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# THE EFFECT OF OXYGEN CONCENTRATION ON THE STEADY-STATE KINETICS OF THE SOLUBILIZED CYTOCHROME *c* OXIDASE

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## Summary

1. The steady-state kinetics of ascorbate oxidation as a function of oxygen concentration was measured with a solubilized cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) preparation.

2. Linear double reciprocal plots were obtained at various fixed concentrations of ascorbate, cytochrome *c* and cytochrome $aa_3$.

3. The results are interpreted in terms of an oxidase model similar to that put forward by Minnaert in 1961 (Minnaert, K. (1961) Biochim. Biophys. Acta 50, 23—34).

4. The $K_m$ for oxygen at infinite cytochrome *c* concentration is 0.95 μM and the intramolecular rate constant for the transfer of electrons from cytochrome *c* to cytochrome $aa_3$ is 400 $s^{-1}$. According to the model, this implies that the second order rate constant for the reaction between oxygen and the oxidase is $9.5 \times 10^7\ M^{-1} \cdot s^{-1}$.

## Introduction

Cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) catalyzes the reaction:

$$4c^{2+} + O_2 + 4H^+ \rightarrow 4c^{3+} + 2H_2O \quad (1)$$

where $c^{2+}$ and $c^{3+}$ is reduced and oxidized cytochrome *c*. The cytochrome *c* steady-state kinetics in excess oxygen of this reaction have been studied extensively (see ref. 2 for review). The rate as a function of the cytochrome *c* concentration, measured by various methods, follows the rate equation (Eqn. 2)

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first derived by Minnaert [1]:

$$v = \frac{V[c^{2+}]}{K_m + [c^{3+} + c^{2+}]} = \frac{V[c^{2+}]}{K_m + C_T} \quad (2)$$

A comprehensive investigation of the steady-state kinetics of the reaction (1) should however also deal with the concentration of the second substrate, oxygen. While the cytochrome *c* kinetics are most easely studied with submitochondrial particles and solubilized oxidase, previous work on the oxygen kinetics has mostly been concerned with intact mitochondria [3—7]. In the few existing studies, where an isolated oxidase preparation was used [5,8] the effect on the oxygen kinetics of varying the cytochrome *c* concentration was not studied.

In the present paper, the steady-state kinetics of the solubilized cytochrome *c* oxidase is described, and the effect of varying the concentration of both substrates, cytochrome *c* and oxygen is investigated.

## Materials and Methods

### *Analytical methods*

The technique of Degn and Wohlrab [6] for measurement of steady-state values of oxygen concentration and respiration rate was used. The respirograph system was extended and adapted as previously described [7]. The oxygen concentrations in both liquid and gas phases were measured with Clark electrodes. Additional modifications of the technique were made as follows.

The on-line computer system, previously employed to plot $1/v_R$ against $1/[O_2]$ automatically during the experiment, is now also used to control the oxygen concentration in the gas phase by means of a feed-back loop designed to give a linear increase with time in the oxygen concentration in the liquid ($T_L$). The slope of the time gradient of oxygen concentration ($dT_L/dt$) in an experiment was 0.005 $\mu$M/s. The computer substracts $dT_L/dt$ from the rate of oxygen transfer to give the respiration rate. However, the above magnitude for $dT_L/dt$ is negligible compared to the usual respiration rate.

The increasing gradient is not started before the zero current of the electrode has stabilized. The electrode is not exposed to high oxygen concentrations during the experiment and for that reason the electrode current will immediately return to this same zero value at any moment during the gradient if nitrogen is introduced as the gas phase. The addition of reagents such as ascorbate, cytochrome *c* etc. at the concentrations used in the experiments, did not significantly change the constant ($K_T$) which determines the diffusion of oxygen into the liquid phase.

Control experiments carried out with a conventional 'closed chamber' oxygen electrode system gave similar maximal rates of respiration.

### *Materials*

Beef heart submitochondrial particles were isolated according to the procedure of Keilin and Hartree [9] as modified by Brodie and Nicholls [10], and finally suspended in 0.66 M sucrose, 1 mM histidine and 50 mM Tris sulphate

(pH 8.0). The cytochrome $aa_3$ preparation was obtained from this suspension following the initial steps of the method described by Fowler et al. [11] until the "green residue" fraction was obtained [12]. In order to avoid the decline in the molecular activity of the oxidase which occurs during purification [13,14] this fraction was used without further purification. The cytochrome $aa_3$ concentration was determined using a $\Delta A_{605-630}$ (reduced minus oxidized) of 27 $mM^{-1} \cdot cm^{-1}$. Cytochrome *c* was Sigma type VI. Potassium ascorbate was obtained by neutralisation of ascorbic acid with KOH solution.

