Effect of 'binary mitochondrial heteroplasmy' on respiration and ATP synthesis: implications for mitochondrial diseases

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Respiratory-chain-complex subunits in mitochondria are encoded by nuclear or mitochondrial DNA. This property might have profound implications for the phenotypic expression of mutations affecting oxidative phosphorylation complexes. The aim of this paper is to study the importance of the origin of the mutation (nuclear or mitochondrial) on the expression of mitochondrial defects. We have therefore developed theoretical models illustrating three mechanisms of nuclear or mitochondrial DNA mutation giving rise to a deficiency in the respiratory-chain complex: (1) a partial deficiency, homogeneously distributed in all of the mitochondria; (2) a complete deficiency, only affecting some of the mitochondria ('binary mitochondrial heteroplasmy'); and (3) a partial deficiency, affecting only some of the mitochondria. We show that mutations affecting oxidative phosphorylation complexes will be expressed in different ways depending on their origins. Although the expression of nuclear or mitochondrial mutations is evidence of a biochemical threshold, we demonstrate that the threshold value depends on the origin and distribution of the mutation (homogeneous or not) and also on the energy demand of the tissue. This last prediction has been confirmed in an experimental model using hexokinase for the simulation of the energy demand and a variation in mitochondrial concentration. We also emphasize the possible role of 'binary mitochondrial heteroplasmy' in the expression of mitochondrial DNA mutations and thus the importance of the origin of the deficit (mutation) for the diagnosis or therapy of mitochondrial diseases.

Key words: metabolic control analysis, oxidative phosphorylation, threshold effect.

INTRODUCTION

Mitochondrial oxidative phosphorylation is responsible for several cell functions; for this reason, defects in oxidative phosphorylation complexes can lead to pathologies affecting different physiological systems in human beings [1–5]. The study of mitochondrial pathologies has revealed some precise mutation sites on mitochondrial DNA (mtDNA) or nuclear DNA but at the same time great variability in the phenotypic expression of these mutations [6–9]. This underlines the complexity of the mechanisms by which mutations in oxidative phosphorylation complexes affect mitochondrial functions. Several explanations have been proposed, such as a sporadic mutation in a given stem cell during embryogenesis [6,7,9], mitotic segregation and the degree of heteroplasmy that are responsible for the different levels of an enzymic deficit, and the presence of a biochemical threshold [10–18].

However, the double origin of the oxidative phosphorylating complexes might also have profound implications for the expression of mutations. Indeed, the complexes of oxidative phosphorylation present the unique property of being composed of subunits encoded by either a nuclear or a mitochondrial genome (reviewed in [6,7,9,19]). The aim of this paper is to study the importance of the origin of the mutation on the expression of mitochondrial defects.

Although mtDNA mutations are well documented from the clinical and molecular points of view, the fundamental mechanism of their expression remains difficult to study. In contrast, although few nuclear mutations have been described up to now, their expression is more predictable.

Indeed, a nuclear mutation in a subunit of an oxidative phosphorylation complex will generally lead to a modulation of the activity of this complex with a homogeneous distribution of the deficit among all mitochondria. Experimentally, on isolated mitochondria, this effect has been mimicked with a specific inhibitor, which enables us to decrease the concentration of the active fraction of a given complex gradually from a normal value to zero [10–12,14–18,20].

Such studies have demonstrated a threshold in the degree of inhibition of the complex before which the fluxes (of respiration and of ATP synthesis) are scarcely affected but after which the fluxes rapidly decrease to zero along with the activity of the inhibited complex. The existence of such a threshold has important physiological consequences: (1) the metabolic expression of the mutation is not sensitive until a substantial deficit of the complex (often more than 70% deficit) has been reached; (2) the threshold value for a given oxidative phosphorylation complex is highly dependent on the tissue; (3) these differences in threshold values could explain, at least partly, the tissue specificity of mitochondrial diseases (for a given oxidative phosphorylation complex, the higher the threshold value in a tissue, the more sensitive this tissue is to a defect in this complex). In this case of a homogeneously distributed defect, we developed a computer model of oxidative phosphorylation in isolated mitochondria [21]. The model was able to fit the experimental threshold curves, allowing us to use it to predict the threshold pattern of oxidative phosphorylation under more physiological conditions (low oxygen concentrations and state 3.5 with hexokinase), which are difficult to assess experimentally [21,22]. The model was also used to study the regulation of ATP supply during muscle exercise [23,24]. In this way the combination of theoretical and experimental tools led us to a better understanding of the expression of homogeneous deficits affecting oxidative phosphorylation.

Abbreviation used: mtDNA, mitochondrial DNA.

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The study of the metabolic expression of mutations affecting mtDNA is more difficult, owing to the particularities of mitochondrial genetics [6,7,9,25–28]: (1) one cell usually contains several thousand copies of mtDNA, and thus several thousand copies of mtDNA-encoded genes, in comparison with the two examplars of the nuclear DNA-encoded genes; (2) in mitochondrial pathology it is possible to have a heteroplasmy of mtDNA, i.e. the coexistence in the same cell of wild-type and mutated mtDNA; (3) mitotic segregation of the mtDNA can change the degree of heteroplasmy in the different tissues of the same patient. Experimentally, the difficulty of mastering these parameters makes the study of the expression of these mutations almost impossible.

