CONTROL OF RESPIRATION BY CYTOCHROME C OXIDASE IN INTACT CELLS: ROLE OF THE MEMBRANE POTENTIAL

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Running title: \(\Delta \psi\) and cytochrome oxidase in cell

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Metabolic control analysis was applied to intact HepG2 cells. The effect on the control coefficient of cytochrome c oxidase (CcOX) over cell respiration of both the electrical (\(\Delta \Psi\)) and the chemical (\(\Delta \mu_{H^+}\)) component of the mitochondrial transmembrane proton electrochemical gradient (\(\Delta \mu_{H^+}\)) was investigated. The overall \(O_2\)-consumption and the specific CcOX activity of actively phosphorylating cells were titrated with cyanide under conditions in which \(\Delta \Psi\) and \(\Delta \mu_{H^+}\) were selectively modulated by addition of ionophores. In the absence of ionophores, CcOX displayed a high control coefficient (\(C_{IV} = 0.73\)), thus representing an important site of regulation of mitochondrial oxidative phosphorylation. A high control coefficient value (\(C_{IV} = 0.85\)) was also measured in the presence of nigericin, i.e. when \(\Delta \Psi\) is maximal, and in the presence of nigericin and valinomycin (\(C_{IV} = 0.77\)), when \(\Delta \mu_{H^+}\) is abolished. In contrast, CcOX displayed a markedly lower control coefficient (\(C_{IV} = 0.30\)) upon addition of valinomycin, when \(\Delta \Psi\) is converted into \(\Delta \mu_{H^+}\). These results show that \(\Delta \Psi\) is responsible for the tight control of CcOX over respiration in actively phosphorylating cells.

Cytochrome c oxidase (CcOX), the final electron acceptor of the mitochondrial respiratory chain, catalyzes the four-electron reduction of \(O_2\) to \(H_2O\), while actively pumping protons from the matrix into the inter-membrane space (1,2). The electron transfer and \(H^+\) translocation across the mitochondrial inner membrane, that are catalyzed by CcOX and by the other respiratory chain complexes, generate and maintain the transmembrane proton electrochemical gradient (\(\Delta \mu_{H^+}\)), comprising the electrical component, i.e. the membrane potential (\(\Delta \psi\)), and the chemical proton gradient (\(\Delta \mu_{H^+}\)). According to the classical chemiosmotic theory (3), the \(\Delta \mu_{H^+}\) is used by \(F_0F_1\)-ATP synthase (complex V) to synthesize ATP from ADP and phosphate. The activity of the respiratory complexes is down-regulated by \(\Delta \mu_{H^+}\) and mitochondria oscillate between two respiratory energetic states, namely state 3, with ATP synthesis occurring at the expense of \(\Delta \mu_{H^+}\) during active oxidative phosphorylation, and state 4, i.e. the resting state, as originally defined (4).

A number of pathological states have been correlated to mitochondrial dysfunction and alteration of \(\Delta \mu_{H^+}\), with uncontrolled production of free radicals and/or lack of energy (5,6). The metabolic control analysis (MCA) (7,8) is a key tool to estimate, under different metabolic conditions, the relative contribution of each respiratory complex to the control of the respiratory chain electron flux, and in turn of oxidative phosphorylation. MCA has been used to estimate, both in isolated mitochondria and intact cells, the control coefficient of each respiratory complex; a value that has been shown to vary depending on tissues and on metabolic conditions leading to distinct mitochondrial energization states (9,10).

Using the MCA approach a large CcOX control over respiration has been observed in human hepatoma HepG2 cells (11,12). Piccoli et al. (11) have recently addressed the role of \(\Delta \mu_{H^+}\) on the control coefficient of CcOX (\(C_{IV}\)), performing the investigation under non-phosphorylating conditions, i.e., in state 4. They found a low \(C_{IV}\) value in the presence of either oligomycin, blocking ATP synthase, or oligomycin plus valinomycin, converting \(\Delta \psi\) into \(\Delta \mu_{H^+}\).

The relative effect of \(\Delta \psi\) and \(\Delta \mu_{H^+}\) on the activity of CcOX, has been extensively investigated on the isolated enzyme. Experiments carried out using CcOX reconstituted into...
artificial phospholipid vesicles (13,14) proved that, if the electron delivery to the enzyme is not limiting, as in the presence of excess reducing substrates, CeOX activity is controlled predominantly by $\Delta \psi$, rather than by $H^+$ re-equilibration, but see also (15-17).

