Oxygen dependence of flux control of cytochrome *c* oxidase – implications for mitochondrial diseases

Falk R. Wiedemann, Wolfram S. Kunz*

Neurobiochemisches Labor, Klinik für Neurologie, Universitätsklinikum der Otto-von-Guericke Universität Magdeburg, Leipziger Str. 44, D-39120 Magdeburg, Germany

Received 27 October 1997; revised version received 11 December 1997

Abstract The oxygen dependence of cytochrome *c* oxidase control on succinate oxidation was investigated in saponin-permeabilized muscle fibers and isolated mitochondria from mouse quadriceps muscle applying metabolic control analysis. For this cyanide titrations of the oxygen consumption in the presence of succinate+rotenone were performed at different oxygen concentrations in the medium. While with isolated mitochondria high flux control coefficients were detected only at oxygen concentrations close to the K_M value of cytochrome *c* oxidase, with saponin-permeabilized fibers a significant increase of cytochrome *c* oxidase flux control was already observed below 130 μM oxygen. The result is in line with the high oxygen sensitivity of maximal respiration of saponin-permeabilized muscle fibers (P_{50} = 33 μM) caused most probably by oxygen diffusion gradients through the fiber lattice. The oxygen dependence of cytochrome *c* oxidase flux control in muscle fibers can explain the pathological phenotype of mild cytochrome *c* oxidase deficiencies in mitochondrial myopathies.

Key words: Oxygen; Cytochrome *c* oxidase; Metabolic control analysis; Mitochondrion; Saponin-permeabilized fiber; Mitochondrial disease

1. Introduction

Cytochrome *c* oxidase is the terminal enzyme of the mitochondrial respiratory chain which reduces molecular oxygen to water in a reaction coupled to proton translocation (for review see [1]). Three subunits of this enzyme are known to be mitochondrially encoded whose expression is affected in several mitochondrial DNA diseases [2,3]. However, the implication of mild cytochrome *c* oxidase deficiencies observed in some of the reported cases remains not understood [4–6]. This is due to the fact that the activity of cytochrome *c* oxidase is 2–3-fold higher than the activities of the other enzymes of the respiratory chain [7]. Therefore, a rather low flux control coefficient of this enzyme on mitochondrial succinate oxidation rate (about 0.2) was observed [7,8]. A threshold value (at approximately 30% of its maximal activity) was detected [9], above which changes in cytochrome *c* oxidase activity should not affect ATP synthesis. Therefore, small activity changes reported in mitochondrial myopathies [4–6], aging [10], mouse models [11] and some neurodegenerative diseases [12] should not contribute to the pathological phenotype, since the maximal mitochondrial ATP synthesis rate should remain unaffected. To resolve this apparent contradiction, we investigated the oxygen dependence of cytochrome *c* oxidase flux control in saponin-permeabilized muscle fibers. The treatment of muscle fibers with low concentrations of the plant-origin glycoside saponin causes a selective perforation of the sarcolemma, leaving mitochondria and sarcoplasmic reticulum intact [13,14]. This technique makes it possible to precisely control the extramitochondrial medium and the mitochondria can be investigated under in situ-like conditions. Our results indicate that the actual flux control coefficient of cytochrome *c* oxidase depends not only on the maximal activity of the enzyme but also on the oxygen availability within the tissue. This can serve as explanation for the pathological phenotypes of mild cytochrome *c* oxidase deficiencies.

2. Materials and methods

2.1. Preparation of muscle fibers

About 50 mg of quadriceps muscle from C57BL/10ScSn mice (obtained from Harlan) were used for the preparation of fibers. The quadriceps muscle was rapidly removed, washed and placed in an ice-cold isotonic NaCl solution. Bundles of muscle fibers containing usually about three single fibers were isolated by mechanical dissection. The saponin treatment was performed by incubation of the fiber bundles in a relaxing solution containing a 10 mM Ca-EGTA buffer (free concentration of calcium 0.1 μM), 20 mM imidazole, 20 mM taurine, 49 mM K-MES, 3 mM K_2HPO_4, 9.5 mM $MgCl_2$, 5 mM ATP, 15 mM phosphocreatine and 50 μg/ml saponin (pH 7.1) as described in [14].

