroplasmy around 90%, before which the respiratory rate was completely normal and after which it decreased very rapidly. In other words, 10% of wild-type mtDNA were sufficient to sustain a normal respiratory rate.

In [22] Porteous et al. constructed a series of cybrids containing 0–86% of a CPEO (chronic progressive external ophthalmoplegia) patient with the 4977 bp common deletion (ΔmtDNA). In cybrids containing less than 50–55% of ΔmtDNA, the mitochondrial potential, the rate of ATP synthesis and the ATP/ADP ratio were the same as in the cybrids with intact mtDNA. However, once the proportion of ΔmtDNA exceeded this threshold, these bioenergetic parameters decreased.

The question thus arises of the mechanism of this threshold expression.

Fig. 2 depicts all the steps leading from the genes encoding a complex to the flux of respiration and of ATP synthesis. At each step a compensatory mechanism could occur explaining that the phenotypic expression does not linearly follow the degree of heteroplasmy. To locate the relevant step(s), it is necessary to measure, for a given degree of heteroplasmy, the amount of normal mRNA, possibly of tRNA and rRNA, the amount of each complex, its activity and the global fluxes of oxygen consumption and ATP synthesis. Few studies are available with the simultaneous measurements of all these parameters.

One of them comes from the laboratory of Serge Alziari in Clermont-Ferrand [23,24] and concerns a deletion in the mitochondrial DNA of a *Drosophila* species. The results exhibit a slight elevation of the wild-type compounds at each step of the process: although the percentage of wild-type mtDNA is only 30% of total mtDNA, the percentage of a mRNA corresponding to genes involved in the deletion ranges from to 35 to 55% of the corresponding normal mRNA content. The activities of complexes I and III, some subunits of which are involved in the deletion, are 60% and 70% respectively of the normal activity. Finally, this ends up with a quasi-normal recovery of the respiratory flux (for a complete analysis of this case see [25]).

Another report comes from the laboratory of van den Bogert in Amsterdam [26] in the case of a Pearson syndrome, and essentially shows the same pattern: 40% of mtDNA deletion, an amount of 40 and 50% of two mRNAs involved in the deletion compared to the normal level of these mRNAs, 81–88% of cytochrome *c* oxidase activity and a normal rate of ATP synthesis.

More recently [27], Bai et al. showed that in a series of mouse cell lines carrying a nonsense mtDNA mutation for the ND5 subunit containing between 4 and 100% of the normal number of wild-type ND5 genes, the functional mRNA level

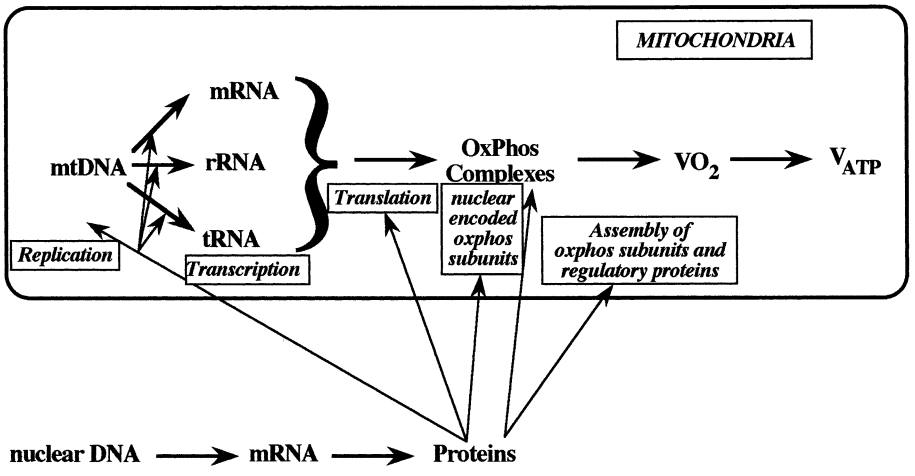

Fig. 2. Expression of mtDNA and nuclear mutations in mitochondria.

was decreased in proportion to the number of wild-type ND5 genes; however, cells carrying 60% of wild-type mRNA were capable of carrying out a normal rate of translation indicating that 40% of ND5 mRNA is in excess.