In the present work, we have extended previous studies in actively respiring HepG2 cells to further investigate the relative effect of $\Delta \psi$ and $\Delta \mu_{H^+}$ on the control coefficient of CeOX, but under phosphorylating state 3 conditions (11), i.e. in the absence of oligomycin. We have shown that in intact cells, where the ATP synthase is not impaired by inhibitors, the metabolic control exerted by CeOX over the respiratory chain depends on $\Delta \psi$. Our results also suggest that the depressing effect over respiration of $\Delta \mu_{H^+}$ affects the distribution of control among the individual steps of the oxidative phosphorylation system.

**Experimental Procedures**

**Chemicals**

Sodium ascorbate, $N,N,N',N'$-tetramethyl-p-phenylenediamine (TMPD), nigericin, valinomycin, oligomycin were from Sigma. The human hepatoma cell line HepG2 was from A.T.C.C.. Dulbecco’s modified Eagle’s medium (DMEM), trypsin, EDTA, penicillin, streptomycin, pyruvate and fetal bovine serum were from Invitrogen.

**Cell cultures**

The HepG2 cell line was maintained in culture with DMEM supplemented with 10% (v/v) fetal bovine serum, 100 U ml$^{-1}$ penicillin, 100 $\mu$g ml$^{-1}$ streptomycin, 1 mM pyruvate, 2 mM L-glutamine. Prior to the experiments, cells were detached by trypsinization, collected by centrifugation and suspended into the medium equilibrated at 37°C, for counting and assessing cell viability by trypan blue exclusion.

**Oxygraphic experiments**

After trypsinization and centrifugation, cells were washed twice, suspended at a final density of 4.5 to 6.5 x 10$^6$ cells ml$^{-1}$ into buffer solution (25 mM HEPES pH 7.2, 118 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO$_4$, 1.2 mM KH$_2$PO$_4$, 20 mM glucose, pre-equilibrated at 37°C) and used immediately for experimentation. Aliquots of the cell suspension were distributed into the 1.5-ml chambers of a dual chamber stand-alone high resolution respirometer (Oxygraph-2k, Oroboros Instruments) to monitor the endogenous cell O$_2$-consumption and, in parallel, the CeOX specific activity at 37°C. When indicated, ionophores were used at the lowest concentration causing maximal stimulation of respiration (i.e. 33 nM valinomycin or 100 nM nigericin or a combination of both substances). Cyanide-sensitivity was assayed by sequential addition of the inhibitor (using 80 mM or 8 mM NaCN stock solutions). According to (11,12,18), under all conditions, the specific activity of CeOX was measured in the presence of 20 nM antimycin A and using as substrates excess ascorbate (10 mM) and non-saturating amounts of TMPD (0.2 mM), thus not at V$_{max}$. At the concentration used, TMPD did not significantly reduces mitochondrial $\Delta \psi$, as assessed using the fluorimetric dye JC1 in cell suspensions (data not shown).

**Determination of the control coefficient**

Based on the metabolic control analysis (19-21), the control coefficient of CeOX ($C_{IV}$) was calculated from the cyanide-titration profile of the global cell respiration and the CeOX specific activity, according to the following equation:

$$C_{IV} = S_1/S_2$$  \hspace{1cm} (EQ1)

where $S_1 = (dJ/dI)_{I \to 0}$ and $S_2 = (dv/dI)_{I \to 0}$, $J$ is the global O$_2$-consumption rate, $v$ is the specific activity of CeOX and $I$ is the cyanide concentration.

The experimental error of $C_{IV}$ ($\varepsilon_{C_{IV}}$) has been evaluated as follows:

$$\varepsilon_{C_{IV}} = C_{IV} \left( \frac{\varepsilon_1}{S_1} + \frac{\varepsilon_2}{S_2} \right)^2$$

where $\varepsilon_1$ and $\varepsilon_2$ are the standard deviations of $S_1$ and $S_2$.

