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Oxygraph assay of cytochrome *c* oxidase activity: chemical O_2 background correction

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Summary: Oxygraphic determination of cytochrome *c* oxidase activity in the presence of TMPD, ascorbate and cytochrome *c* requires consideration of chemical background oxygen consumption. Several compounds are readily oxidized by molecular oxygen when dissolved in water. This leads to a significant chemical oxygen consumption in the absence of any respiring biological sample. The rate of this autoxidation represents a chemical background which strongly depends on experimental conditions, such as temperature, chemical composition, pH and oxygen concentration. Moreover, the rate of autoxidation may be significantly catalyzed by metal traces and metal-containing proteins (e.g. cytochrome *c*). Therefore, high-resolution respirometric measurement of oxygen flux ultimately depends on chemical background correction analyzed under experimental conditions over the entire oxygen range.

1. Chemical background flux in the presence of TMPD, ascorbate and cytochrome *c*

TMPD (*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine) is frequently used in respiratory assays for cytochrome *c* oxidase (Complex IV, CIV) activity as an artificial electron donor. TMPD, however, is rapidly oxidized by

molecular oxygen with formation of radical cations of unusual stability (Wurster's Blue), particularly in alkaline solutions. In the assay for CIV activity, ascorbate is added as regenerating system for reduced TMPD. Importantly, the autoxidation of TMPD is accompanied by the production of superoxide radical, hydrogen peroxide, and can be significantly accelerated by traces of ionic copper, copper or iron-containing proteins. The chelating agent EDTA abolishes the stimulatory effect of copper but not of iron. Thus, iron completely complexed with EDTA also stimulates the reaction of TMPD autoxidation. For the study of cytochrome *c* kinetics of CIV and in respirometric tests of cytochrome *c* depletion of mitochondria (respiratory control by cytochrome *c*), a wide range of cytochrome *c* concentrations is applied during respiration measurements. Although ferricytochrome *c* itself is not capable of autoxidation, it can significantly accelerate autoxidation of ascorbate&TMPD, possibly due to the presence of iron.

As a basis for chemical background corrections, chemical background oxygen flux due to autoxidation of TMPD (0.5 mM) was analyzed in the presence of 2 mM ascorbate, at different concentrations of cytochrome *c* (0 – 87 µM) and over the entire oxygen range. Autoxidation rates were corrected for instrumental background. Autoxidation rates increased with increasing cytochrome *c* concentration (Fig. 1).

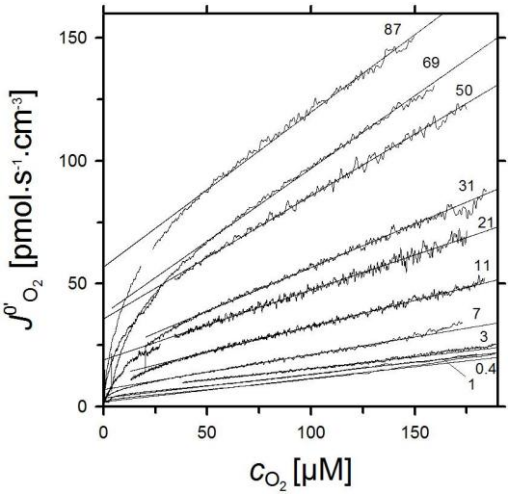

Fig. 1. Continuous records of chemical background flux with 0.5 mM TMPD, 2 mM ascorbate, and various cytochrome *c* concentrations (µM, indicated as numbers at each curve), plotted as a function of oxygen concentration. Measured at 30 °C in mitochondrial respiration medium MiR05 with 280 IU/ml catalase. The linear sections of the curves were used for background corrections at high oxygen >50 µM.

The positive intercept, *a*', indicates a non-linear oxygen dependence in the low oxygen region (with a hyperbolic component at O_2 <25 to <50 µM). Therefore, chemical background flux, must be described by a combination of a hyperbolic function at low oxygen and a linear function at high oxygen concentration (Fig. 1). Only the linear

component (linear autoxidation) is taken into consideration in the case of experimental design where oxygen concentration is kept high (Fig. 2), particularly in experiments with permeabilized cells, fibres or tissue preparation. Incorporation of the hyperbolic low-oxygen region is important in studies of oxygen kinetics.