To cope with this problem and to take these properties of mitochondrial genetics into account, we developed theoretical models illustrating three mechanisms of mtDNA mutation giving rise to a deficiency in respiratory-chain complexes: (1) a partial deficiency, homogeneously distributed in all of the mitochondria; (2) a complete deficiency, only affecting some of the mitochondria ('binary mitochondrial heteroplasmy'); and (3) a partial deficiency, affecting only some of the mitochondria. The first model is equivalent to the model that we developed previously to study the expression of nuclear mutations homogeneously affecting all mitochondria [21]. We show here that the same degree of enzymic deficiency has different effects on mitochondrial respiration depending upon the model. Indeed, as in the first model [21,22], the expression of a mtDNA mutation in the second model still shows a threshold; however, this threshold seems to be more pronounced (higher threshold value of the fraction of active complex necessary to maintain a flux), has the same value for deficiencies in different mitochondrial complexes encoded in mtDNA and depends on the relative energy demand of the tissue. This last theoretical prediction was validated experimentally by using hexokinase for varying the energy demand and different mitochondrial concentrations mimicking the remaining non-affected mitochondria. Finally, the results and their physiological consequences, obtained with isolated mitochondria, are discussed in the framework of mitochondrial diseases.

MODELS

Mitochondrial models

In the present study, oxidative phosphorylation is modelled in the same way as in [21]. The following enzymes, processes and metabolic blocks are taken into account explicitly within the model: substrate dehydrogenation (hydrogen supply to the respiratory chain), complex I, complex III, complex IV (cytochrome *c* oxidase), proton leak, ATP synthase, ATP/ADP carrier, phosphate carrier, adenylate kinase and ATP usage system (experimentally, different amounts of hexokinase in the presence of glucose). The time variations of the metabolite concentrations that constitute independent variables (NADH, ubiquinol, cytochrome *c*, O_2, internal protons, internal ATP, internal P_i, external ATP, external ADP and external P_i) are expressed in the form of a set of ordinary differential equations. The other (dependent) variable values (such as other metabolite concentrations and thermodynamic forces) are calculated from the independent variable values. The set of differential equations is integrated numerically. In each iteration step, new values of rates, concentrations and other parameters are calculated on the basis of the corresponding values from the previous step. The Gear procedure was used for numerical integration and the simulation programs were written in FORTRAN.

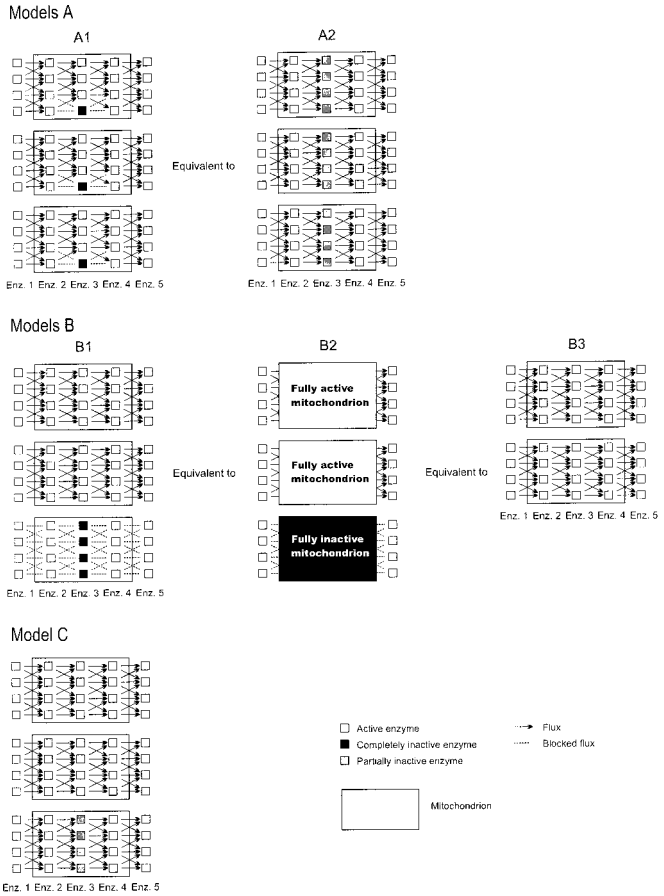

Figure 1 The different models of altered mitochondria metabolism used in this study

In models A, the distribution of inactive enzyme molecules between mitochondria is homogeneous: one enzyme 3 molecule is completely inactive out of four enzyme 3 molecules in each mitochondrion (model A1). The metabolic flux that cannot flow through the defective molecules of a given enzyme bypasses them and flows through the remaining active molecules of this enzyme in the same mitochondrion. This model is equivalent to model A2, in which all the enzyme 3 molecules are inactivated to the same extent (25% in this case). In models B, in some fraction of mitochondria all the molecules of a given enzyme (enzyme 3) are inactive (model B1); the flux cannot bypass them (using, for example, active molecules of this enzyme from other mitochondria) because they are spatially separated. As a result, the mitochondria containing the deficient enzyme are completely inactive and the potentially active molecules of other enzymes encapsulated in these mitochondria cannot work. Model B1 is equivalent to model B2, in which mitochondria are considered as entities, active or inactive ('binary mitochondrial heteroplasmy'), and to model B3, in which the concentration of active mitochondria is simply decreased. Model C is equivalent to model B1 with enzyme 3 only partly inactivated. Abbreviation: Enz. *n*, Enzyme *n* ($n = 1, 2, 3, 4$ or 5).

