Hypothesis

The apparent non-linearity of the relationship between the rate of respiration and the protonmotive force of mitochondria can be explained by heterogeneity of mitochondrial preparations

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Received 15 January 1985

The apparent non-linear relationship between the rate of respiration and the protonmotive force in mitochondria under resting state conditions is an observation which has led to concepts such as (i) non-chute characteristics of the proton leak through the mitochondrial membrane, or (ii) a slippage of proton pumps. We propose that this non-linearity may be a consequence of a heterogeneity of isolated mitochondria concerning the degree of coupling, since a small proportion of totally uncoupled, or loosely coupled, organelles may considerably contribute to the total respiration but not, or only slightly, to the protonmotive force. This hypothesis is supported by a fairly good fitting of computed relationships with those determined experimentally.

Protonmotive force *Respiration* *Resting state* *Proton leak* *Mitochondria*

1. INTRODUCTION

It is well recognized since early studies on oxidative phosphorylation [1,2] that ADP greatly stimulates the rate of mitochondrial respiration. This phenomenon reflects a tight coupling of electron transport with ATP synthesis. Nevertheless, in the absence of ADP or after exhaustion of added ADP, mitochondrial respiration does not come to a stop but remains at a low, though measurable, level designated as *state 4* [2] or *resting state*. This resting state respiration has been initially attributed to residual oxidative phosphorylation due to contamination of mitochondrial preparations with mitochondrial fragments where ATP hydrolysis can proceed. Indeed, it has been shown [3,4] that oligomycin or carboxyatractyloside decreases the rate of this respiration. Also energy-utilizing processes have been claimed to be responsible for at least a part of the resting state respiration. Calcium recycling was found to account for 20% of this respiration under certain conditions [5]. Nevertheless, there always remains quite a significant respiration still to be accounted for.

According to the chemiosmotic hypothesis of energy coupling [6], the proton electrochemical potential, also designated as the *protonmotive force*, forms a link between the respiratory chain and the synthesis of ATP. In these terms, a leak of protons through the inner mitochondrial membrane may also be responsible for the resting state respiration, and such an explanation has been proposed [7]. In experiments with respiratory inhibitors, however, it was observed [7] that the rate of the resting state respiration (equivalent to the proton leak) was not proportional to the magnitude of the protonmotive force throughout its whole range but rapidly increased at higher values of the protonmotive force. This was explained [7] as reflecting non-ohmic characteristics of the mitochondrial inner membrane. More recently, however, a concept of slippage of proton pumps has been proposed and adopted to coupling phenomena in mitochondria [8]. According to Pietrobon et al.

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00145793/85/$3.30

[8–10], the resting state itself and its non-linear dependence on the protonmotive force are manifestations of slipping of proton pump(s) in the respiratory chain. On the other hand, the resting state respiration and its relation to the protonmotive force have also been interpreted in terms of the mosaic protonic coupling hypothesis [11].

2. HYPOTHESIS

In the present paper we propose an explanation for the non-linearity of the dependence between the protonmotive force and the respiration rate under resting state conditions without retreating to these rather sophisticated solutions. Our hypothesis is based on two assumptions: (i) populations of isolated mitochondria are heterogeneous concerning the degree of coupling in individual organelles; and (ii) the control strength of the respiratory chain in the resting state depends on the degree of coupling.

Heterogeneity of mitochondrial preparations is a well known fact. It may reflect the heterogeneity of the cells in the tissue [12] and the heterogeneity of mitochondria in the cell [13], but may also result from the fractionation procedure, for example, as an effect of mechanical damage or chemical and physical alterations of some of the organelles [14–16]. In fact, Ficoll zonal centrifugation reveals subfractions of mitochondria with very different respiratory control indices and rates of respiration [16].

The second assumption originates from the concept of the control strength [17,18]. As described in a recent review [19], the control pattern, i.e., the distribution of control points, differs depending on the functional state of mitochondria. For example, in the active state (state 3 according to the terminology of [2]) the main control strength is distributed among the adenine nucleotide translocator, the substrate carrier and the respiratory chain [20]. In the uncoupled state, practically the total control strength is exerted by the respiratory chain and, perhaps, the substrate carrier. On the contrary, it can be assumed (cf. [21]) that in the resting state the proton leak is the main controlling point for the respiration. Consequently, inhibitors of the respiratory chain will more strongly decrease the respiration rate of mitochondria with low coupling characteristics or fully uncoupled than that of tightly coupled mitochondria.