## Results

The oxidation of ascorbate in the presence of cytochrome *c*, soluble cytochrome $aa_3$, and oxygen was studied as a function of the concentrations of all four components. Fig. 1 shows the Lineweaver-Burk plots of $1/v_R$ against $1/[O_2]$ obtained at various fixed concentrations of soluble cytochrome $aa_3$ and with high concentrations of cytochrome *c* (68 $\mu$M) and of ascorbate (23 mM). The plots are linear, and the lines intersect at a point above the $1/[O_2]$ axis in the second quadrant. This behaviour might be the result of a non-enzymatic step contributing to the overall reaction rate, apparently the reduction of ferricytochrome *c* by ascorbate. This has a second-order rate constant of 5 $M^{-1} \cdot s^{-1}$

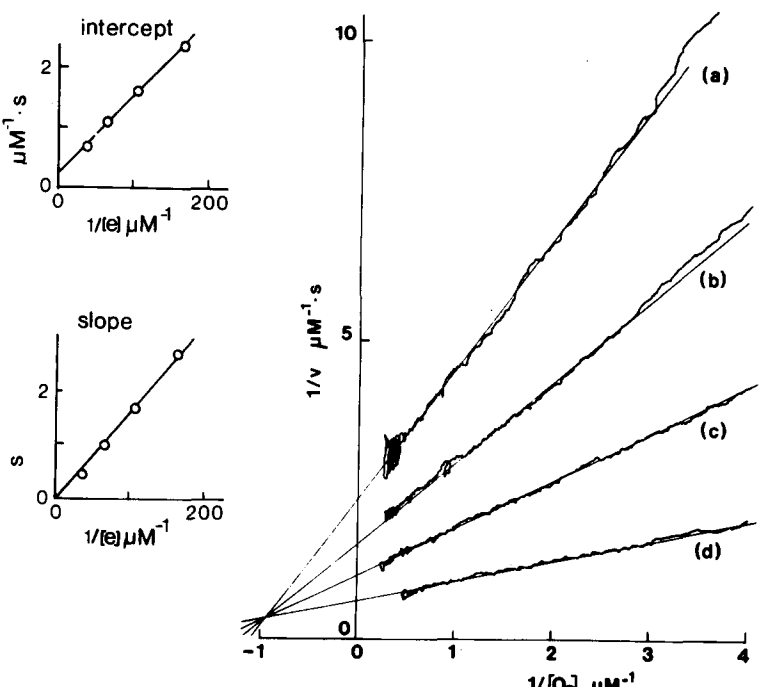



Fig. 1. Reciprocal plots of $[O_2]$ against rate of oxidation by solubilized cytochrome $aa_3$. The effect of varying enzyme concentration. (a) 6.1 nM, (b) 9.2 nM, (c) 15.3 nM, (d) 27 nM cytochrome $aa_3$. The medium contained 70 mM potassium phosphate, 2 mM EDTA, 0.5% Tween-80, pH 7.4; 23 mM ascorbate, and 68 $\mu$M cytochrome *c*.

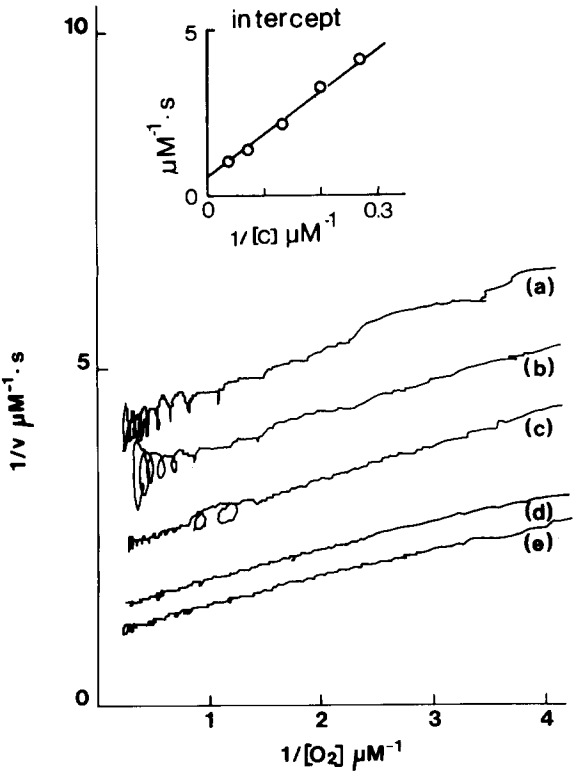



Fig. 2. Reciprocal plots of $[O_2]$ against rate of oxidation by solubilized cytochrome $aa_3$. The effect of varying cytochrome *c* concentration. (a) 3.8 μM, (b) 5 μM, (c) 7.5 μM, (d) 15 μM, (e) 30 μM cytochrome *c*. The medium contained 70 mM potassium phosphate, 2 mM EDTA, 0.5% Tween-80, pH 7.4, 23 mM ascorbate, and 19 nM cytochrome $aa_3$.

(assuming that $v = 2\ k_3$ [ascorbate] · [cytochrome *c*]). The maximum $K_m$ for oxygen is given by the $1/[O_2]$ coordinate of the point of intersection and has a value of 1.1 μM.

