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Early reduction of cytochrome *c* in hypoxia

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Shifts of the steady state of cytochromes *a*, a_3 and *c* at high pO_2 values are cited as evidence of the low O_2 affinity of cytochrome oxidase in vivo [(1971) Brain Res. 108, 143–154; (1985) Adv. Exp. Med. Biol. 191, 833–842; (1987) in: Int. Soc. Oxygen Transp. Tissue, Sapporo, p. 84]. Highly aerobic, small diploid yeast cells show $<0.44\%$ change of steady state in the interval prior to an abrupt reduction of cytochrome *c*. Thus, 'pre-reduction' seems falsified as a physiological response of metabolizing yeast cells that are free of hemoglobin, do not aggregate and maintain a steady state. Pre-reduction may be due to spectroscopic interference, for example hemoglobin deoxygenation, to oxygen gradients in aggregated cells and tissues or to non-steady states of substrate and metabolic controls, as contrasted to an altered cytochrome oxidase oxygen affinity in vivo.

Steady state; Cytochrome *a*; Cytochrome a_3; Cytochrome *c*; Cytochrome oxidase; Oxygen affinity; (Yeast cell)

1. INTRODUCTION

The relationship between the steady-state oxidation-reduction level and the rate of respiration in cytochrome oxidase is fixed by the solutions of the differential equations for the respiratory chain for the particular condition of steady state [4]. Since it is generally recognized that the utilization of oxygen or production of products by the respiratory chain is of zero order until very low oxygen tensions are reached [5], the changes of the steady state of cytochromes prior to significant deviations from zero order kinetics are exceptional. Jobsis [2] has indicated that the steady state of cytochrome oxidase varies continuously with inspired oxygen over the physiological range of brain tissue based upon measurements in the near infrared (NIR) region attributed to the copper *a* component. Rosenthal et al. [1] came to a similar conclusion based upon measurement of absorption in the visible region of the heme $a+a_3$ component at 605 nm. Computer algorithms are stated to correct for the overlapping absorption changes of hemoglobin at 830 and 605 nm. Wilson and Rumsey [3] have correlated the respiration rate with the early reduction of cytochrome *c* in cells and isolated mitochondria. The magnitude of the in vivo-in vitro discrepancy is approximately two orders of magnitude (0.1 to 13 μM) [3]. Since the idea of 'early warning' of impending hypoxia by cytochrome or 'oxygen sensing' by cytochrome is an attractive one [6–10], the hypothesis is here evaluated at high precision with intact respiring cells that do not contain hemoglobin, do not adhere to form aggregates [11] and maintain steady states.

Two approaches seem appropriate: (i) precise measurements of the rate of respiration down to the 'break point' between zero and first order [5,12]; and (ii) measurements of the changes of the steady-state oxidation-reduction level of cytochrome *c* of the respiratory chain. The latter is evaluated here.

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2. EXPERIMENTAL

2.1. *Methods*

In order to clarify whether the hypothesis that cytochrome oxidase is responsive to high O_2 concentrations (~50 μM), we have re-examined our observations made in the early 1950's on the constancy of the steady state of cytochrome *c* in suspensions of baker's yeast [13] during respiration from air-saturated medium until anoxia abruptly occurs, accompanied by rapid reduction of the cytochrome *c* [14].

A dual-wavelength spectrophotometer, a pair of Beckman DU quartz monochromators and a 60 Hz chopper as used in earlier experiments on baker's yeast [14], heart muscle preparation [15] and mitochondria [16] were employed for this particular study since this apparatus is of very high sensitivity and stability. The dual-wavelength output (550–540 nm) was plotted versus time on a strip chart recorder; the sensitivity of full scale is equal to 6.0×10^{-3} *A* (absorbance). The average value of the noise was $2.7 \times 10^{-5} A$. The response time was 1 s obtained by a single time constant RC circuit.

2.2. *Materials*

The choice of materials for this experiment is of vital importance. 24–36 h aerated baker's yeast (*Saccharomyces cerevisiae*) is employed: (i) this yeast is diploid and thus small (~2 μm diameter); (ii) microscopic observations show no aggregation of cells [17]; (iii) the cytochrome content is high (0.3 μmol/kg) [14]; (iv) the redox state of cytochrome *c* in this condition is oxidized as has been observed [14]; (v) the cells contain no hemoglobin [18]; (vi) the cells have a high endogenous ATP value (1.8 mM) [19], which is maintained in aerobiosis in the glucose-free state (as clearly shown in fig.15-3 of [20]); (vii) the respiration rate is high, the turnover number of cytochrome *c* being 20 s^{-1}, typical for many cells and tissues [16]; (viii) a similar hemoglobin containing yeast has been shown to have no detectable intracellular oxygen gradient [18].

3. RESULTS

A typical result is shown in fig.1 where the steady state of cytochrome *c* is observed from air saturation (240 μM) to fully reduced as checked independently by the O_2 cathode. In the steady-state interval of 6 min, the deviation of the cytochrome *c* redox state from the steady-state value up to its abrupt reduction is within the standard error from the mean (2.7×10^{-5}) in absorbance (*A*).