In the present study we consider three models of mtDNA mutation leading to differences in the compartmentation and intensity of the enzymic deficit. In the first model, model A1 in Figure 1, a completely deficient step is homogeneously distributed in all mitochondria (one deficient molecule per four total molecules of enzyme 3 in the example in Figure 1); this model corresponds to an intra-mitochondrial heteroplasmy of the mtDNA mutation. It also corresponds to the titration of enzyme 3 with a specific inhibitor [21] and is entirely equivalent to a model in which the activity of enzyme 3 would be decreased (to 75% in our example) in all molecules (model A2). This last form of model A corresponds to the case of a nuclear mutation affecting homogeneously the activity of enzyme 3 or to a

homoplasmic mtDNA mutation partly affecting enzyme 3 activity. This deficit is simulated in the model by decreasing the rate constant of the corresponding complex (k_{C3} or k_{C4}; see Figure 2).

At the opposite extreme, model B1 describes the case in which all completely deficient enzyme 3 molecules are concentrated in some of the mitochondria. In mitochondrial pathologies, this situation can be due to the mtDNA segregation in 'normal' mitochondria (without any mutated mtDNA molecules) and 'deficient' mitochondria (containing only mutated mtDNA molecules) (likely to take place as the result of random transfer of a few mtDNA molecules present in each mitochondrion to daughter mitochondria during mitochondria divisions; see the Results and discussion section). Indeed, the accumulation of all the inactive enzyme 3 molecules in some of the mitochondria blocks all of the fluxes in those mitochondria, despite the fact that the other respiratory-chain enzymes have normal activities. Thus mitochondria can be divided into two types: fully active mitochondria, operating with normal flux (white rectangles), and fully inactive mitochondria (black rectangles), with zero flux (model B2). For this reason we named this model 'binary mitochondrial heteroplasmy' or simply 'mitochondrial heteroplasmy'.

Because in model B2 a fraction of mitochondria is completely non-operating, this model, from the point of view of the absolute flux values, is equivalent to model B3 in which the concentration of fully operating mitochondria is decreased. Model B3 is the basis of our theoretical simulations in which we decrease all rate constants by the same factor (see Figure 4), and of our experiments in which we modulate the concentration of mitochondria (see Figure 5).

Finally, we developed a third model (model C) in which we considered a partly active enzyme 3 (Figure 1). In this model, which is a combination of models A2 and B1, we distinguish two populations of mitochondria: one population with all enzyme 3 molecules fully active and one population with all enzyme 3 molecules carrying partial activity. In the simulations (see Figure 3) we varied the fraction of fully active and defective mitochondria as well as the degree of inactivation of the defected enzyme molecules (by decreasing the particular rate constant of the defective complex in the defective mitochondrial subpopulation). This allowed us to describe the simultaneous effect of these two factors on the impairment of oxidative phosphorylation. Model C corresponds to an mtDNA-encoded mutation leading to the partial activity of an oxidative phosphorylation complex (enzyme 3 in our example) and to the segregation of mtDNA (and hence of enzyme 3 activity) as in model B.

Simulations

Different levels of energy demand (used in the simulations; see Figure 4) were established by fixing different values of the rate constant of ATP usage (k_{UT}) [21]: from 0 (state 4) to 12 arbitrary units (state 3). In particular, the energy demand in state 3.5 (resulting in approx. 32% of state 3 respiration) used in the simulations shown in Figures 2 and 3 corresponded to a k_{UT} of 1.5.

In the simulations of the effect of enzyme deficiencies performed for model A (see Figure 2), a decrease in the concentration or activity of a given enzyme by some factor was equivalent to a decrease in the value of the rate constant of this enzyme: in Figures 2 and 3, k_{C3} and k_{C4} are decreased to different degrees between 0 (no activity) and 1 (full activity). Simulations of enzyme deficiencies in model B (see Figures 2 and 4) involved an identical decrease in the rate constants of all mitochondrial complexes, which was equivalent to a decrease in the quantity of (active) mitochondria. In model C (see Figure 3), which is, in a sense, a mixture of model A and model B, we consider two subpopulations of mitochondria, one active and one deficient, in a proportion indicated on the *a*-axis (the fraction of fully active mitochondria between 0 and 1). In the deficient subpopulation, the rate constant of complex III (k_{C3}) was decreased to different degrees (between 0 and 1 of its initial value) plotted on the *b*-axis, whereas in the remaining fraction of mitochondria the activity of complex III remained unchanged. Each subpopulation is described by a separate set of equations and a separate set of intramitochondrial metabolites. This is necessary because internal pools of metabolites from different mitochondria do not communicate with each other; thus their concentrations are different in different mitochondrial subpopulations. In contrast, the external pool (ATP, ADP and P_i) and ATP usage are common.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 200–300 g and having free access to water and standard laboratory diet were used for this study. After 1 day of starvation, animals were killed by cervical shock and decapitation.

Chemicals

Hexokinase (from baker's yeast) and chemicals were obtained from Sigma (St Louis, MO, U.S.A.).