2.2. Isolation of mitochondria

The mitochondria from about 1 g of total hindlimb skeletal muscle from one mouse were isolated according to a procedure developed for rat skeletal muscle [15]. Using 10 mM glutamate and 5 mM malate as substrates respiratory control ratios of higher than 6 were routinely obtained.

2.3. Respiration measurements

The oxygen consumption of permeabilized fibers was measured at 25°C using an Oroboros oxygraph (Anton Paar, Graz) in a medium containing 5 mM $MgCl_2$, 60 mM KCl, 110 mM mannitol, 10 mM KH_2PO_4, 0.5 mM Na_2EDTA and 60 mM Tris-HCl (pH 7.4). The respiration of isolated mitochondria was measured in the same medium containing additionally 2 mg/ml bovine serum albumin.

2.4. Determination of flux control coefficients

Using specific non-competitive inhibitors, the flux control coefficient of an enzyme *i* can be determined experimentally [7,8,15,16] according to the following equation:

$$C_i = -(dJ/J)/(dp_i/I_{max}) \quad (1)$$

where J is the flux through the pathway, p_i the concentration of a specific inhibitor of enzyme i and I_{max} is the maximal amount of inhibitor binding sites.

Eq. 1 was used for the calculation of flux control coefficients of cytochrome *c* oxidase. The maximal amount of cyanide binding sites in the samples (I_{max}, in nmol/mg protein or in nmol/mg dry weight) was determined from cyanide titration curves (performed under normoxic conditions) using non-linear regression analysis, accounting for the dissociation equilibrium of the inhibitor as described in [15,16]. The initial slopes of the titration curves (dJ/dp_i) were obtained by linear regression of the first three points of the titration. Different

*Corresponding author. Fax: (49) (391) 6715228.

PII S0014-5793(97)01586-X

oxygen concentrations were adjusted by argon/oxygen equilibration of the medium. In order to keep almost identical oxygen concentrations during the entire titration experiment, the potassium cyanide solutions used in the titrations were made with oxygen-equilibrated water (and kept in an oxygen atmosphere).

3. Results and discussion

In Fig. 1 the maximal respiration rates of saponin-permeabilized mouse quadriceps fibers oxidizing succinate+rotenone are plotted versus the actual oxygen concentration in the measurement chamber (open circles, dotted line, left scale). It is remarkable that with these fibers already at 33 μM oxygen the respiration rate is about one half of its normoxic value. This is about 100-fold higher than the K_m value of cytochrome *c* oxidase for oxygen. For comparison, we determined the oxygen dependence of maximal respiration of mouse muscle mitochondria under identical conditions. It can be seen that this rate depends to a much smaller extent on the actual oxygen concentration (filled circles, solid line, right scale), with a P_{50} value of about 2 μM. These data, being in line with previous reports for permeabilized cardiac myocytes and isolated rat heart mitochondria [17], point to the extreme importance of oxygen gradients within the muscle tissue.

To determine if this oxygen dependence of maximal respiration is caused by cytochrome *c* oxidase and to study the metabolic consequences of this phenomenon in detail, we applied metabolic control analysis and determined the flux control coefficient of cytochrome *c* oxidase at different oxygen concentrations. For this we performed cyanide titrations of the succinate (+rotenone) oxidation rate in the presence of 1 mM ADP. To keep the concentration of oxygen in the measurement chamber at a constant value we used oxygen-equilibrated inhibitor solutions for the titration experiments. Typical titrations of succinate respiration of saponin-permeabilized fibers at two different oxygen concentrations in the chamber are shown in Fig. 2. While under nearly normoxic conditions (190 μM oxygen, open circles) the initial slope of the titration curve is rather flat, indicating a low flux control coefficient, already at about 130 μM oxygen (filled circles) a steeper initial slope was detected. Moreover, the