**RESULTS**

The effect of $\Delta \mu_{H^+}$ on the metabolic control of cytochrome $c$ oxidase over cell respiration was investigated by performing cyanide titrations of both the endogenous respiration (global activity) and the O$_2$ consumption sustained by the sole CeOX (specific activity) in intact HepG2 cells. To gather information on the relative effect of the electrical and the chemical component of $\Delta \mu_{H^+}$, measurements were carried out in the absence and presence of ionophores specifically collapsing either $\Delta \psi$ (valinomycin) or $\Delta \mu_{H^+}$ (nigericin). Data reported in Table 1 indicate that, in the absence of ionophores, cell respiration sustained by endogenous substrates proceeds at a rate of 62.8 ±
20.8 pmol O₂ · s⁻¹ · 10⁶ cells⁻¹ that is almost completely (≥ 95 %) abolished by 1 mM NaCN or 20 nM antimycin A (data not shown). This rate is only slightly stimulated in the presence of 100 nM nigericin, whereas it increases by ~2-3 folds upon addition of 33 nM valinomycin alone or in combination with nigericin (132.7 ± 55.1 and 182.3 ± 69.1 pmol O₂ · s⁻¹ · 10⁶ cells⁻¹, respectively). No significant changes were observed at the level of specific activity regardless of the presence of ionophores.

Figure 1 shows typical sets of data acquired with untreated intact HepG2 cells (top panels) and in the presence of valinomycin (bottom panels). As expected, upon sequential additions of cyanide to the respiring cells, the O₂-consumption rate decreases in a concentration dependent manner. In the absence of ionophores, both the global (Fig.1A) and the specific activity (Fig. 1B) are affected by cyanide to a similar extent and particularly in the low cyanide concentration range (0-16 µM), critical for estimation of the CeOX control coefficient (Cᵥ). In particular, at 16 µM NaCN (addition ‘d’ in the figure), the global and the CeOX specific residual activities are comparable (88 % and 83% of the corresponding initial values, respectively), strongly indicating that the control coefficient approaches the unity under these conditions. In the presence of valinomycin, converting ΔΨ into ΔpH, the global activity is less sensitive to cyanide than the CeOX specific activity (compare panel C and D in Fig. 1); i.e. at 16 µM KCN, the residual global activity is up to 95% whereas the specific activity of CeOX is about 81 %. In other words, low concentration values of cyanide, while inhibiting CeOX, are still insufficient to significantly affect the endogenous respiration of valinomycin-treated cells, pointing to a low control coefficient of the enzyme (Table 2). The effect of valinomycin is better appreciated in Figure 2 summarizing the results of cyanide titration experiments carried out with untreated cells (A) or after addition of valinomycin (B), nigericin (C) or both the ionophores (D). For each condition, the residual global and CeOX specific activities (open and closed circles, respectively), measured in the presence of increasing amounts of cyanide, are expressed as percentages of the uninhibited respiration rate and reported as a function of the inhibitor concentration. The data were obtained by averaging the results of a series of titration experiments similar to those reported in Fig.1.

Figure 2 clearly shows that the data points corresponding to the global and the CeOX specific activities markedly diverge only in valinomycin treated cells (panel B, when ΔΨ is converted into ΔpH). Indeed, under that condition, the CeOX specific activity (closed circles) is more sensitive to cyanide than the global one (open circles) whereas under all the other conditions explored, the initial slopes of the curves are similar. As a result, the flux control coefficient, calculated as the ratio of the initial slope in the curve profile of the global and specific (CeOX) activities (see EQ1), is low in the presence of valinomycin, whereas it takes higher values approaching unity in the other cases (cf. Tab. 2). This finding strongly suggests that Δψ increases the control coefficient of CeOX over cell respiration.

The role of ΔpH on the control coefficient of CeOX was investigated by carrying out cyanide titrations in the presence of nigericin, a K⁺/H⁺ antiporter which converts the mitochondrial ΔpH into Δψ; the titration profiles of both the global and specific activities in Fig. 2C and the corresponding flux coefficient values are comparable to those acquired in the untreated cells.

The abolishment of ΔµH⁺ by the simultaneous addition of nigericin and valinomycin to the cell suspension was also achieved. As shown in Fig. 2D, under these conditions CeOX exerts a high metabolic control over respiration, based on the finding that the specific CeOX activity and the endogenous cell respiration display similar cyanide sensitivity, particularly in the low cyanide concentration range.

Figure 3 shows the threshold plots relative to our data; the residual endogenous cell respiration rate at any given cyanide concentration is plotted as a function of the percentage of cyanide-inhibited CeOX in the sample. Two different threshold profiles have been observed: the profile for valinomycin treated cells has a markedly curved shape and 50% inhibition of CeOX by cyanide results in only 15-20% decrease of the global flux. Under all the other conditions (i.e. untreated cells or both in the presence of nigericin alone and in combination with valinomycin) the profiles appear significantly less curved. Particularly, under fully uncoupled conditions the CeOX inhibition correlates almost linearly with that of the global respiratory flux. Therefore, in untreated cells the CeOX control over cell respiration is fairly high whereas, when ΔΨ is converted into ΔpH, the CeOX control is markedly reduced.
DISCUSSION