Preparation of liver mitochondria

Rat liver mitochondria were isolated by differential centrifugation as described by Johnson and Lardy [29], with some modifications. Liver was collected in isolation medium A [250 mM sucrose/10 mM Tris/HCl (pH 7.6)/1 mM EGTA] and homogenized. The homogenate was centrifuged at 1000 ***g*** for 5 min; the supernatant was strained on gauze and recentrifuged at 7000 ***g*** for 10 min. The resulting pellet was resuspended in ice-cold isolation medium B [250 mM sucrose/10 mM Tris/HCl (pH 7.6)/0.1 mM EGTA] and a new series of centrifugations (1000 ***g*** and 7000 ***g***) was performed. The mitochondrial pellet was resuspended in a minimum volume of isolation medium B and laid down on a discontinuous sucrose gradient (10%, 30% and 50%, w/v). The mitochondria were collected from the 30%/50% sucrose interface, diluted in excess medium B and centrifuged at 7000 ***g*** for 10 min. The last mitochondrial pellet was resuspended in a minimum volume of isolation medium B to obtain a mitochondrial concentration between 50 and 70 mg/ml. Protein concentration was estimated by the biuret method [30] with BSA as standard.

Oxygraphic measurements

Mitochondria oxygen consumption was monitored at 30 °C in a 1 ml thermostatically controlled chamber equipped with a Clark oxygen electrode (Hansatech Instruments Ltd, Kings Lynn, Norfolk, U.K.), in respiration buffer consisting of 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 substrates (10 mM pyruvate in the presence of 10 mM malate). State 4 was recorded in the absence and in the presence of 1 mM ATP. Intermediate states and state 3 (as defined by Chance and

Williams [31]) were obtained by successive additions of hexokinase (0.1, 0.2, 0.3 and 0.45 unit respectively, in 1 ml) in the presence of 20 mM glucose. The mitochondrial concentration used for this study was decreased from 1.4 to 0.1 mg/ml. Respiratory rates are expressed in n-atom of O/min.

RESULTS AND DISCUSSION

Comparison of models A and B

In the simulation of Figure 2, we compared the dependence of the respiratory rate as a function of the fraction of active complex III or complex IV in the two models A and B. In both cases we obtained curves exhibiting a threshold as previously described in both experimental work [10–13,14–18,20] and simulations [13,21,22]. In a homogeneous distribution of the inhibited complex molecules (models A), the threshold curves (Figure 2, open circles) are different for both complexes and exhibit different thresholds: 0.03 and 0.13 for complexes III and IV respectively. These low thresholds mean that complex III and complex IV can be inhibited to a very large extent without affecting the respiratory rate; this reflects the low control of the these steps over the oxygen consumption flux [10,13,21].

A quite different situation occurs in 'binary mitochondrial heteroplasmy' (models B). In this model, the threshold curves for complexes III and IV are the same (Figure 2, filled circles); this can be explained by the fact that in model B the affected mitochondria contain only fully inactive complex III, which completely blocks electron transfer in the respiratory chain and consequently blocks the respiratory flux. As a result, these mitochondria cannot work at all, although they contain active molecules of other complexes. A complete defect in complex IV molecules will lead to the same effect (model B1): the filled-circle curve in Figure 2 represents the unique threshold curve depicting the dependence of the respiration rate on any oxidative phosphorylation deficiency. Moreover, the threshold value is much greater, 0.4, than for any respiratory-chain complex in model A. It means that, generally, the same level of enzyme deficiency can have a significantly greater effect in 'mitochondrial heteroplasmy' than in a homogeneous distribution of the inactive (defected) enzyme. For example, at a relative activity of complex III or complex IV equal to 20 % of the wild-type value, essentially no effect on the respiratory rate can be observed in a homogeneous distribution, whereas in 'mitochondrial heteroplasmy' the respiratory rate falls below half of its initial value (Figure 2). The results shown above for complexes III and IV of the respiratory chain apply equally well to other steps of oxidative phosphorylation in mitochondria (results not shown).

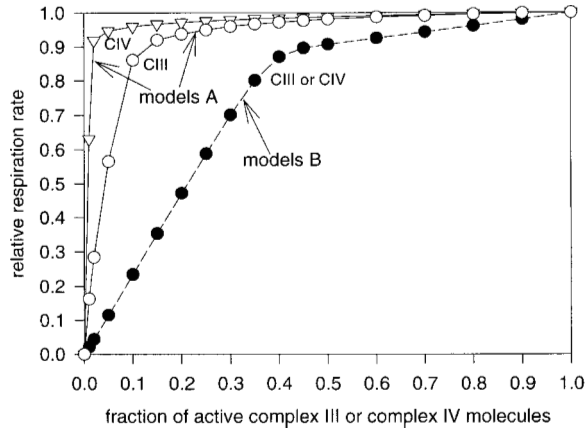

Figure 2 Simulated threshold curves of complex III (CIII) and complex IV (CIV) for a homogeneous distribution of inactive enzyme molecules (○, ▽) and for 'binary mitochondrial heteroplasmy' (●)

The simulations were performed in state 3.5 (approx. 32% of the state 3 respiration rate) with $k_{UT} = 1.5$ (k_{UT} is the rate constant of ATP usage). The homogeneous changes in complex III or IV activities (○, ▽) were obtained by varying k_{C3} and k_{C4} respectively. For the description of 'binary mitochondrial heteroplasmy' (●) all the rate constants were varied simultaneously by the same factor (k_{C3} and k_{C4} are the rate constant of complex III and IV respectively).

