19 Metabolic control analysis and threshold effect in oxidative phosphorylation: study in an intermediate state of respiration

M. Malgat, T. Letellier, R. Rossignol and J.-P. Mazat
INSERM EMI 99-29 - Université Victor Segalen-Bordeaux II, 146 rue Léo-Saignat, F-33076 Bordeaux Cedex, France

Introduction

The metabolic control analysis (MCA) [1–3] has often been used for quantitative studies of the regulation of mitochondrial oxidative phosphorylations (OXPHOS) [4–23]. The main contribution of these works has been to show that the control of mitochondrial metabolic fluxes (oxygen consumption and ATP synthesis) can be shared among several steps of the oxidative phosphorylation process, and that this distribution can vary according to the steady state and the tissue.

However, these studies in mitochondrial energetic metabolism, are performed in state 3 (maximum respiration of mitochondria with excess ADP) or in state 4 (non-phosphorylating respiration) while it is proposed that *in vivo*, mitochondria respiration could occur in an intermediate steady state between state 3 and state 4 (state 3.5).

In the present work, such an intermediate steady state of respiration was obtained experimentally in rat muscle isolated mitochondria by delivering a stable intermediate ATP/ADP ratio using adenylate kinase in presence of a high AMP concentration (20mM) and different ATP concentrations. This enabled us to determine the control coefficients for complex I, complex III, complex IV, ATP synthase and adenine nucleotide translocator on the oxygen consumption flux at intermediate state. For this, we applied the so-called inhibitor method to those five OXPHOS complexes. To know if there really exists a variation in the distribution of flux control coefficients between state 3 and intermediate state of respiration, we have also determined the control coefficients of the same OXPHOS complexes at state 3 on the same isolated rat muscle mitochondria.

The specific inhibitors used to determine the flux control coefficients for various OXPHOS complexes can be used to mimic a physiological defect in the re-

spective activity of these complexes. In this case, we have evidenced a biochemical threshold effect concerning the expression of an OXPHOS deficiency on mitochondrial respiration [10,12,13,15,24,25] and we have shown that this biochemical threshold can be predicted in the framework of the metabolic control theory [1–3]. To assess the differences in the metabolic expression of a defect in an OXPHOS complex between state 3 and intermediate state, we have determined the threshold curves for the adenine nucleotide translocator at this two steady-state of respiration. On these curves, it is observed that the threshold value can vary according to steady state of respiration, changing the sensitivity of mitochondrial respiration to a defect in the adenine nucleotide translocator. This observation will be discussed in the field of mitochondrial diseases.

Materials and methods

Chemicals Antimycin, carboxyatractyloside (CATR), cyanide (KCN), α-cyano-4-hydroxycinnamate (C4H), N,N,N′,N′-tetramethyl-p-phenylenediamine, oligomycin, rotenone, (TMPD) were from Sigma. Fresh solutions were used throughout this study.

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

Preparation of rat muscle mitochondria To have enough muscle mass to obtain a sufficient mitochondrial protein content, the hind limb muscles were used, i.e., the *gastrocnemius* (G) the *plantaris* (P) and the *soleus* (S). Rapidly removed from the bones, the muscles were immersed in high EDTA buffer and freed from tendons and paratendinous tissue, visible fat and connective tissue. Rat muscle mitochondria were isolated by differential centrifugations as described by Morgan-Hughes *et al.* [26]. To avoid any contamination by ATP-consuming activities, we have added to this classical isolation method a new step of purification. For this, the crude mitochondrial pellet was resuspended in a final volume of 10 ml in a 3% Ficoll medium (3% Ficoll, 250 mM sucrose, Tris/HCl 10 mM pH 7.4 and 0.5 mM K^+ EDTA). This suspension was carefully layered onto 20 ml of a 6% Ficoll medium (6% Ficoll, 250 mM sucrose, Tris/HCl 10 mM pH 7.4 and 0.5 mM K^+ EDTA) and centrifuged for 30 min at 11,500 g. The mitochondrial pellet was resuspended in medium II (225 mM mannitol, 75 mM sucrose, 0.1 mM EDTA, 10 mM TRIS pH 7.2) and recentrifuged for 10 min at 12,500 g. The mitochondria were made up to a concentration of 60 to 70 mg of protein/ml in medium II. Protein concentration was estimated by the Biuret method [27] using bovine serum albumin as standard.