For the sake of simplicity let us assume that the mitochondrial population is composed of two subpopulations: a tightly coupled one and a completely uncoupled one. The specific respiration rate of the uncoupled subpopulation is higher than that of the coupled subpopulation by a factor equal to the respiratory control ratio of the latter subpopulation. Therefore, the respiration of the uncoupled subpopulation may have a considerable share in the overall oxygen uptake, even though this subpopulation constitutes only a small portion of the total population. Thus, when such a heterogeneous mitochondrial population is titrated with a respiratory inhibitor, the respiration of uncoupled particles is first decreased, whereas the protonmotive force, related exclusively to coupled mitochondria, is hardly affected, and only much higher concentrations of the inhibitor will substantially inhibit the respiration of the coupled subpopulation and, as a result, also decrease its protonmotive force.

This description is substantiated by the following mathematical considerations. The relationship

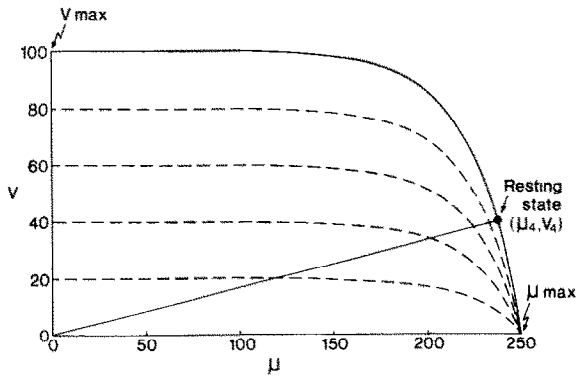

Fig.1. Theoretical relationship between the rate of electron transport and the protonmotive force in mitochondria. The solid (upper) curve is computed according to eqn 1 for $V_{max} = 100$ and $\mu_{max} = 250$. Dashed lines represent the same dependence for mitochondria in which the respiratory chain was inhibited to its 80, 60, 40 and 20% activity, respectively. The straight line depicts the linear dependence between the proton leak and the protonmotive force. Its intersection with the upper curve marks the rate of the resting state (state 4) respiration (V_4) and the corresponding value of the protonmotive force (μ_4). Its intersections with the dashed lines indicate resting state parameters for partially inhibited mitochondria.

between the electron transport rate and the protonmotive force can be expressed by the following exponential function [22–24]

$$V = V_{max}\left\{1 - \exp\left[(\mu - \mu_{max})\frac{\epsilon}{kT}\right]\right\} \quad (1)$$

where V is the electron flow rate; V_{max} is the maximum flow capacity of the respiratory chain (practically equal to electron flow rate under uncoupled conditions); μ is the protonmotive force; and μ_{max} is the highest attainable protonmotive force at which the electron flow completely stops (in ideally coupled mitochondria); ϵ is the elementary charge; k is Boltzmann's constant; and T is the absolute temperature. This relationship for V_{max} arbitrarily taken as 100 and μ_{max} as 250 is presented by the upper curve in fig.1. Assuming the proton leak to be proportional to the protonmotive force, it can be represented by a straight line, as also shown in fig.1. Its intersection with the exponential curve indicates the magnitude of the resting state respiration (V_4). The corresponding value of the protonmotive force (μ_4) can by calculated by transforming eqn 1

$$\mu_4 = \mu_{max} + \frac{kT}{\epsilon}\ln\left(1 - \frac{V_4}{V_{max}}\right) \quad (2)$$

or

$$\mu_4 = \mu_{max} + \frac{kT}{\epsilon}\ln\frac{R-1}{R} \quad (3)$$

where R is the respiratory control ratio (V_{max}/V_4).