In a very interesting paper, Heddi et al. [28] show that in a case of a MELAS patient, the analysis of transcripts in several tissues shows a strong increase only when the percentage of mutant mtDNA is 90% or more. This increase not only concerns the mitochondrial transcript but also some nuclear transcripts such as the ones of ATP synthase β subunit, the heart muscle isoform of the adenine nucleotide translocator, muscle mitochondrial creatine kinase, muscle glycogen phosphorylase, hexokinase I, muscle phosphofructokinase, the E1α subunit of pyruvate dehydrogenase and the ubiquinone oxidoreductase. In this patient's kidney, the percentage of mutant mtDNA is only 73% and the amount of messenger RNA is similar to that of the controls. The transcript levels in another MELAS patient with 50% mutant mtDNAs and in a MERRF patient with 78% mutant mtDNAs were not particularly elevated. These few reports, and particularly the last one, seem to indicate that until a large threshold in mutant mtDNA heteroplasmy (70%) – a threshold until which the respiratory flux and the ATP synthesis are weakly affected – the increase of the normal mitochondrial transcripts is not particularly elevated (if any). Thus the main compensatory mechanism responsible for the maintenance of the fluxes has to be searched probably after these steps, in the oxidative phosphorylation network itself.

1.2. *The threshold in the expression of complex deficiencies*

It is rather easy to mimic the effects of oxidative phosphorylation complex deficiencies because specific inhibitors are known for all of them. In other words, it is thus possible to observe the effects on respiration or on ATP synthesis of a modulation of respiratory chain complex activities induced by specific inhibitors. So we did for all the different complexes of respiratory chain in rat muscle mitochondria [29,30]. Fig. 3 shows the effect of cyanide inhibition on cytochrome *c* oxidase activity alone and on the respiration. It can be seen that even at 50% cytochrome *c* oxidase inhibition one only observes a very weak inhibition of the whole flux and one has to go as far as 80–90% inhibition of the isolated step in order to obtain a substantial inhibition of the respiration. This is more obvious on the representation in Fig. 3B, called 'threshold plot', where we have plotted the percentage of the respiratory flux as a function of the cytochrome *c* oxidase inhibition for the same cyanide concentrations. What we observe is a clear-cut threshold: until 70% inhibition of cytochrome *c* oxidase activity the respiratory rate decreases slowly and linearly, but beyond 70% of cytochrome *c* oxidase inhibition the respiratory rate abruptly falls to reach the zero level.

A similar pattern is observed for all the oxidative phosphorylation complexes [31,32].

This behavior is clearly explained in the framework of the metabolic control theory [33–35]. The ratio of the initial slopes of the two curves of Fig. 3A measures the control coefficient of cytochrome *c* oxidase on oxygen consumption flux; this control coefficient is also measured by the initial slope at the 'threshold curve' of Fig. 3B (the control coefficient is defined as the change in the flux divided by the causal change in the step; these changes are reflected in the initial slopes to the inhibition curves in Fig. 3A; the control coefficient is also exactly the initial slope to the threshold curve in Fig. 3B). Because most of the control coefficients are found experimentally small (of the order of 0.1–0.2 in the case of muscle [29], presumably partly due to the summation theorem), such threshold curves with an initial plateau are inescapable: we must have (see Fig. 3), at the beginning, a weak slope due to the low control coefficient. On the contrary, at a very low activity of the step both curves must meet again, due to the fact that the flux becomes zero when the step is being inactivated. This unavoidably leads to a sigmoid inhibition shape of the flux inhibition curve and to a threshold effect when the flux is plotted as a function of the inhibition of one of its steps.

This behavior has already been observed for the cytochrome *c* oxidase activity (COX activity). For instance, Bindoff and Turnbull in Newcastle [36] observed that both in a patient with cytochrome *c* oxidase deficiency and in an animal model, a copper-deficient rat, lowering the activity of complex IV by over 50% did not affect the respiratory flux. More