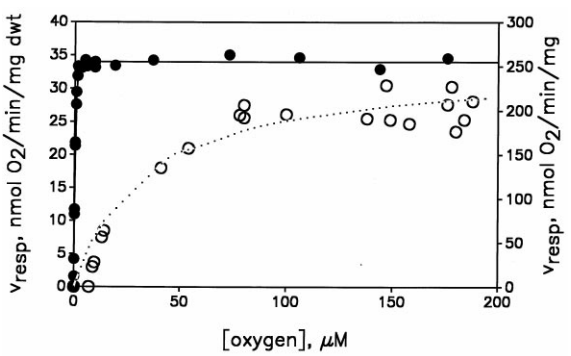

Fig. 1. Oxygen dependence of succinate oxidation of isolated mouse muscle mitochondria (filled circles, right scale) and saponin-permeabilized skeletal muscle fibers (open circles, left scale). 0.025 mg/ml mitochondria or 1 mg dry weight fibers in the medium for respiration measurements, respectively, containing additionally 10 mM succinate and 10 μM rotenone. The different oxygen concentrations were adjusted by equilibration of the medium with an oxygen/argon mixture. The maximal respiration was adjusted by the addition of 1 mM ADP.

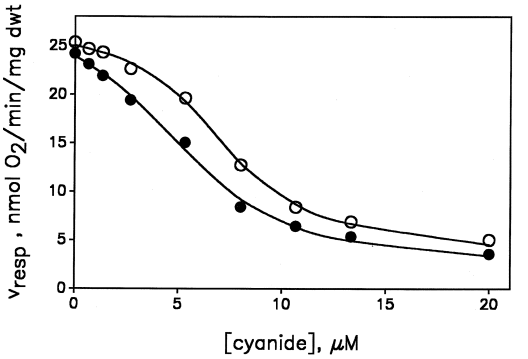

Fig. 2. Cyanide titration of the maximal succinate oxidation rate of saponin-permeabilized mouse quadriceps fibers at two different oxygen concentrations in the medium. 1 mg dry weight saponin-permeabilized fibers at 190 μM oxygen (open circles) or at 130 μM oxygen (filled circles) in the medium for respiration containing additionally 10 mM succinate, 10 μM rotenone and 1 mM ADP. To keep the oxygen concentration in the chamber nearly constant oxygen-equilibrated cyanide solutions were used for the titration experiments. Using non-linear regression [16] titration curves were fitted to the experimental points with the following parameters: Open circles – flux control coefficient (C_i) = 0.14, maximal amount of inhibitor (I_{max}) = 6.9 μM, dissociation constant of the inhibitor (K_d) = 0.35 μM, initial rate of respiration (J) = 25.0 nmol O_2/min/mg dry weight; filled circles – C_i = 0.38, I_{max} = 6.6 μM, K_d = 0.4 μM, J = 23.9 nmol O_2/min/mg dry weight.

amount of cyanide necessary to cause complete inhibition of respiration decreased. For the calculation of flux control coefficients according to Eq. 1 we determined the initial slopes of the titration curves by linear regression of the first three points and used the value for the maximal amount of cyanide binding sites calculated by non-linear regression analysis of the complete titration curve under normoxic conditions (according to [16]). Using this procedure we observed at 190 μM oxygen a flux control coefficient of cytochrome *c* oxidase of 0.14 and at 130 μM oxygen a flux control coefficient of 0.38.

Similar experiments at different oxygen concentrations in the chamber were performed also with isolated mitochondria. From all titration curves we calculated the cytochrome *c* oxidase flux control coefficients and plotted the values versus the oxygen concentration (Fig. 3). It is remarkable that the flux control coefficient of cytochrome *c* oxidase on maximal respiration flux (succinate+rotenone) in isolated mitochondria above 10 μM oxygen is below 0.3, approaching under normoxic conditions, in agreement with previous reports [7,8], a value of about 0.2 (filled circles, solid line). For saponin-permeabilized fibers this dependence of cytochrome *c* oxidase flux control is shifted to much higher oxygen concentrations (open circles, dotted line). Already at about 130 μM oxygen in the measurement chamber the flux control of cytochrome *c* oxidase approaches values of 0.4.