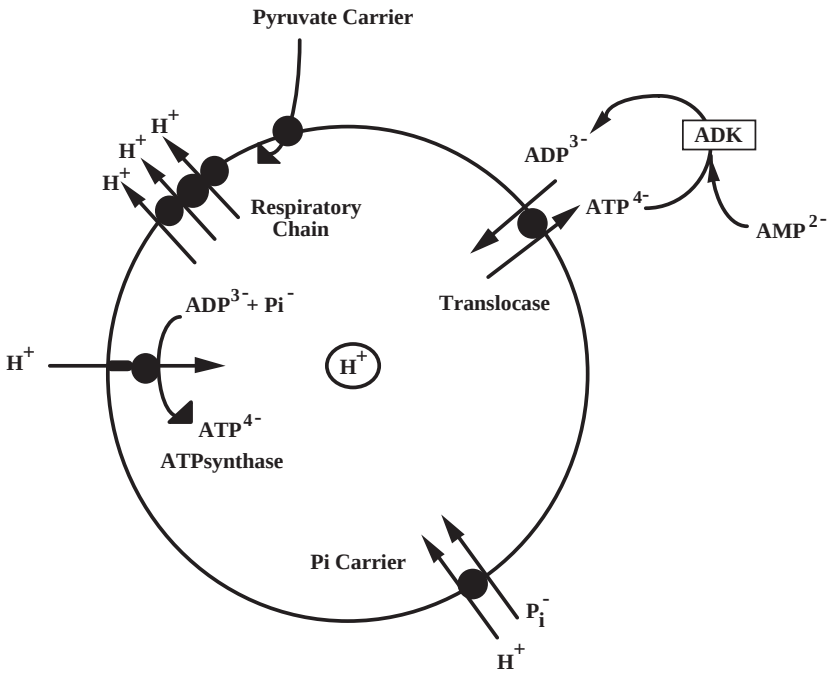

Fig. 19.1 Mitochondrial oxidative phosphorylation and the adenylate kinase ADP regenerating system

Oxygraphic measurements Mitochondrial oxygen consumption was monitored at 30 °C in a 1 ml thermostatically controlled chamber equipped with a Clark oxygen electrode, in the following respiration buffer: mannitol 75 mM, sucrose 25 mM, KCl 100 mM, Tris Phosphate 10 mM, Tris/HCl 10 mM pH 7.4, EDTA 50 μM plus respiratory substrates (pyruvate 10 mM in presence of malate 10 mM or succinate 25 mM when precised). The mitochondrial concentration used for this study was 1 mg/ml and the respiratory rates were expressed in natom O/min/mg proteins.

Intermediate state measurements In order to obtain a mitochondrial respiration in an intermediate state we used the adenylate kinase system (Fig. 19.1) to maintain a stable ATP/ADP intermediate ratio. Indeed, adenylate kinase catalyses the phosphorylation of AMP to produce ADP according to the equation:

$$\mathrm{AMP} + \mathrm{ATP} \rightleftharpoons 2\,\mathrm{ADP} \tag{19.1}$$

Firstly we have determined the concentration of adenylate kinase necessary to measure the same state 3 respiration rate as obtained in excess of ADP. This was done with ADK (0.2 U), ATP (1 mM) and AMP (20 mM) in the presence of $MgCl_2$ (3mM) with pyruvate/malate (10 mM) as respiratory substrates. In order to establish an intermediate state of respiration, we modulated the ATP/ADP ratio by

varying the ATP concentration in presence of a fixed AMP concentration (10 mM), until a respiratory rate corresponding to half the state 3 was reached. This was done for an ATP concentration around 5 mM.

State 3 measurements The state 3 (according to Chance [28]) was obtained in the presence of 10 mM pyruvate and 10 mM malate by addition of 2 mM ADP.