Because in model B1 a complete deficit in any complex of oxidative phosphorylation leads to a completely blocked oxidative phosphorylation and consequently to a full inactivation of some of the mitochondria, the quantity of inactive mitochondria is the pertinent parameter (model B2), rather than the degree of deficiency in the complex.

In fact, the main difference resulting from comparison of models A and B, namely the shift in the threshold value, can be understood intuitively as follows. Let us assume that 80 % of molecules of some complex X are completely inactive. In models B, this would be equivalent to a complete inactivation of 80 % of mitochondria; 20 % of mitochondria would remain completely active. In contrast, in models A, all the complexes, except that with decreased activity, are fully active and can partly compensate, through intermediate metabolites variations, for the 80 % decrease in activity in the X complex. In models B, because the whole mitochondria are fully inactive, no compensation can occur: mitochondria are either fully active or fully inactive. Thus, in models B, the threshold value is higher than in models A for the same degree of enzyme deficiency.

Study of model C

In the simulations in Figure 2, particularly for model B ('binary mitochondrial heteroplasmy'), it was assumed that the mtDNA mutation led to a complete complex deficiency. However, many mutations (in mtDNA) can cause only a partial inactivation of an enzyme.

We therefore checked, in a theoretical way (model C and Figure 3), the influence of the combination of both factors: the fraction of 'normal' mitochondria (*a*-axis) at different degrees of activity of defective enzyme molecules (*b*-axis). The simulated results for complex III are presented in Figure 3. It must be pointed out that the two curves of the models A and B of Figure 2 are plotted on the two sides of the three-dimensional graph (the curves identified by filled and open circles in Figure 3 exactly correspond to the curves of Figure 2 with the same symbols). Indeed, they correspond to (1) the variation of a homogeneous deficit in all mitochondria (open circles, 0 on the *a*-axis with variation in the deficit on the *b*-axis) and (2) a complete deficit in a variable fraction of mitochondria (full circles, 0 on the *b*-axis with variation in the fraction of fully active mitochondria on the *a*-axis). Thus, in model C, the effect of a mutation on the respiratory flux is the result of two contributions that both present a threshold: the fraction of 'normal' mitochondria and the degree of the defect. This leads to a delay in the effect of the mutation and contributes to maintaining a quasi-normal flux for most perturbations (of active mitochondria on the *a*-axis and of the amount of deficit on the *b*-axis). This is shown in Figure 3 by the wide plateau area on the top of the three-dimensional graph.

Threshold and energy demand

The simulations described in Figures 2 and 3 were performed at an intermediate state of respiration (state 3.5, corresponding to

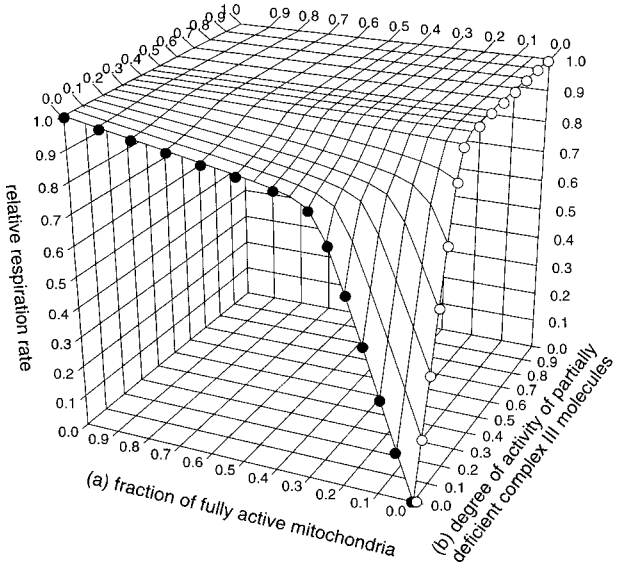

Figure 3 Simulated dependence of the respiration rate on the amount of fully active mitochondria (*a*-axis) as well as on the relative degree of activity of partly deficient complex III molecules (*b*-axis) for 'binary mitochondrial heteroplasmy' (model C)

The simulations were performed in state 3.5 (approx. 32% of the state 3 respiration rate; $k_{UT} = 1.5$). The curve on the *a*-axis (●) is equivalent to the curve with filled circles in Figure 2; the curve on the *b*-axis (○) is equivalent to the corresponding curve with open circles in Figure 2. On the *a*-axis, two subpopulations of mitochondria (one active and one deficient) are distinguished (each is described by a separate set of equations in the model) and the fraction of active mitochondria on the *a*-axis varies between 0 and 1. On the *b*-axis, the rate constant of complex III k_{C3} in the deficient subpopulation of mitochondria is multiplied by a factor between 0 and 1 describing the degree of activity of complex III (1 and 0 on the *b*-axis corresponding to full activity and full inactivity respectively).