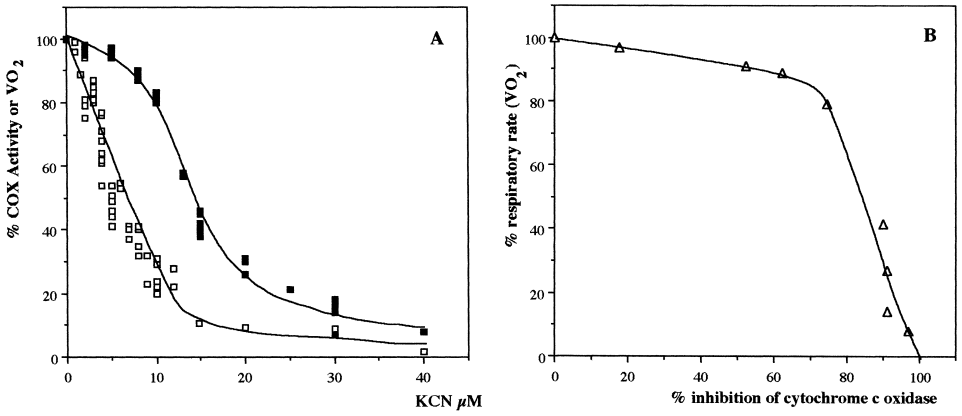

Fig. 3. (A) KCN inhibition of respiratory rate (■) with 10 mM pyruvate+10 mM malate as respiratory substrate and of cytochrome *c* oxidase activity using ascorbate/TMPD as substrate (□) in the presence of 2 mM ADP. (B) Respiratory rate as a function of cytochrome *c* oxidase inhibition. The points are the means of the data of panel A, corresponding to the same KCN concentrations. (Reproduced with permission from the authors, Biochemical Journal 302 (1994) 171–174. Copyright holder: the Biochemical Society [30].)

recently, Kuznetsov and Kunz [37] showed that, in a mouse mutant with a severe copper deficiency, the activity of COX is only about one half of the normal activity, but that no difference was found in maximal rates of respiration; however, the control coefficient was higher in the mutants (0.8 instead of 0.30 for the control value), indicating a greater sensitivity to any variation.

Davey and Clark [38] also evidenced a distribution between several steps of the control of oxidative phosphorylation in non-synaptic rat brain mitochondria. They found a control coefficient of 0.14 for complex I, of 0.15 for complex III and of 0.24 for complex IV. More interestingly, they drew the 'threshold curves' and found high threshold values of 72%, 70% and 60% respectively. In a further work [39,40] they compared the control of non-synaptic and synaptic rat brain mitochondria and reported big changes in the activities, control and threshold. When passing from non-synaptosomal to synaptosomal mitochondria, the activity of complex I (scaled by citrate synthase activity) is reduced by a factor of 1.2. On the contrary, complex IV activity (also scaled by citrate synthase activity) is increased by a factor of 2. They observed, as expected, a strong increase of the flux control coefficient of complex I (from 0.13 to 0.30) and a decrease of complex IV control coefficient (from 0.20 to 0.15). A very low threshold (25%) is associated to the higher control coefficient of complex I in synaptosomal mitochondria, suggesting that even for weak decrease of complex I activity energy metabolism should be severely impaired [40,41].

It should be pointed out that this study of a biochemical threshold applies equally well to mitochondrial mutations and to nuclear ones; it only takes into account the defect in a given complex and not the origin of the defect itself.

1.3. Tissue specificity in the control of oxidative phosphorylation

These studies emphasize one of the important consequences of the summation theorem in metabolic control theory, namely the fact that the distribution of the control of a pathway among its different steps can vary according to several factors and more particularly to the steady state, the tissue and the comparative amount of the different activities involved in the pathway. All these three factors can vary when comparing different tissues. That is the reason why we undertook a systematic study of the control coefficients and the associated threshold of seven steps of oxidative phosphorylation in five tissues: muscle, heart, brain, liver and kidney [31,32]. We evidenced two tissue groups, each characterized by similar threshold values: the muscle and the heart on the one hand, which are more controlled by the respiratory chain, and the kidney and the brain on the other hand which are more controlled by the ATP synthesis machinery (ATP synthase, P_i carrier and ATP/ADP translocator). The liver can be associated to