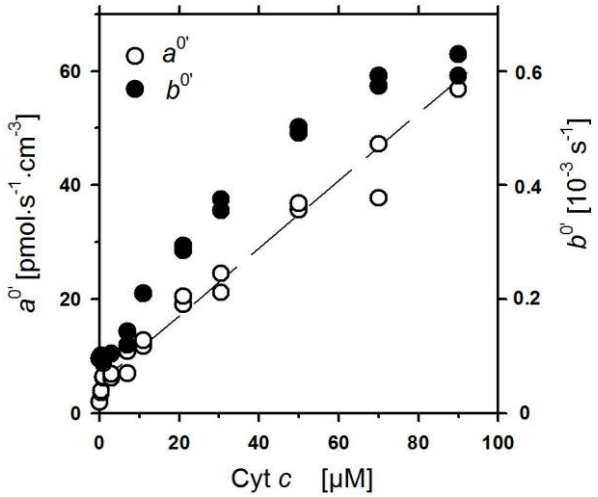

Fig. 2. Parameters of chemical background oxygen flux with ascorbate&TMPD as a function of cytochrome *c* concentration (Cyt *c* [µM)]. *a*°' intercept and *b*°' slope of linear part of oxygen dependence, from Fig. 1.

For each concentration of cytochrome *c*, the parameters of linear dependence of autoxidation rate on oxygen concentration (down to 25 µM O_2) are described by the equation,

$$J'_{O2} = a' + b' \cdot c_{O2} \quad (1)$$

Eq.(1) was used as a basis for the chemical background correction (c_{O_2}, oxygen concentration [µM]). The chemical background data are summarized in Table 1. The intercept, *a*', was a linear function of cytochrome *c* concentration, whereas the slope *b*' showed a nearly hyperbolic dependence (Fig. 2).

Table 1. Parameters of the linear dependence of ascorbate&TMPD autoxidation on oxygen concentration, c_{O_2} [µM], in the presence of different concentrations of cytochrome *c*.[a]

Cytochrome *c* concentration [µM]	*a*' [pmol·s^{-1}·ml^{-1}]	*b*' [10^{-3} s^{-1}]
0.0	1.99 ± 0.035	0.095 ± 0.001
0.4	3.74 ± 0.22	0.099 ± 0.003
1.0	6.29 ± 0.13	0.091 ± 0.004
3.0	6.49 ± 0.52	0.104 ± 0.00
7.0	8.90 ± 2.73	0.131 ± 0.017
10.9	12.22 ± 0.70	0.210 ± 0.001
20.8	19.78 ± 1.02	0.289 ± 0.006
30.5	22.83 ± 2.36	0.365 ± 0.014
49.7	36.25 ± 0.78	0.496 ± 0.008
68.6	42.49 ± 6.69	0.583 ± 0.012
87.0	59.88 ± 4.27	0.611 ± 0.027

[a] Oxygen consumption was measured at 30 °C in mitochondrial medium R05 with 0.5 mM TMPD, 2 mM ascorbate and 280 IU/ml catalase. *a*', intercept and *b*', slope of linear part of oxygen dependence included O_2 concentrations in the range from 25 to180 µM.

2. Effect of catalase on chemical background flux with ascorbate & TMPD & cytochrome *c*

To prevent accumulation of hydrogen peroxide in the course of TMPD autoxidation, and to mimic catalase activity of cardiac mitochondria or fibres, 280 IU/ml of bovine liver catalase were added to the experimental medium.

3. Test for chemical background correction

To distinguish cellular respiration from autoxidation of TMPD & ascorbate & cytochrome *c,* Complex IV (CIV) respiration is inhibited by potassium cyanide. Since autoxidation of TMPD dramatically increases with increase of pH, KCN stock solution (normally very alkaline) must be carefully neutralized (pH 7.1 – 7.2) with concentrated (1 M) potassium phosphate buffer. Note: Simultaneous application of pyruvate and other keto acids or ketons must be avoided since pyruvate masks the expected CIV inhibition by KCN due to formation of cyanohydrins and reversal of cyanide inhibition.

4. References

Gnaiger E, Lassnig B, Kuznetsov AV, Rieger G, Margreiter R (1998) Mitochondrial oxygen affinity, respiratory flux control and excess capacity of cytochrome *c* oxidase. J exp Biol 201: 1129-39. »

Lassnig B (1999) Hypoxia and regulation of mitochondrial respiration. Diplomarbeit, Univ. Innsbruck.

Munday R (1988) Generation of superoxide radical, hydrogen peroxide and hydroxyl radical during the autoxidation of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine. Chem-Biol Interactions 65: 133-43.

Renner K, Kofler R, Gnaiger E (2002) Mitochondrial function in glucocorticoid triggered T-ALL cells with transgenic Bcl-2expression. Molec Biol Rep 29: 97-101. »

Cittadini A, Galeotti T, Terranova T (1971) The effect of pyruvate on cyanide-inhibited respiration in intact ascites tumor cells. Experientia 27: 633-5.