In Table 1 the calculated P_{50} values for oxygen from maximal rate measurements (according to Fig. 1) or from flux control determinations of cytochrome *c* oxidase (according to Fig. 3) are summarized. Obviously, the P_{50} values are about 3-fold greater when calculated from the oxygen dependence of flux control coefficients (P_{50mito} = 6.6 μM and $P_{50fiber}$ = 95 μM) than from the oxygen dependence of maximal rates (P_{50mito} = 2 μM and $P_{50fiber}$ = 33 μM). This result is in line with the concept of metabolic control analysis that the effect of an enzyme activity change on the maximal flux rate through a linear metabolic pathway becomes detectable if the flux control coeffi-

Table 1
P_{50} values of maximal respiration rates and flux control coefficients of cytochrome *c* oxidase for oxygen

	Isolated mitochondria	Saponin-permeabilized fibers
Maximal rate of respiration	2 ±0.5 μM	33 ± 13 μM
Flux control coefficient	6.6 ± 1.4 μM	95 ± 15 μM

The P_{50} values of maximal respiration rates and flux control coefficients of cytochrome *c* oxidase were calculated from the plots of the oxygen dependence of maximal rates in Fig. 1 and the oxygen dependence of flux control coefficients in Fig. 3, respectively.

cient of this enzyme approaches values close to unity [18,19]. Therefore, Fig. 3 suggests that effects on the maximal rates should be observed in mitochondria at oxygen concentrations below 3 μM and in fibers at oxygen concentrations below 60 μM, which is in agreement with the results presented in Fig. 1.

These data indicate that the influence of small activity changes of cytochrome *c* oxidase on tissue energy metabolism crucially depends on the oxygen concentration within the tissue. In agreement with earlier reports with isolated cardiac myocytes [17] we observed a strong oxygen dependence of maximal rates of respiration of saponin-permeabilized muscle fibers. This phenomenon is most probably caused by oxygen diffusion gradients within the fiber lattice [17,20]. In agreement with the larger diffusion distances in skeletal muscle fibers (the diameter of a bundle of three mouse muscle fibers is about 100 μm, the diameter of a cardiomyocyte is in the order of 20 μm [20]) the P_{50} values for maximal respiration of fibers (33 ± 13 μM) are about 5-fold higher than of isolated cardiomyocytes (6–8 μM [17]). This oxygen dependence strongly affects the controlling influence of cytochrome *c* oxidase on mitochondrial oxidative phosphorylation at physiological oxygen concentrations in the muscle tissue. Below 50 μM oxygen the flux control coefficient of the enzyme becomes close to unity, e.g. cytochrome *c* oxidase is nearly exclusively rate controlling (cf. Fig. 3, open circles).

Since saponin-permeabilized muscle fibers are a model of mitochondrial function in skeletal muscle under in situ-like conditions [13,14], the observed oxygen dependence of cytochrome *c* oxidase flux control can explain the pathological phenotypes of mild cytochrome *c* oxidase deficiencies in muscle, reported for mitochondrial diseases [4–6], aging [10], neurodegenerative diseases [12] and cytochrome *c* oxidase deficient mice [11]. Under normoxic conditions these deficiencies (about 50% of the control activity) do not affect the maximal rate of respiration of saponin-permeabilized skeletal muscle fibers [6,11]. Only a two-fold increase of the flux control coefficient of cytochrome *c* oxidase was reported [6,11]. However, at lower physiological oxygen concentrations, when the flux control exerted by cytochrome *c* oxidase increases, mild deficiencies of this enzyme obviously can affect the mitochondrial ATP production rate. In addition, our data indicate that at these oxygen concentrations modulators of cytochrome *c* oxidase activity (like NO [21] or adenine nucleotides [22]) should contribute to the regulation of mitochondrial oxidative phosphorylation in skeletal muscle.

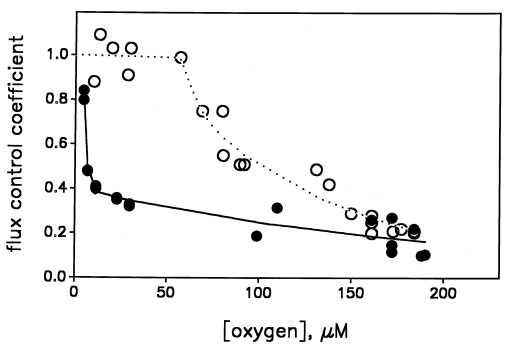

Fig. 3. Oxygen dependence of the flux control of cytochrome *c* oxidase on succinate oxidation in isolated mouse muscle mitochondria (filled circles) and saponin-permeabilized skeletal muscle fibers (open circles). The flux control coefficients were calculated from cyanide titration experiments at different oxygen concentrations in the measurement chamber (cf. Fig. 2). The calculations were performed on the basis of Eq. 1.