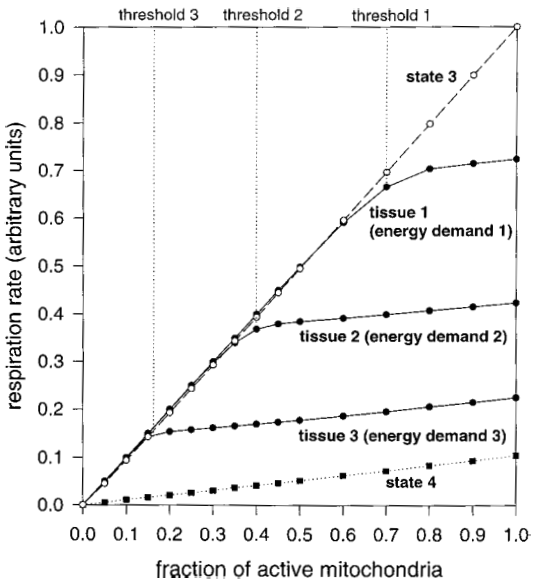

Figure 4 Simulated dependence of the respiration rate on the quantity of mitochondria (model B2 or B3), or on the fraction of active mitochondria in 'binary mitochondrial heteroplasmy' (model B1), at different levels of energy demand (simulated with different rate constants of ATP usage)

$k_{UT} = 12$ for state 3, $k_{UT} = 4.2$ for tissue 1, $k_{UT} = 2.25$ for tissue 2, $k_{UT} = 0.9$ for tissue 3 and $k_{UT} = 0$ at state 4.

32% of state 3 respiration). However, in intact tissues the energy demand can change depending on conditions, for example on work load in skeletal muscle [32]. Different tissues can also exhibit different energy demands in relation to their oxidative phosphorylation capacities (determined by the amounts and activities of their mitochondria). Therefore, to understand the role of energy demand in the tissue specificity observed in mitochondrial cytopathies, we used model B (involving a completely deficient step) to follow the effect, on the respiratory rate, of the variation in the amount of inactive mitochondria as a function of the energy demand. Figure 4 presents the different threshold curves obtained: one curve corresponds to state 3 (maximal energy demand), one curve corresponds to state 4 (no energy demand, all oxygen consumption due to proton leak) and the remaining three curves correspond to intermediate energy demands. The curve for state 4 exhibits no threshold: the respiration falls monotonously to zero as the fraction of active mitochondria decreases. In phosphorylating mitochondria, it can be clearly seen that the threshold value strongly depends on the energy demand level. As the energy demand increases, the threshold value shifts towards higher fractions of active mitochondria: for energy demands causing respiratory rates equal to 22%, 42% and 72% of the maximal (state 3) respiration rate, the threshold values are 0.17, 0.38 and 0.69 respectively. It can be seen that the threshold value increases linearly with the energy demand.

To check the above theoretical predictions, we designed an experimental system corresponding to model B3. We incubated isolated mitochondria in the presence of an ATP-usage system consisting of hexokinase plus glucose. For a set of hexokinase concentrations, corresponding to different energy demand levels, the concentration of mitochondria was changed over a wide range and the oxygen consumption rate was measured for each mitochondrial concentration. Indeed, 'titration' of the respiratory rate with mitochondrial concentration (model B3) exactly mimics a variation in the fraction of 'normal' mitochondria (Model B2). Zero concentration of hexokinase gives state 4 at each concentration, whereas the highest concentration of hexokinase was just saturating for the respiratory flux at the highest concentration of mitochondria used (state 3 at 1.4 mg/ml). Intermediate concentrations of hexokinase correspond to intermediate states of respiration for the highest mitochondrial concentration. The results are plotted in Figure 5, which agrees well with the predictions of Figure 4. The results presented are a representative example of several experiments performed with different preparations of mitochondria isolated from different tissues: liver, skeletal muscle and heart. All are in accordance with the predictions of model B3. The 'titration curves' (by mitochondrial concentration) are straight lines in state 4 and state 3, whereas in intermediate states they have a quasi-plateau region at higher mitochondrial concentrations and exhibit, as a function of the mitochondrial concentration, a threshold value that increases with energy demand.

This change in threshold value with energy demand can be explained: at the highest energy demand all the mitochondria are fully involved and control the respiratory rate; in this situation, a decrease in mitochondrial concentration is followed by the same relative decrease in the flux. In contrast, when the demand is decreased (by decreasing the amount of hexokinase), mitochondria are not fully saturated and their concentration can be decreased (to a certain extent) without significantly affecting the global flux. Such a phenomenon has been analysed in terms of enzyme deficiency [11].