MCA applied to isolated mitochondria showed that the control of the respiratory rate is distributed among several steps, depending on fluxes and availability of the mitochondrial substrates (10). Besides the respiratory chain complexes, proton leakage, phosphate carrier, pyruvate carrier, ATP synthase and adenine nucleotide carrier have been found to exert some control over oxidative phosphorylation. The control exerted by the electron transport chain complexes increases, however, when evaluated in experimental systems closer to in vivo conditions (22,23) and in intact cells (11,12,24,25). The higher control coefficient value in cells rather than in isolated mitochondria (11) and the low inhibition threshold level (20% for several human cell lines (25)) measured for CcOX under near physiological conditions point to a tight control of the enzyme in intact cells (25). Since CcOX activity is reportedly influenced by the proton electrochemical gradient (see below), we have focussed our attention on the role of ΔμH⁺ and its electrochemical components (ΔΨ and ΔpH) on the activity of CcOX and the control of the enzyme over cell respiration in whole HepG2 cells.

The main result herein presented is that, under near physiological conditions, the control of CcOX over cell respiration predominantly depends on the electrical component (ΔΨ) of the proton electrochemical gradient. This finding is fully consistent with a large body of previous spectroscopic (1,13,14,26) and electrometric (27,28) measurements carried out using CcOX reconstituted into artificial phospholipid vesicles (COV). Those experiments provide a possible mechanistic explanation of how the membrane potential controls the activity of the enzyme: the rate limiting step in the CcOX catalytic cycle, i.e. the internal electron transfer from heme a to the oxidized heme a₃/CuB site, was shown to be coupled to both a vectorial H⁺ uptake from the mitochondrial N-phase and H⁺ translocation to the P-phase, synchronous with the redox reaction (28). The electron transfer process is thus electrogenic and consistently it slows down remarkably in the presence of a suitable ΔΨ (≥ 180 mV in COV, (13)). All together these findings support the original proposal by Brunori et al. (3) (14) that ΔΨ stabilizes CcOX in a state characterised by a lower rate of internal electron transfer and thus a lower turnover rate. This stabilization is removed by valinomycin, selectively converting ΔΨ into ΔpH, and allows the enzyme to turnover more rapidly (molecular basis of the respiratory control ratio in COV).

Consistently, we show that the addition of valinomycin to HepG2 cells decreases the control coefficient from CIV = 0.73 to CIV = 0.30 indicating that ΔΨ contributes to the control exerted by CcOX in the cell. This finding might appear in contrast with the report of Piccoli et al. (11) who showed minor effects of valinomycin on the CcOX control coefficient evaluated in the same cell line. The discrepancy, however, is only apparent since those experiments, at difference with the present work, were carried out in the presence of the ATP-synthase inhibitor oligomycin, i.e. in state 4 condition. Under these conditions ΔμH⁺ is likely maximal and most of the control over respiration is exerted by the H⁺ back-leak into the matrix rather than by CcOX or by any other respiratory chain complex (11,29); therefore the collapse of ΔΨ by valinomycin can not affect significantly the control exerted by CcOX. Conversely, our data show that, in actively phosphorylating cells, ΔΨ is responsible for the CcOX tight control over respiration, whereas under the same condition ΔpH seems irrelevant in this respect, as the addition of nigericin is essentially ineffective.

The effect of valinomycin on the CIV appears fully consistent with the results of rapid mixing experiments (carried out using COV (13,14). According to the MCA theory, all the control coefficients of the steps involved in an integrated process should sum to one (summation theorem (21)). Thus in the presence of valinomycin, in response to a decrease of the CcOX flux control, a compensatory increase of the control level of other steps is expected. The chemical H⁺ gradient (ΔpH) is expected to increase at the expense of the electrical component, and to balance the dissipation of mitochondrial ΔΨ. Under these conditions the respiratory complexes whose activity is more sensitive to ΔpH (30,31), i.e. those upstream CcOX, likely increase their control over respiration. When both ΔpH and ΔΨ collapse, as in the presence of both valinomycin and nigericin, the enzymes upstream CcOX may lose such control. Consistently, our and previous data (11,12) show that the abolishment of ΔμH⁺ leads to a high CIV over respiration, and suggest that other factors, such as substrates concentration and level of effectors, may limit CcOX activity under these conditions (25) and account for the high control over respiration.
We observed two different types of threshold plots (Fig.3). In the presence of valinomycin alone, the data show a plateau phase in which inhibition of up to 30-40 % CcOX modestly affects the overall respiratory flux. Thus, when $\Delta \psi$ is converted into $\Delta \text{pH}$, there is an excess of functional capacity of CcOX. Under all the other conditions examined (control cells alone or plus nigericin in the presence/absence of valinomycin), a clear threshold is not detected consistent with a higher control of CcOX corresponding to a smaller threshold value (10).