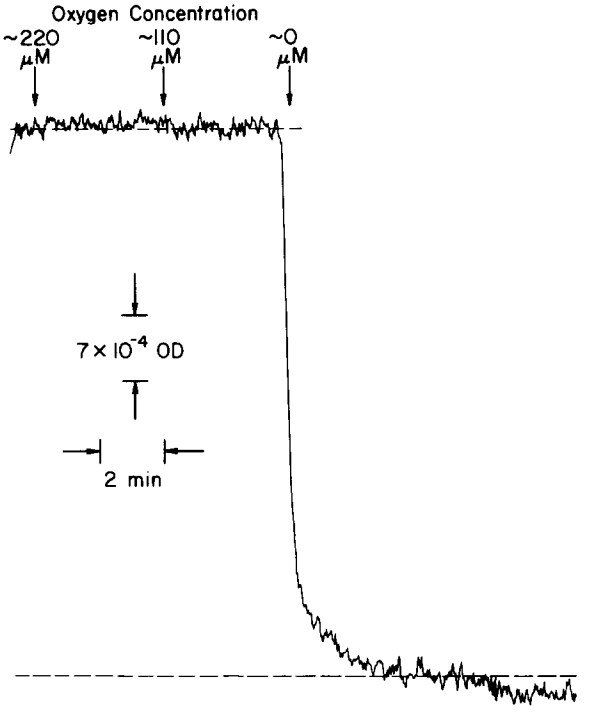

Fig.1. Illustrating the constancy of oxidation-reduction steady state of cytochrome *c* in baker's yeast prior to the abrupt reduction at low oxygen concentrations. Monitoring in a separate experiment of the oxygen concentration gave the values indicated in the top line, time proceeding from left to right. The steady-state interval of approx. 6 min is abruptly terminated by the downward deflection of the trace indicating cytochrome *c* reduction. The wavelengths employed are 550–540 nm and time calibrations are included with horizontal lines appropriate to identifying any change of the steady-state levels prior to the abrupt reduction. 2% baker's yeast, prepared as described in the text, the oxidase reduced change is 6×10^{-3} cm^{-1}.

Table 1 summarizes the results of four iterations of the experiment as in fig.1. On the top, line 1, is the number of the test and line 2 the duration of the steady state in min. Line 3 gives the standard (root mean squared) error of an 80–90 point

Table 1

Accuracy evaluation

1 Test	1	2	3	4
2 Duration (min)	9	6	8	6
3 Standard error ($A \times 10^5$)	3.0	2.7	2.5	2.4
4 Standard error (% ox-red)	0.50	0.45	0.42	0.40
5 Standard error in conc. (nM)	1.6	1.4	1.3	1.3
6 Deviation from zero slope	n.s.	n.s.	n.s.	n.s.

statistical analysis of the fluctuations of the data of each test about the mean value (2.7×10^{-5}) in absorbance. The error in percent of the oxidized-reduced change is 0.44 (line 4) and the error is equivalent to 1.4 nM in cytochrome *c* (line 5). In line 6, the best fit line over the 6–9 min time interval shows no significant trend from zero slope.

4. DISCUSSION

Three factors may afford a reconciliation with these observations and those of a 'low-affinity' cytochrome oxidase.

(i) Artificial oxygen gradients may be present in the case of respiratory measurements of cells [11,21]. In this case, factors of 10^2 in apparent shifts of K_m are readily obtainable. In order to avoid this artifact, it is extremely important to provide microscopic data that cells do not clump and are intrinsically small enough to avoid artificial O_2 gradients. Microscopic and microfluorometric observations of the yeast cells verify that they do not clump in respiratory activity [17].

(ii) Pigments other than cytochromes may be present in the mitochondrial and submitochondrial particles prepared from blood perfused organs [5]. For example, spectroscopic interference of the deoxygenation of HbO_2 would give an apparent K_m of 30 μM for cytochrome oxidase. In vivo studies of brain, etc., which rely upon algorithms based upon hemoglobin free perfusion of the hypoxic brain, are considered by us to contaminate cytochrome aa_3 with HbO_2 or some identified pigment of similar K_m for oxygen in the Hb perfused organ [22,23]. Our 'uncorrected' data for HbO_2 and cytochrome aa_3 show a sharp break at 80 to 90% HbO_2 deoxygenation, that is appropriately attributed to cytochrome aa_3 reduction which has been found to coincide with the fall in the ratio of phosphocreatine/inorganic phosphate (PCr/P_i) [24].

(iii) Maintenance of a steady state of adenine nucleotides and substrates over the period of oxygen depletion is essential to avoid alterations of the steady state of cytochromes not related to their O_2 sensitivity. Continuous decreases of ATP following re-activation of respiration in hyperemic reflow following hypoxia are well documented [25,26] and cause changes of the cytochrome steady state.

In observations of the steady state of carriers of the respiratory chain, NADH fluorescence shows a steady state until hemoglobin is >70% deoxygenated in rat brain [27]. In dog and cat brain, cytochrome oxidase copper (Cu_a) (782–830 nm) is reduced when 90% deoxygenation of hemoglobin is observed and phosphocreatine falls in concert with cytochrome aa_3.

In summary, explanations of cytochrome responses to high pO_2 (30–100 μM O_2) are provided by the following factors: (i) interference from hemoglobin deoxygenation; (ii) high O_2 gradients particularly in rapidly metabolizing tissues and aggregated cells; (iii) non-steady states of control chemicals, and mitochondrial substrates; and (iv) another pigment, not cytochrome aa_3, is present.

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