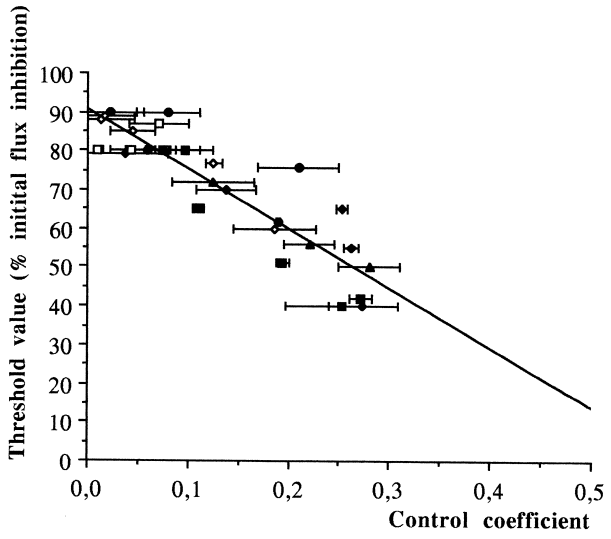

Fig. 4. Threshold values as a function of control coefficient values. The data are taken from [31] and [32]. ♦, complex I; ●, complex III; ◇, complex IV; ■, ATPase; □, ATP/ADP translocator; ▲, P_i carrier. The correlation coefficient is 0.90.

either one or the other of these two groups according to the complex. A correlation can be obtained when plotting the threshold value as a function of the corresponding control coefficient for the same complex in the same tissue. Although there is no mathematical reason for such a correlation, it is not unexpected that the lower the control coefficient, the greater the threshold value. However, it can be seen that different threshold values can be associated with the same control coefficient; this is due to the fact that control coefficients are defined locally in the linear neighbourhood of the initial steady state, while threshold values correspond to bigger changes (Fig. 4).

We observed that the threshold curves can present two kinds of shape, which we called type I and type II [31]. Type I curves are characterized by a large plateau associated with a great threshold value. In type II curves, a plateau is no longer apparent and a threshold is hardly defined. The plateau of type I curves is due to an excess of enzyme capacity which has to be suppressed before an effect on the flux would be appreciable. In type II curves, the enzyme reserve is small and the curve only reflects the adjustment of the metabolites inside the network which buffer the changes in the complex activity. As a matter of fact, type II curves correspond to the last part of type I curves beginning just before the threshold point (Fig. 5).

The variation in biochemical threshold values for a given complex according to the tissue origin of the mitochondria suggests an explanation (among others) of the tissue specificity observed in mitochondrial cytopathies. For a given oxidative phosphorylating complex, the lower the threshold value in a tissue, the more sensitive this tissue to a defect of this complex. For example, we showed [31] that an 80% decrease in cytochrome *c* oxidase activity will induce a small decrease in mitochondrial respiration in liver, while the respiration will be decreased to 40% in heart mitochondria. This work also shows that it is possible to pass from one type of threshold curve to the other for the same complex (here the ATP/ADP translocator in liver) depending upon the respiratory substrate (pyruvate or succinate). This has an obvious application to mitochondrial diseases because different tissues do not use preferentially the same respiratory substrate (pyruvate, fatty acids, ketone bodies, etc.) and thus for the reason stated above will not be affected by the same defect to the same extent. This adds another reason to observe tissue specificity in mitochondrial diseases.

It must be noted that most of the threshold values are high ($>50\%$). This phenomenon could be a way to provide a safety margin for oxidative phosphorylation against a defect in one or several of its complexes. This observation can be correlated with the fact that in most patients with clinical features of mitochondrial cytopathies of mtDNA mutation ori-

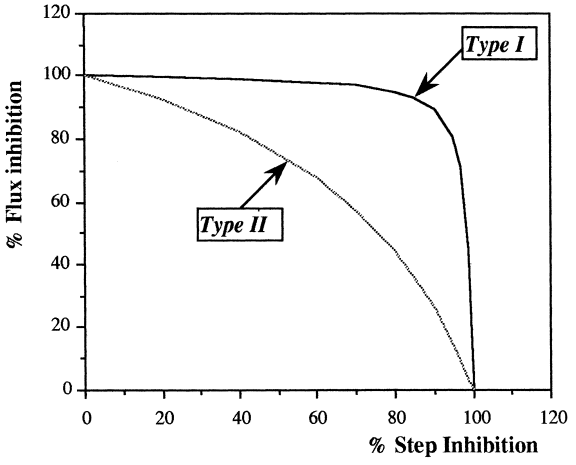

Fig. 5. Threshold curves of types I and II.

gin, the proportion of mutant mitochondrial DNA has to be rather high in order to observe a phenotypic effect.