Control coefficient determination The control coefficients of various steps involved in oxidative phosphorylation were determined using the inhibitor method [7]. This method is based on the determination of the global flux (oxygen consumption or respiratory rate) and the isolated step (OXPHOS complex) activity titration curves, performed with specific inhibitors. In this case the control coefficient of the step i on the flux J is calculated by :

$$C_i^J = \left(\frac{\partial \ln J}{\partial I} \Big/ \frac{\partial \ln v_i}{\partial I} \right)_{\text{steady state I=0}} \tag{19.2}$$

where the control C_i is equal to the ratio of the initial slope of the inhibition curve of the global flux J over the initial slope of the inhibition curve of the isolated step activity v_i determined in the same conditions as for the global flux.

The specific inhibitors used were: rotenone for complex I, antimycin for complex III, KCN for complex IV, oligomycin for ATP synthase, CATR for adenine nucleotide translocator, mersalyl for phosphate carrier and α-cyano-4-hydroxycinnamate for pyruvate carrier.

For complexes I, III and IV, the inhibition curves of the enzymatic complexes activity (isolated step activity) were determined experimentally. However, in some cases, it was impossible to determine the activity of the isolated step in the same conditions as for the global flux. This was the case for the ATP synthase, the adenine nucleotide translocator, the phosphate carrier and the pyruvate carrier, the activities of which are dependent on the amount of the $\Delta\mu_{H^+}$ generated by the respiratory chain. In these cases, the method presented by Gellerich *et al.* [29] was used. This method uses a non-linear regression that fits the respiratory rate inhibition curve. Non-linear fitting was done using the program Simfit [30]. From the parameters obtained by this fitting procedure and the model equations, we drew the inhibition curve of the isolated step activity with the program TK Solver Plus (Universal Technical Systems, Rockford, IL, U.S.A.).

The flux control coefficient values of the various steps involved in oxidative phosphorylation were also calculated using this model.

For the complexes I, III and IV, a second method to calculate the control coefficients was also used. Indeed, in those cases where the inhibition curve of the isolated step activity can be experimentally determined, we used a graphical method based on the initial slope determinations by a linear regression on the first point of both the global flux and the isolated step inhibition curves.

Results

Control coefficients on the oxygen consumption flux for various OXPHOS steps at state 3 and intermediate state

Only the titration curves obtained for the adenine nucleotide translocator at state 3 and intermediate state of respiration are presented in Fig. 19.2. The inhibition of the isolated step activity being inaccessible experimentally, we have employed the fitting method to determine the control coefficient of the ANT on the oxygen consumption flux (Fig. 19.2). The values obtained are given in Table 19.1.

Table 19.1 Control coefficient values of different OXPHOS complexes at two steady states of respiration.

	State 3	State 3.5
Complex I	0.14	0.10
Complex III	0.23	0.27
Complex IV	0.19	0.07
ATP Synthase	0.10	0.04
A.N. Translocator	0.08	0.39
Phosphate Carrier	0.08	ND
Pyruvate Carrier	0.2	ND
Σ	1.09	0.87

For each control coefficient determination, we used the same respiratory buffer and the same experimental conditions in respect of the steady state studied. In these conditions, the different respiratory rates (state 4 and 3 according to Chance [28]) and the respiratory control ratio were determined. We have used mitochondrial preparations only when the respiratory control ratio was closed to values reported in the same conditions.The control coefficients on the oxygen consumption flux were measured for seven steps of the OXPHOS (complexes I, III, IV, ATP synthase, adenine nucleotide translocator (ANT), phosphate carrier and pyruvate carrier) on rat muscle isolated mitochondria. In our experimental conditions and whatever the steady state of respiration, the control is largely distributed between the different OXPHOS steps indicating that there is no limiting step as it can be predicted from the metabolic control theory. Nevertheless, it has been impossible to determine the control coefficient for the phosphate carrier and the pyruvate carrier at intermediate state because the specific inhibitors used for this purpose had also an inhibitory effect on the adenylate kinase (data not shown).