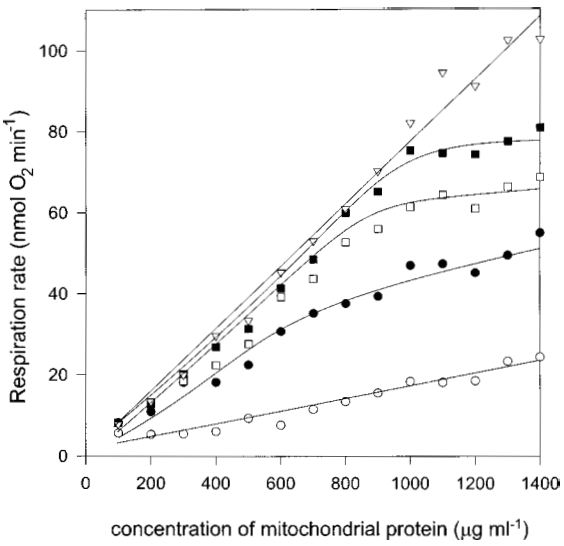

Figure 5 Experimentally measured dependence of respiration on the quantity (concentration) of mitochondria at different levels of hexokinase and in the presence of 20 mM glucose

The concentrations of hexokinase were respectively 0 (state 4, ○), 0.1 (●), 0.2 (□), 0.3 (■) and 0.45 (∇) unit/ml (state 3) respectively (1 ml final volume). Other conditions were as described in the Materials and methods section.

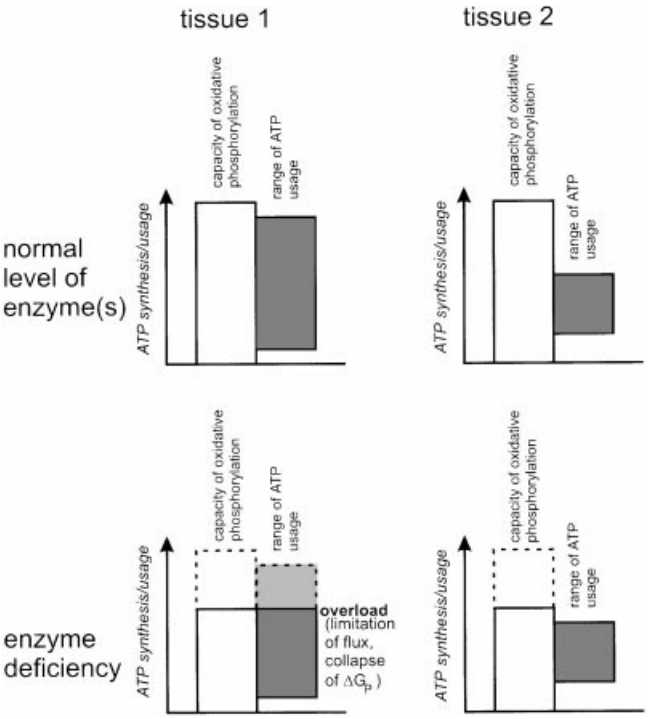

Figure 6 Illustration of different hypothetical oxidative phosphorylation capacities and energy demands in different tissues

In tissue 1, where the maximal ATP usage is normally near the maximal oxidative phosphorylation capacity, a decrease in oxidative phosphorylation capacity affects the ATP availability, whereas at the same time in tissue 2 the same decrease does not.

Wallace [3,5,7] has proposed a classification of tissues on the basis of their relative energy demands (brain > muscle > heart > kidney > liver) and has shown that this classification correlates most of the time with the affected tissues in patients carrying mtDNA mutations [33]. Figure 4 shows that, for example, 50% of working mitochondria can cause a significant impairment of oxidative phosphorylation in tissues with high energy demand (tissue 1) but at the same time it can be above the threshold value and can apparently exert no effect in tissues with lower energy demands (tissues 2 and 3). This is also illustrated schematically in Figure 6 (which is merely an illustration rather than any particular experimental or theoretical results), in which it can be seen that quantitatively the same dysfunction of oxidative phosphorylation can cause a severe effect (limitation of the ATP consumption flux) in a tissue with a high relative energy demand, but there is apparently no effect at all in a tissue with a low energy demand.

We stress that we have only considered in our models and in our experiment the energy supply function of mitochondria because it is a simple way of shifting oxidative phosphorylation from state 4 to state 3 both theoretically and experimentally. Although this function is probably one of the most important in some tissues (such as skeletal muscle and heart), other mitochondrial functions (most of which are energy-consuming) could also be differentially affected in the same way as energy supply by the expression of mutations, depending on their localization.

Model B, a model for mtDNA mutations

Models B have been developed to study the influence of the expression of mtDNA mutations on oxidative phosphorylation fluxes. It is based on several assumptions: First, the model assumes the segregation of all mtDNA mutations in some of the mitochondria. Indeed, if there is no physiological bias in a random segregation of mtDNA into the daughter mitochondria, the small number of mtDNA molecules in each individual mitochondrion (2–10) favours the occurrence, after a few divisions, of a homogeneous mtDNA distribution inside each mitochondrion, an irreversible situation that is transmitted to the daughter mitochondria. This will lead to two pure mitochondrial subpopulations: one with only wild-type DNA and one with only mutated DNA. This justifies the name of 'binary (0 or 1) mitochondrial heteroplasmy' or simply 'mitochondrial heteroplasmy' that we gave to this situation.

Secondly, this model assumes that there is no communication between mitochondrial units (especially between active and inactive ones). It means that there is no complementation between mtDNA molecules situated in different mitochondria, nor any transfer of intermediate metabolites between mitochondria. This has been shown in [34,35], with evidence for an absence of complementation between two mtDNA point mutations separately introduced in the same cybrids [36]. However, this point is still a matter of discussion. Indeed, although they do not prove completely in all cases that the two types of mtDNA are located within the same organelle, several authors [34,35,37–41] have shown such complementation. In this case, a model B with communication between mitochondria is equivalent to model A; experimentally, a quantitative study of the expression of mtDNA mutation should permit discrimination between these situations.