1.4. The control of oxidative phosphorylation in whole cells

Villani and Attardi [42,43] analyzed the control of respiration by cytochrome *c* oxidase in intact cultured human osteosarcoma 143B.TK$^-$ cells, the hepatoma HepG2 cell line, lung carcinoma A-549, the neuroblastoma SK-N-SH cell line, the SKO-007 (J3) myeloma cell line and in mitochondrial DNA mutation-carrying human cell lines. They compared the uncoupled endogenous respiration of the whole chain to the respiration of cytochrome *c* oxidase isolated from the rest of the chain with antimycin and fed with ascorbate and TMPD (*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine). Surprisingly they found low threshold values (20% or less) in all types of normal cell lines, especially in the 143B.TK$^-$, indicating that cytochrome *c* oxidase capacity could be in low excess in intact cells and thus exert a tighter control on oxidative phosphorylation than could be assumed on the basis of experiments carried out on isolated mitochondria. They confirm these results on digitonin-permeabilized cells respiring on glutamate/malate.

Wisniewski et al. [44] studied the control of oxidative phosphorylation on saponin-skinned fibers. They stimulated state 3 respiration with calcium which activates actomyosin ATPases. In these conditions they found control coefficient values of 0.16 for mitochondrial ATP synthase, 0.34 for adenine nucleotide translocase, 0.08 for phosphate carrier, 0.01 for complex I and 0.09 for cytochrome *c* oxidase. The inhibition of cytochrome *c* oxidase itself by KCN (Fig. 7 in their paper) evidenced a strong sigmoicity with a large plateau and was very similar to the inhibition of respiration by KCN of isolated muscle mitochondria in Fig. 3A. One can infer from this curve a similar threshold value for the corresponding threshold curve. It means that in these experiments, mitochondria in permeabilized fibers do not behave differently from isolated mitochondria.

The discrepancy between the results reported by the group of Attardi and the group of Kunz [44] may have different reasons. First, the material on which they work is very different. Attardi's group mainly works with tumor cells, which are probably more glycolytic than aerobic; they may have a perturbated oxidative metabolism with particularly low levels of some of the respiratory chain complexes. Second, they stimulate respiration by uncoupling, unlike Kunz's group who stimulates the respiration of their permeabilized fibers by the demand in increasing ATP turnover with actomyosin ATPase activity. This is probably a more physiological situation; it can certainly be thought that, in these conditions, the demand in ATP is a strongly controlling step and indeed, Wisniewski et al. measured a control coefficient of 0.5 for actomyosin ATPase. Third, one cannot exclude that in permeabilized fibers the organization of the cytoskeleton or the binding of mitochondria to the cytoskeleton has been perturbed so that the control in the permeabilized fibers resembles more that of isolated mitochondria and is not representative of the physiological situation; however, all the data on permeabilized fibers, including electron microscopy [45,46] and the different accessibility of ADP and other substrates for mitochondria [47,48], do not favor this hypothesis.

Barrientos and Moraes [49] used a drug-induced model (rotenone-dependent complex I inhibition in a human osteosarcoma-derived cell line) and a genetic model of human xenomitochondrial cybrids (HCX lines) to quantify the physiological consequences of a partial inhibition of complex I. At 1 nM rotenone incubation for 4 h in the cell culture medium, they observed a 19% complex I inhibition but still 98% of cell respiration rate. Only when complex I was inhibited by 35–40%, cell respiration began to decrease. HXC have an approx. 40% complex I inhibition and an approx. 80% residual cell respiration. This indicates a comparable control of complex I in whole cells and in isolated mitochondria.

2. Conclusion

2.1. The control of oxidative phosphorylation in isolated mitochondria

Although MCA does certainly not explain all the particular features of the expression of mutations affecting mitochondria, and perhaps does not satisfy

all the criteria in order to be applied to oxidative phosphorylation (see below), it, however, gives a framework in which these features can be logically accommodated or at least discussed and even in some cases rejected. The analysis of the expression of the deficiencies affecting mitochondria in the light of MCA allowed to understand several, at first sight paradoxical, properties.