To summarize, we have demonstrated that, in intact cells under phosphorylating conditions (i.e., with fully active ATP synthase), CcOX activity and its control over oxidative phosphorylation are dependent on $\Delta \Psi$. The $\Delta \Psi$ dependence suggests that specific mutations associated to a diminished CcOX efficiency and/or an altered $\Delta \psi/\Delta \text{pH}$ balance (32) may change the distribution of control in the respiratory metabolic pathway and therefore the specific tissue susceptibility to diseases.

**ACKNOWLEDGMENTS**

Work partially supported by Ministero dell’Istruzione, dell’Università e della Ricerca of Italy to P.S. and to G.L.

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FIGURE LEGENDS

Figure 1: Cyanide titration of O2-consumption by HepG2 cells: effect of valinomycin
Oxygraphic traces (thick line refs. to left axis scale) shown together with their first derivative curves, indicating the rate of oxygen consumption, (thin, step-wise, line refs. to right axis scale, 100% taken as the activity measured in the absence of the inhibitor). Endogenous respiration (A, C) and CcOX specific activity (B, D) measured in untreated (top panels) or valinomycin-treated (bottom panels) cells in the presence of different cyanide concentrations (μM, from a to l): 2.7; 5.3; 10.7; 16; 26.7; 80; 133.3; 189.4; 295.7; 829.4. Cell density (cells·ml⁻¹): 3.3 x 10⁶ (A); 3.9 x 10⁶ (B) and 2.3 x 10⁶ (C, D).

Figure 2: Cyanide titration profiles
Cyanide titration profiles of the global (open circle) and the CcOX specific (closed circle) activity, as measured in intact HepG2 cells under the following conditions: (A) control, n = 8; (B) valinomycin, n = 9; (C) nigericin, n = 9; (D) valinomycin and nigericin, n = 7. Data points ± standard deviations. Insets show data in the low cyanide concentration range, together with the linear regression analysis.

Figure 3: Threshold plots
Threshold plots of the data shown in Fig. 2. Control (1, closed circles); valinomycin (2, open squares); nigericin (3, open circles); valinomycin and nigericin (4, open triangles).
Table 1 – Rate of oxygen consumption (± S.D.) in HepG2 cells in the absence and in the presence of ionophores. Independent experiments number, n ≥ 10.

|                        | Endogenous Respiration | CcOX activity |
|------------------------|------------------------|---------------|
|                        | (pmol O₂ · s⁻¹ · 10⁶ cells⁻¹) |               |
| control                | 62.8 ± 20.8            | 151.6 ± 30.5  |
| + valinomycin          | 132.7 ± 55.1           | 135.5 ± 20.3  |
| + nigericin            | 70.3 ± 18.3            | 126.7 ± 34.8  |
| + valinomycin and nigericin | 182.3 ± 69.1         | 125.9 ± 30.5  |
Table 2: Flux control coefficients of CcOX over endogenous respiration in HepG2 cells in the absence and in the presence of ionophores. Error analysis in the Experimental Procedures.

| Control coefficient | (C_n) |
|---------------------|-------|
| control             | 0.73 ± 0.06 |
| + valinomycin       | 0.30 ± 0.03 |
| + nigericin         | 0.85 ± 0.07 |
| + valinomycin and nigericin | 0.77 ± 0.10 |
Fig. 1

A  CTR (global)  

B  CTR (specific)  

C  VAL (global)  

D  VAL (specific)  

[O₂] (μM)  

ACTIVITY (%)  

TIME (min)
Fig. 2

![Graphs showing activity against [NaCN] and [CN] concentrations for different conditions.](http://www.jbc.org/)

- **A** (CTR): Graph showing activity against [NaCN] concentration.
- **B** (VAL): Graph showing activity against [NaCN] concentration.
- **C** (NIG): Graph showing activity against [CN] concentration.
- **D** (VAL/NIG): Graph showing activity against [CN] concentration.
Control of respiration by Cytochrome c Oxidase in intact cells: Role of the membrane potential
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J. Biol. Chem. published online September 23, 2009

Access the most updated version of this article at doi: 10.1074/jbc.M109.050146

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