Thirdly, model B describes the behaviour of a population of isolated mitochondria. Nevertheless, this model should be applied with caution to the expression of mtDNA mutations in tissues composed of populations of cells. Indeed, this model does not take into account the following: (1) the mitotic segregation of mitochondria during embryogenesis, leading to different proportions of inactive mitochondria in each individual cell (in this

case the tissue flux is the sum of the individual cell fluxes obeying models B but with different proportions of inactive mitochondria); and (ii) additional tissue regulations (for example, account must be taken of the difference in regulation of ATP supply in intact tissues and in isolated mitochondria). The changes in fluxes and intermediate metabolite concentrations observed in cells and tissues *in vivo* after stimulation of respiration (and ATP synthesis) cannot be explained only by the stimulation of respiratory rate after a decrease in the ATP-to-ADP ratio [23,24,42], which is the mechanism by which ATP supply meets energy demand in the isolated system containing mitochondria and hexokinase studied here. In skeletal muscle, for instance, during the transition from resting state to maximal work, a great increase in the respiration rate takes place, accompanied by only a weak decrease in the ATP-to-ADP ratio [32], in contrast with the great decrease in the ATP-to-ADP ratio used to stimulate state 3 respiration in isolated mitochondria. Such observations demonstrate the necessity of complex regulatory mechanisms, among which a direct stimulation of most of the oxidative phosphorylation complexes is compulsory [23,24].

Although model B has been developed to account for mutations in mtDNA, it can also be used for some nuclear mutations such as those affecting the amount of mtDNA itself (mtDNA depletion). For instance, for a nuclear mutation affecting mtDNA replication, leading to a 99% decrease in mtDNA in comparison with the normal situation, the remaining mtDNA molecules (between 10 and 20) cannot be homogeneously distributed in all the hundreds of mitochondria in a cell. This case necessarily leads to a majority of mitochondria without any mtDNA molecule ('fully inactive mitochondria') which corresponds to model B2, although the mutation could be of nuclear origin.

Application to mitochondrial pathologies

The study of patients with mitochondrial diseases shows that the effect of mutations (nuclear or mitochondrial) is, most of the time, a decrease in the activities of one or several oxidative phosphorylation complexes (we do not consider here other types of mutation, for instance those that affect the quantity of reactive oxygen species); in these cases, a decrease in the activity of a given complex can be represented by a modulation of the rate constant in its rate equation.

Because the changes in the parameters of our model cover the whole range (0–100%) in oxidative phosphorylation complex activity and in relative energy demand, they are representative of the effects of any mutation in the mitochondrial or nuclear genomes: the effect of any given particular mutation is represented by a point in our theoretical figures (Figures 2–4) or in our experimental data (Figure 5).

This study emphasizes the role of 'binary mitochondrial heteroplasmy' in the expression of mtDNA mutations on oxidative phosphorylation fluxes. Although this work has been done, both theoretically and experimentally, on isolated mitochondria, the results obtained could have important implications for the diagnosis and study of mitochondrial diseases: the same defect is less deleterious when homogeneously distributed in all mitochondria than when concentrated in some of them. Indeed, 100% of the mitochondria with a 80% deficit, homogeneously distributed (model A) have much less effect on the fluxes than a full deficit in 80% of the mitochondria (model B) (see Figure 2), although both situations lead globally to an 80% deficit when assessed biochemically. It means that, in the diagnosis of mitochondrial diseases, it is not sufficient to study the global amount of a deficit, as usually done, but it is also important to know the cellular distribution of the deficit. One should also bear in mind that a possible effect of 'binary mitochondrial heteroplasmy' would be quantitatively the same for each mtDNA-encoded complex of oxidative phosphorylation, regardless of the control of this complex over the respiration flux in normal mitochondria.

Conclusion

The present paper shows that different mutations affecting oxidative phosphorylation complexes are not expressed in the same manner due to a different distribution of mutations among mitochondria. The pattern of distribution depends on the origin of the mutation: a nuclear mutation usually affects a particular oxidative phosphorylation complex homogeneously in all mitochondria (models A), whereas an mtDNA mutation tends to be expressed in a fraction of mitochondria in which all the mutated mtDNA species segregate (models B and C). For mtDNA mutations, we have shown that the same decrease in the global activity of a given complex affects respiratory flux and ATP synthesis differently depending on the distribution of inactive complex molecules (Figure 2): in the complete inhibition of a fraction of the molecules of some complex, the effect is much more pronounced (and is identical for each complex) when all the inactive complex molecules are gathered together in a fraction of the mitochondria (mtDNA mutations, model B1) than when inactive complex molecules are equally distributed between all mitochondria (model A1, corresponding to a titration curve with a specific inhibitor and equivalent to model A2 of a homogeneously distributed partial defect).

In addition, we have shown that the expression of nuclear or mitochondrial mutations exhibits a threshold that depends on the origin and distribution of the mutation (homogeneous or heterogeneous) and also on the relative energy demand of the tissue.

Although our models are oversimplifications of a complex reality, they show, particularly when models A and B are compared, the importance of the compartmentation of deficient enzymes to the diagnosis or treatment of mitochondrial diseases.

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