All the control coefficient values that we have obtained are listed in Table 19.1. The control coefficient determined at state 3 present the same values whenever the state 3 is obtained by an excess of ADP or in presence of ADK. A striking difference concerns the Adenine nucleotides translocator which exerts a strong con-

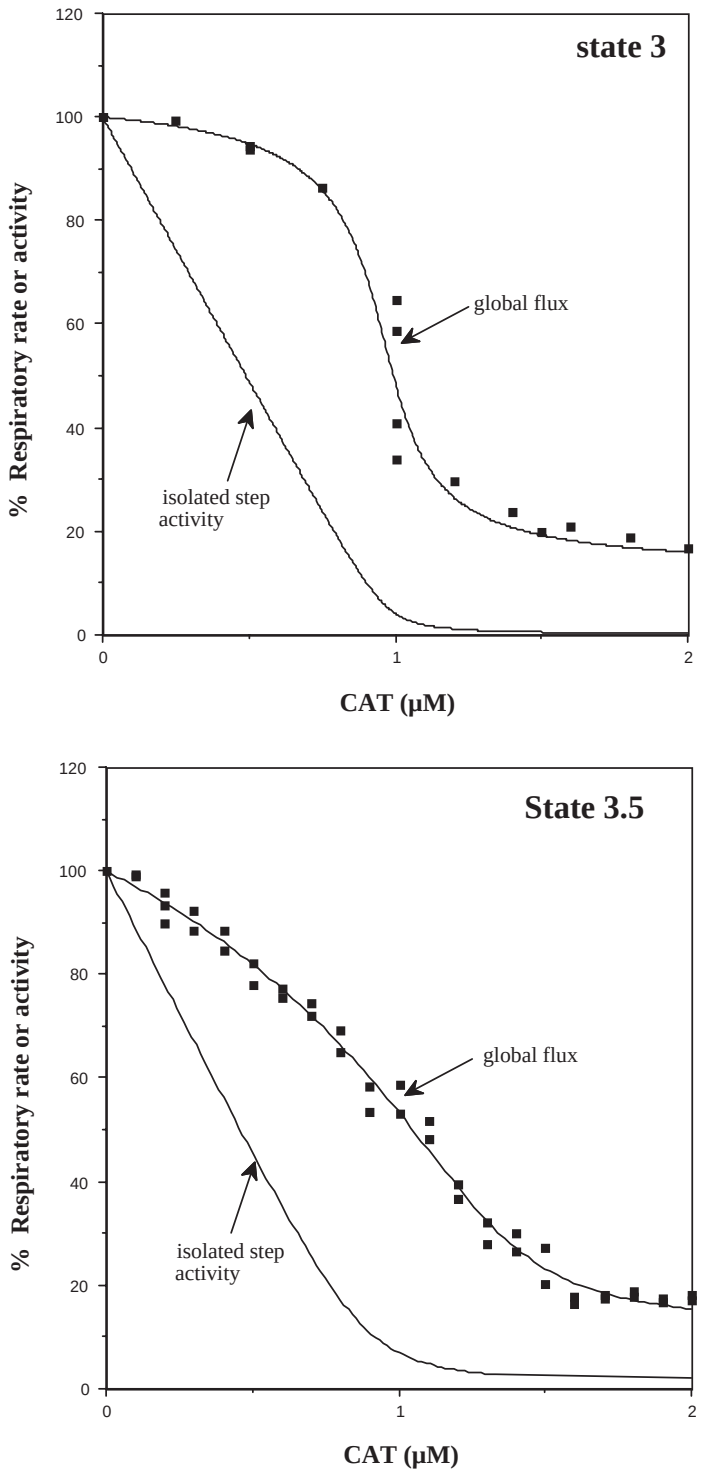

Fig. 19.2 Adenine nucleotides translocator titration curves at state 3 and 3.5

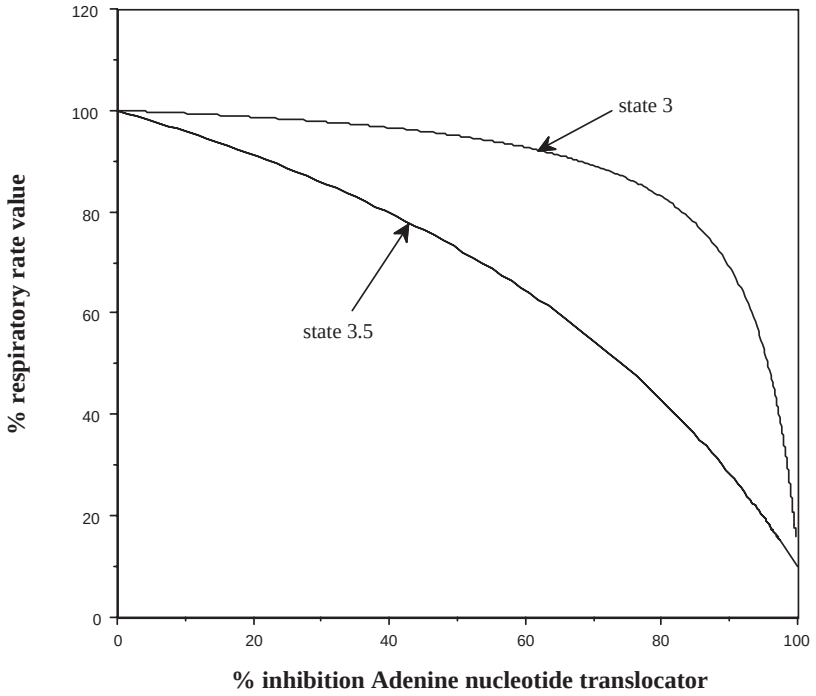

Fig. 19.3 Adenine nucleotides translocator threshold curves at state 3 and 3.5

trol on mitochondrial respiration at intermediate state (0.39) compared to state 3 (0.08). Complex I and complex III appear to exert the same control on the oxygen consumption flux whatever the steady state of respiration. Indeed, the control coefficient values of complex I and complex III at state 3 (0.14 and 0.23) are comparable to the values determined at intermediate state of respiration (0.10 and 0.27). The control of cytochrome c oxydase is low at intermediate state of respiration (0.07) compared to state 3 (0.19), and the same observation can be done for the ATP synthase (0.04 at intermediate state versus 0.10 at state 3). Concerning the phosphate carrier and the pyruvate carrier, it has not been possible to determine their flux control at intermediate state of respiration because the specific inhibitors used (respectively the mersalyl and C4H) had an inhibitory effect on adenylate kinase activity. However, the pyruvate carrier appears to be a major controlling step (0.20) at state 3 while the phosphate carrier has a low control (0.08). The sum of flux control coefficients at state 3 is around 1 (1.09) as predicted by the metabolic control theory but at intermediate state this sum is less than 1 (0.87). This can be partly explained by the fact that the control coefficient of pyruvate and phosphate carriers has not been assessed.

Threshold curves for adenine nucleotide translocator at state 3 and intermediate state