By gradually decreasing the amount of functionable respiratory chain, e.g., by titration with a respiratory inhibitor, one obtains a series of curves represented by dashed lines in fig.1. Their intersections with the straight line showing the proton leak indicate respective rates of the resting state respiration and corresponding values of the protonmotive force. It can be seen that initial steps of the inhibition of the respiratory chain result in only a slight diminution of the resting state respiration. This is in accordance with the fact that the respiratory chain has a negligible part in the control strength under resting state conditions (cf. [21]). Only inhibition of a large proportion of the respiratory chain produces a substantial decrease of the respiration. It can be shown that the corresponding protonmotive force (μ_a) fulfills the following equation

$$\mu_a = AR\left(\mu_{max} + \frac{kT}{\epsilon}\ln\frac{R-1}{R}\right) \times\left\{1 - \exp\left[(\mu_a - \mu_{max})\frac{\epsilon}{kT}\right]\right\} \quad (4)$$

where A indicates the fraction of the non-inhibited respiratory chain. The corresponding respiration rate (V_a) is

$$V_a = AV_{max}\left\{1 - \exp\left[(\mu_a - \mu_{max})\frac{\epsilon}{kT}\right]\right\}. \quad (5)$$

Now, let us assume that the mitochondrial population contains a bulk of tightly coupled organelles (with the respiratory control ratio R) and a small proportion of completely uncoupled particles. The resulting respiration rate is then the sum of the respiration of both subpopulations. On inhibition of the respiratory chain, the respiration rate of the uncoupled subpopulation diminishes linearly (see ordinate in fig.1), whereas that of the coupled subpopulation changes in a more complex way as described above. If B indicates the proportion of the coupled subpopulation, the respiration rate of the total heterogeneous population (V_{total}) can be expressed as follows

$$V_{total} = ABV_{max}\left\{1 - \exp\left[(\mu_a - \mu_{max})\frac{\epsilon}{kT}\right]\right\} + A(1-B)V_{max}. \quad (6)$$

By eliminating A, which can be calculated from eqn 4, one obtains the following relationship between the respiration and the protonmotive force of a heterogeneous mitochondrial population titrated with a respiratory inhibitor

$$V_{total} = \frac{\mu_a V_{max}\left\{1 - B\exp\left[(\mu_a - \mu_{max})\frac{\epsilon}{kT}\right]\right\}}{R\left(\mu_{max} + \frac{kT}{\epsilon}\ln\frac{R-1}{R}\right)\left\{1 - \exp\left[(\mu_a - \mu_{max})\frac{\epsilon}{kT}\right]\right\}} \quad (7)$$

In this equation μ_a indicates the protonmotive force of the coupled subpopulation. However, the measurable (apparent, μ_{app}) protonmotive force of the total population is smaller, since the protonmotive force of the uncoupled subpopulation is zero. It can be expressed as

$$\mu_{app} = \mu_a + \frac{kT}{\epsilon} \ln B. \tag{8}$$

This equation was deduced by assuming that the intramitochondrial concentration of probes used to determine the membrane potential and the transmembrane pH difference is zero in totally uncoupled mitochondria, whereas in fact it is equal to the extramitochondrial concentration. Nevertheless, such an approximation is acceptable for high values of μ_a, and only for low protonmotive force values does it result in erroneous calculations.

Substituting μ_{app} for μ_a, one obtains

$$V_{total} = \frac{V_{max}\left(\mu_{app} - \frac{kT}{\epsilon} \ln B\right)\left\{1 - B\exp\left[(\mu_{app} - \mu_{max})\frac{\epsilon}{kT} - \ln B\right]\right\}}{R\left(\mu_{max} + \frac{kT}{\epsilon} \ln \frac{R-1}{R}\right)\left\{1 - \exp\left[(\mu_{app} - \mu_{max})\frac{\epsilon}{kT} - \ln B\right]\right\}}. \tag{9}$$

Fig.2A shows a series of curves for the relationship described by eqn 9, assuming the respiratory control ratio of the coupled subpopulation as 10. It can be seen that even a small admixture of uncoupled mitochondria, e.g. 5%, produces a considerable deviation from linearity. For comparison, the results obtained from an experiment with rat liver mitochondria are also shown in fig.2B. In the latter figure only the main constituent of the protonmotive force, i.e. the membrane potential (ψ), was measured. However, since its ratio to the total protonmotive force remains practically constant under these experimental conditions [21], such a comparison may be acceptable.

A similar relationship for a fixed proportion of uncoupled particles but various degrees of coupling of the coupled subpopulation is presented in fig.3A, whereas fig.3B shows a series of curves for rat liver mitochondria whose respiratory control was decreased by small amounts of carbonyl cyanide *m*-chlorophenylhydrazone.