Acknowledgements: The excellent technical assistance of Mrs. K. Kaiser and J. Witzke is gratefully acknowledged. This work was supported by a grant of the Land Sachsen-Anhalt (1795A/0084).

References

[1] Babcock, G.T. and Wikström, M. (1992) Nature 356, 301–309.
[2] Wallace, D.C. (1992) Annu. Rev. Biochem. 61, 1175–1212.
[3] Moreas, C.T., DiMauro, S., Zeviani, M. and Schon, E.A. (1989) New Engl. J. Med. 320, 1293–1299.
[4] Shanske, S., Moraes, C.T., Lombes, A., Miranda, A.F., Bonilla, E., Lewis, B.A., Whelan, M.A., Ellsworth, C.A. and DiMauro, S. (1990) Neurology 40, 24–28.
[5] Geromel, V., Parfait, B., von Kleist-Retzow, J.-C., Chretien, D., Munnich, A., Rötig, A. and Rustin, P. (1997) Biochem. Biophys. Res. Commun. 236, 643–646.
[6] Kuznetsov, A.V., Winkler, K., Kirches, E., Lins, H., Feistner, H. and Kunz, W.S. (1997) Biochem. Biophys. Acta 1360, 142–150.
[7] Letellier, T., Malgat, M. and Mazat, J.P. (1993) Biochim. Biophys. Acta 1141, 58–64.
[8] Taylor, R.W., Birch-Machin, M.A., Bartlett, K., Lowerson, S.A. and Turnbull, D.M. (1994) J. Biol. Chem. 269, 3523–3528.
[9] Letellier, T., Heinrich, R., Malgat, M. and Mazat, J.-P. (1994) Biochem. J. 302, 171–174.
[10] Müller-Höcker, J. (1992) Brain Pathol. 2, 149–158.
[11] Kuznetsov, A.V., Clark, J.F., Winkler, K. and Kunz, W.S. (1996) J. Biol. Chem. 271, 283–288.
[12] Benecke, R., Strumper, P. and Weiss, H. (1993) Brain 116, 1451–1463.
[13] Veksler, V.I., Kuznetsov, A.V., Sharov, V.G., Kapelko, V.I. and Saks, V.A. (1987) Biochim. Biophys. Acta 892, 191–196.
[14] Kunz, W.S., Kuznetsov, A.V., Schulze, W., Eichhorn, K., Schild, L., Striggow, F., Bohnensack, R., Neuhof, S., Grasshoff, H., Neumann, H.W. and Gellerich, F.N. (1993) Biochim. Biophys. Acta 1144, 46–53.
[15] Wisniewski, E., Kunz, W.S. and Gellerich, F.N. (1993) J. Biol. Chem. 268, 9343–9346.
[16] Gellerich, F.N., Kunz, W.S. and Bohnensack, R. (1990) FEBS Lett. 274, 167–170.
[17] Kennedy, F.G. and Jones, D.P. (1986) Am. J. Physiol. 250, C374–C383.
[18] Kacser, H. and Burns, J.A. (1973) In: Rate Control of Biological Processes (Davies, D.D., Ed.), pp. 65–104, Cambridge University Press, London.
[19] Heinrich, R. and Rapoport, T.A. (1974) Eur. J. Biochem. 42, 97–105.
[20] Jones, D.P. and Kennedy, F.G. (1986) Am. J. Physiol. 250, C384–C390.
[21] Brown, G.C. (1995) FEBS Lett. 369, 136–139.
[22] Kadenbach, B., Barth, J., Akgun, R., Freund, R., Linder, D. and Possekel, S. (1995) Biochim. Biophys. Acta 1271, 103–109.