The fact that in many cases there is not a linear correspondence between the degree of the deficiency and the decrease in the fluxes, particularly the flux of ATP synthesis. MCA explains particularly well the concept of threshold which is now well recognized in the expression of complex deficiencies, in relationship with the concept of control coefficients, although the two concepts are not identical. This concept of threshold is general in the metabolism as stressed by Kacser and Burns [50].

Because in MCA the dependence of the control coefficients on the steady state and on the elasticities of the different steps of a metabolic network has always been emphasized, it prompted us to recognize that the functional and structural differences in the mitochondria from various tissues or organisms can modulate the value of the thresholds; this is an explanation (certainly not the only one) of the observed tissue variation in the expression of mutations affecting mitochondria.

This situation is certainly not specific to mitochondria. A long time ago, it was described by Kacser and Burns [50] on a variety of systems; they have explained in this way the apparent evolutionary paradox of the heterozygote, carrying roughly half of the normal activity of the wild-type homozygote and nevertheless maintaining normal fluxes and they conclude that in the metabolism it cannot be avoided that most of the enzymes appear, each, as if they were in excess.

Note that, in mitochondria, the steady state cannot only change in response to the ATP demand (or in response to other functions) in the cell, but also in response to changes in the respiratory substrate used (or the combination of respiratory substrate (see the paper of Kunz in this issue)).

2.2. Control of oxidative phosphorylation in vivo

One can wonder whether the distribution of the control and the large enzymatic reserves observed in isolated mitochondria can be applied to the in vivo situation. If metabolic control analysis applies to the in vivo situation one can predict that the control values and the threshold may have different values from those in isolated mitochondria but that the main results of the studies on isolated mitochondria remain, i.e. the control will be shared associated with large threshold and large enzymatic reserve of most of the steps in oxidative phosphorylation. The observation in the cases of mitochondrial mutations of the necessity of a large degree of heteroplasmy of mutant mtDNA (often higher than 90%) supports this prediction.

Another prediction of metabolic control analysis for the in vivo situation which should be similar to what has been observed in isolated mitochondria is that the control, the threshold and the enzymatic reserves are certainly different from one tissue to another, due to the different functions ensured by their mitochondria, the different respiratory substrates they use and the different steady state at which they work.

In addition, as shown by Wisniewski et al., it can be expected that in vivo part of the control will be attached to the demand in ATP (control coefficient of 0.5 in their experiments [44]) and also to the supply of respiratory substrates (most of the time the control coefficient for the pyruvate carrier in isolated mitochondria is around 0.2 [32]). Because the sum of the control coefficients is 1, this leaves few control for oxidative phosphorylation complexes themselves. Consequently the thresholds in vivo should be higher than for isolated mitochondria and also the reserve in oxidative phosphorylation complexes.

However, as stressed by Arnold and Kadenbach [51] attention must be paid to a possible loss of essential metabolites and to the absence of cytosolic substrate and coenzyme environment in isolated mitochondria.

2.3. Validity of MCA for analyzing the control of oxidative phosphorylation

These predictions are only valid assuming that MCA applies to describe the control of oxidative phosphorylations. Three assumptions, which lie at the basis of MCA development, may not be com-

pletely fulfilled in the case of oxidative phosphorylation.

The first one supposes that the enzymes catalyzing each step of the network and the metabolites are all free to interact. This is not true for mitochondria, which realize a compartmentation of individual sets of the network. For instance in the case of mtDNA mutations, it is not known if all mitochondria uniformly contain the same number of mutant mtDNA, and this is probably not the case, especially for the high degrees of heteroplasmy. It is also known from histo-enzymology patterns that the distribution of mutant mtDNA is different in different cells of the same tissue. These constraints will modify the expression of the complex deficiencies.

The second one supposes that the enzymes in the network are independent of each other, i.e. do not interact. The existence in some conditions of supercomplexes of respiratory complexes has recently been shown in yeast and in mammals [52,53]. In these conditions the defect in one complex will prevent the electron to proceed in all the pathways attached to the deficient complex. The 'apparent' control coefficients in these cases will be higher, the threshold lower and the enzymatic reserve of any complex participating to the supercomplex will appear lower.