It should be noted that the resultant (or apparent) respiratory control ratio (R_{app}) is a function of the real respiratory control of the coupled subpopulation (R) and the proportion of that subpopulation (B)

$$R_{app} = \frac{R}{R(1-B)+B}. \tag{10}$$

3. CONCLUSION

The considerations described here explain the apparent non-linear dependence between the respiration rate and the protonmotive force in resting state mitochondria, assuming a linear dependence between the proton leak and the protonmotive force ('ohmic' leak) and a heterogeneity of the mitochondrial population. The formulas presented in section 2 are based on the simplest assumption that a heterogeneous population is composed of only two subpopulations, one of which is tightly coupled, whereas the other is completely uncoupled. In fact, it is more likely that a broad spectrum

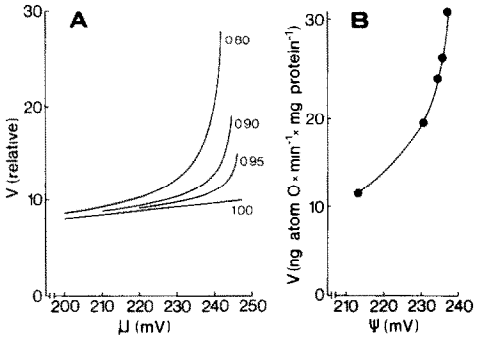

Fig. 2. Dependence between the resting state respiration and the protonmotive force in mitochondria titrated with a respiratory inhibitor. (A) Theoretical curves calculated from eqn 9 for mitochondria containing a mixture of coupled organelles exhibiting a respiratory control ratio (R) of 10 and totally uncoupled particles. The proportion of the coupled subpopulation (B) is indicated by the numbers beside the curves. Values for V_{max} and μ_{max} are those given in fig.1. (B) Experimentally obtained relationship for rat liver mitochondria respiring with succinate (+rotenone) and titrated with malonate, at 25°C. Oxygen uptake was measured with a Clark type electrode and the membrane potential (ψ) by a tetraphenylphosphonium-sensitive electrode [25].

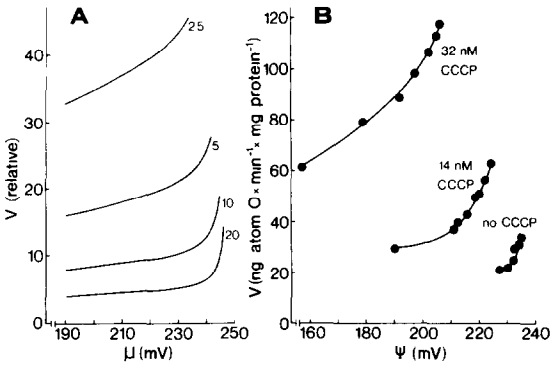

Fig.3. Dependence between mitochondrial respiration and the protonmotive force at various degrees of coupling. (A) Theoretical curves calculated according to eqn 9 for a mitochondrial population containing 90% ($B = 0.90$) of particles of various degrees of coupling, characterized by their respiratory control ratios (R) of 20, 10, 5 and 2.5, as indicated, and 10% of uncoupled mitochondria. The resultant respiratory control ratio (R_{app} in eqn 10) is 6.90, 5.26, 3.57 and 2.17, respectively. (B) Experimental results with rat liver mitochondria respiring with succinate (+rotenone) and titrated with malonate in the absence or presence of two concentrations of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), as indicated. The resultant respiratory control ratio was 4.5 in the absence of the uncoupler and 2.4 and 1.3 in the presence of 14 and 32 nM CCCP, respectively. Oxygen uptake and the membrane potential were determined as described for fig.2B.

of the degree of coupling applies for a real mitochondrial population. This makes a mathematical description of the events more difficult, if at all possible, but does not change the main feature of such a population, namely that the relationship between the resultant respiration and the protonmotive force becomes non-linear. For this reason, one should not expect a perfect fitting of experimental results with computed curves. The comparison of theoretical relationships with experimental findings, as illustrated in figs 2 and 3, is only aimed to stress a general similarity of the shape of respective curves.

The aim of the present considerations is neither to question non-ohmic properties of mitochondrial membranes under certain conditions nor to deny the existence of slips in proton pumps but to propose a simple and, to our opinion, very likely explanation for the non-linear interdependence between the resting state respiration and the protonmotive force.

ACKNOWLEDGEMENT

We wish to thank Anna Zółkiewska for determinations of oxygen uptake and the membrane potential shown in figs 2B and 3B.

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