The threshold curves come from the titration curves. Each point of a threshold curve represents the respiratory rate inhibition percentage as a function of inhibition percentage of the isolated step activity for the same inhibitor concentration. In Fig. 19.3, are presented the threshold curves obtained for the Adenine nucleotide translocator at state 3 and at intermediate state. On this figure we can distinguish the two types of threshold curves that we have already evidenced in mitochondria isolated from different tissues [25].

In fact, the curve obtained at state 3 is a type I threshold curve which present a plateau phase followed by a steep breakage, while the curve obtained at intermediate state correspond to a type II characterized by smoother curves where the breakage is no longer evident, and where a precise threshold value is far more difficult to determine.

The variation observed in the biochemical threshold for the adenine nucleotide translocator between state 3 and intermediate state evidences a difference in the metabolic expression of a biochemical defect in the adenine nucleotide translocator. In fact, mitochondrial respiration appears to be more sensitive to a defect in the adenine nucleotide translocator at intermediate state compared to state 3. Thus, when considering, a defect of this step, it will not necessarily lead to a significant decrease in the mitochondrial metabolism at state 3 while, the same defect could impair mitochondrial respiration at intermediate state. This could suggest that in patients with clinical features of mitochondrial cytopathies, the impairment in mitochondrial energetic could be expressed only at some particular steady state of respiration.

References

1. Kacser, H. and Burns, J.A. (1973) *Symp. Soc. Exp. Biol.* **32**, 65–104.
2. Heinrich, R. and Rapoport, T.A. (1974) *Eur. J. Biochem.* **42**, 89–95.
3. Reder, C. (1988) *J. Theor. Biol.* **135**, 175–201.
4. Brand, M., Hafner, R. and Brown, G. (1988) *Biochem. J.* **255**, 535–539.
5. Doussiere, J., Ligeti, E., Brandolin, G. and Vignais, P.V. (1984) *Biochim. Biophys. Acta* **766**, 492–500.
6. Gellerich, F.N., Bohnensack, R. and Kunz, W. (1983) *Biochim. Biophys. Acta* **722**, 381–391.