The third one concerns the regulation of oxidative phosphorylation complexes. For a long time, Kadenbach et al. [51] have demonstrated that cytochrome *c* oxidase is regulated by intramitochondrial ATP, accompanied by a change of hyperbolic into sigmoidal kinetics which can strongly inhibit the cytochrome *c* oxidase activity, thus dramatically reducing the reserve in this complex. This situation, which is far from the measurement on isolated mitochondria (with state 3 reached via addition of ADP), can well occur in vivo. More recently, [54], Kadenbach et al. showed that cytochrome *c* oxidase could be phosphorylated in a cAMP dependent manner and dephosphorylated with a mitochondrial protein phosphatase calcium dependently. The phosphorylated cytochrome *c* oxidase once more shows a sigmoidal kinetic as a function of cytochrome *c* concentration, leading to complete inhibition of the activity at low concentration of cytochrome *c*. Once more, one must admit that this situation is not taken into account in measurements on isolated mitochondria in which cytochrome *c* oxidase appears non-phosphorylated.

Another criticism can be addressed to MCA. In principle MCA only applies to small variations of activities, which is not the case for the description of threshold curves. In fact, MCA is the study of sensitivity to perturbations of metabolic networks in the linear neighbourhood of a steady state. The dimension of this neighbourhood depends upon the network and the rate function around the steady state. It can be rather large; in fact the threshold point indicates the limit of this neighbourhood. In Fig. 3B for instance it is very clear that the decrease of respiratory flux is linear until the threshold (70% of COX inhibition) which extends the validity domain of MCA rather far from the steady-state point defined by step activity = 100% and flux = 100%.

2.4. What are mitochondria doing in the cell?

Finally, mitochondrial diseases question the very cellular functions of mitochondria: if in affected tissues the flux of ATP synthesis is unchanged, is actually ATP synthesis the main function of mitochondria? As a matter of fact it appears that in many tissues a great part of ATP can be synthesized by glycolysis; this is the case in liver where, in some conditions, 50% of ATP is synthesized by glycolysis [55], and it is of course the case for rho^0 cells. Thus, the damaging effect of complex deficiencies has to be looked for elsewhere. Once more MCA gives us a working hypothesis: if, for a complex activity change, the fluxes remain unchanged it is not the case for the intermediate metabolites, the variations of which have precisely the role of maintaining the steady state at the same level; in the case of oxidative phosphorylation it means that the redox centers upstream from the defect are more reduced and thus are able to produce more free radicals, engaging the mitochondria and the cell in a vicious circle amplifying the free radical production linked to metabolic defects. Preliminary indications are now reported showing that this could well be the case. Thus even a mild chronic impairment in a complex activity could act as a starting point engaging the cell in the vicious circle of free radicals damaging mitochondria. This point was well demonstrated in the work of Barrientos and Moraes [49]. In their examples of

xenomitochondrial cybrids (human-apes) and of a rotenone-induced model of complex I deficiency, they showed a significant increase in free radical production, which triggers an apoptotic program in conditions where there is no decrease in ATP synthesis. In another work, Esposito et al. [56] demonstrated on mitochondria isolated from mice lacking the heart/muscle isoform of adenine nucleotide translocator (ANT1), that oxidative phosphorylation was inhibited and that markedly increased amounts of reactive oxygen species (ROS) were produced in skeletal muscle, heart and brain, consistently with ANT1 expression in these tissues. On the contrary liver mitochondria, which express a different ANT isoform, produced normal low levels of ROS. This increased production of ROS in muscle and heart was associated with a strong increase in MnSOD (mitochondria specific) in muscle and no increase in heart mitochondria, leading to an increase in mtDNA rearrangements in the heart and not in muscle. This work shows two things: first the role of ROS in damaging mitochondria in conditions where oxidative phosphorylation is inhibited; second a differential tissue susceptibility to ROS due to their different capacity to protect against free radicals.

3. Summary

In summary, MCA is probably too simple to account for the complexity of the expression of oxidative phosphorylation mutations in mitochondrial diseases. However, it afforded the way to understand two particular features of this expression. First the existence of a great threshold in the expression of the amount of the defect on the fluxes associated with an enzymatic reserve, which could or could not exist in vivo (but the problem is now clearly set). Second the existence of differences in the expression of oxidative phosphorylation defects in different tissues, emphasizing the differences in mitochondria for different tissues in their different structural properties and functions.

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