7. Groen, A.K., Wanders, R.J.A., Westerhoff, H.V., Van der Meer, R. and Tager, J.M. (1982) *J. Biol. Chem.* **257**, 2754–2757.

8. Groen, A.K., Van der Meer, R., Westerhoff, H.V., Wanders, R.J.A., Akerboom, T.P.M. and Tager, J.M. (1982) in *Metabolic Compartmentation* (Sies, H., ed.), pp. 9–37, Academic Press, New York.

9. Jouaville, L., Ichas, F., Letellier, T., Malgat, M. and Mazat, J.P. (1993) Modern Trends in Biothermokinetics (Schuster, S., Rigoulet, M., Ouhabi, R. and Mazat, J.-P., eds.) **2**.

10. Korzeniewski, B. and Mazat, J. (1996) *Acta Biotheoretica* **44**, 263–269.

11. Kunz, W., Gellerich, F.N., Schild, L. and Schonfeld, P. (1988) *FEBS Lett.* **233**, 17–21.

12. Letellier, T., Malgat, M. and Mazat, J.P. (1993) *Biochim. Biophys. Acta* **1141**, 58–64.

13. Letellier, T., Malgat, M., Rossignol, R. and Mazat, J.P. (1998) *Mol. Cell. Biochem.* **148**, 409–417.

14. Mazat, J.P., Jean-Bart, E., Rigoulet, M. and Guerin, B. (1986) *Biochim. Biophys. Acta* **849**, 7–15.

15. Mazat, J.P., Letellier, T., Bédes, F., Malgat, M., Korzeniewski, B., Jouaville, L.S. and Morkuniene, R. (1997) *Mol. Cell. Biochem.* **174**, 143–148.

16. Moreno-Sanchez, R. (1985) *J. Biol. Chem.* **260**, 12554–12560.

17. Moreno-Sanchez, R., Devars, S., Lopez-Gomez, F., Uribe, A. and Corona, N. (1991) *Biochim. Biophys. Acta* **1060**, 284–292.

18. Hafner, R., Brown, G. and Brand, M. (1989) *Eur. J. Biochem.* **188**, 313–319.

19. Tager, J.M., Wanders, R.J.A., Groen, A.K., Kunz, W., Bohnensak, R., Kuster, U., Lekto, G., Boehme, G., Duszynski, J. and Wojtczak, L. (1983) *FEBS Lett.* **151**, 1–9.

20. Taylor, R.W., Birch-Machin, M.A., Bartlett, K., Lowerson, S.A. and Turnbull, D.M. (1994) *J. Biol. Chem.* **269**, 3523–3528.

21. Rolfe, D.F.S., Hulbert, A.J. and Brand, M.D. (1994) *Biochim. Biophys. Acta* **1118**, 405–416.

22. Wisniewski, E., Gellerich, F.N. and Kunz, W.S. (1995) *Eur. J. Biochem.* **230**, 549–554.

23. Wisniewski, E., Kunz, W.S. and Gellerich, F.N. (1993) *Eur. J. Biochem.* **268**, 9343–9346.

24. Letellier, T., Heinrich, R., Malgat, M. and Mazat, J.P. (1994) *Biochem. J.* **302**, 171–174.

25. Rossignol, R., Malgat, M., Mazat, J.P. and Letellier, T. (1999) *J. Biol. Chem.* **247**, 33426–33432.

26. Morgan-Hughes, J.A., Darveniza, P., Kkahn, S.N., Landon, D.N., Sherratt, R.M., Land, J.M. and Clark, J.B. (1977) *Brain* **100**, 617–640.

27. Gornall, A.G., Bardawill, C.J. and David, M.M. (1949) *J. Biol. Chem.* **177**, 751–766.

28. Chance, B. and Williams, G.R. (1956) *Adv. Enzymol.* **17**, 65–134.

29. Gellerich, F.N., Kunz, W.S. and Bohnensack, R. (1990) *FEBS Lett.* **274**, 167–170.

30. Holzhüter, H.G. and Colosimo, A. (1990) *Comput. Appl. Biosci.* **6**, 23–38.