In Fig. 2 the corresponding plots obtained at various fixed cytochrome *c* concentrations are shown. These plots are linear and parallel. When the maximal velocities (extrapolated to an infinite concentration of oxygen) at the various concentrations of cytochrome *c* are plotted against the latter concentrations (inset of Fig. 2), an apparent $K_m$ for cytochrome *c* of 20 μM is found, closely similar to the values obtained by other methods (see ref. 2). The $K_m$ for oxygen at infinite cytochrome *c* concentrations is 0.95 μM.

Fig. 3 illustrates the effect of varying the ascorbate concentration. Again linear and parallel plots are obtained. From the results of Figs. 1, 2 and 3 we derive the empirical rate equation:

$$\frac{1}{v} = \frac{1}{2k_a A C_T} + \frac{K_m + C_T}{C_T}\frac{1}{k_b e} + \frac{1}{4k_c e}\frac{1}{[O_2]} \qquad (3)$$

where $v$ = rate of respiration, $A$ = [ascorbate], $C_T$ = [total cyt. *c*], $K_m$ = apparnet $K_m$ for cytochrome *c* (above), $e$ = [total $aa_3$] and in which $k_a$, $k_b$ and $k_c$ are constants.

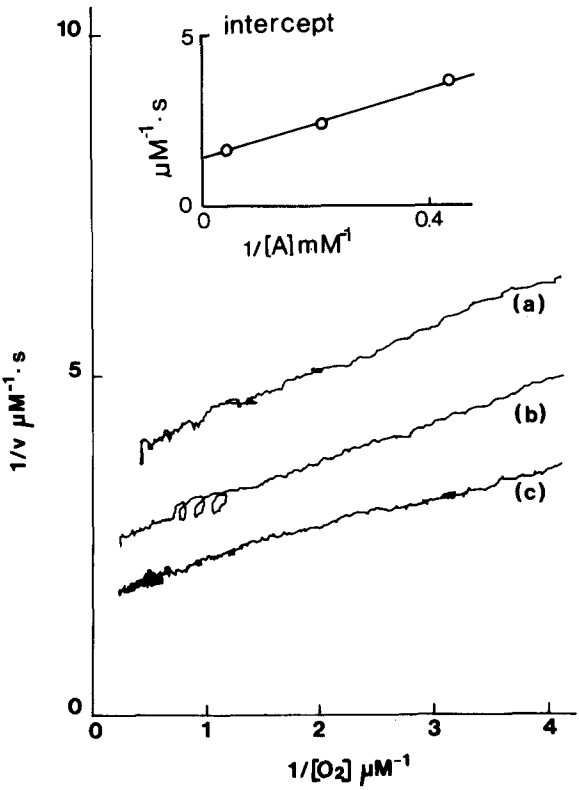
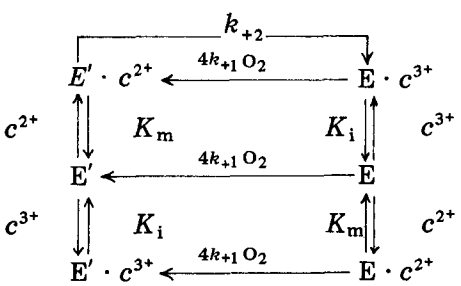



Fig. 3. Reciprocal plots of $[O_2]$ against rate of oxidation by solubilized cytochrome $aa_3$. The effect of varying ascorbate concentration. (a) 2.3 mM, (b) 4.6 mM, (c) 23 mM ascorbate. The medium contained 70 mM potassium phosphate, 2 mM EDTA, 0.5% Tween 80, pH 7.4, 23 μM cytochrome *c*, 19 nM cytochrome $aa_3$.

## Discussion

The simples model capable of accounting for the kinetics of the uncoupled ascorbate-cytochrome *c*-cytochrome $aa_3$-oxygen system [15] is Minnaert's "mechanism IV" [1], a slightly extended version of which is given in Eqn. 4a:




(4a)

$$c^{3+} + AH_2 \xrightarrow{k_{+3}} c^{2+} + AH\cdot \qquad (4b)$$

$$c^{3+} + AH\cdot \xrightarrow{\text{fast}} c^{2+} + A \qquad (4c)$$

where E is reduced and E′ oxidized cytochrome $aa_3$ and where $K_m = K_i$, [2]. From Eqn. 4a we obtain the rate equation given in Eqn. 5:

$$\frac{1}{v} = \frac{K_m + C_T}{e \cdot k_{+2} \cdot C_S \cdot C_T} + \frac{1}{e \cdot 4 \cdot k_{+1}} \frac{1}{[O_2]} \tag{5}$$

where $C_T$ is the total concentration of cytochrome *c* and $C_S$ is the steady-state value of cytochrome *c* reduction ([cyt. $c^{2+}$]/[cyt. *c*] total). If ascorbate ($AH_2$) reduces cytochrome *c* according to Eqn. 4b and 4c the following rate Eqn. 6 is derived from Eqn. 5:

$$\frac{1}{v} = \frac{1}{2k_{+3}AC_T} + \frac{C_T + K_m}{C_T} \frac{1}{k_{+2}e} + \frac{1}{4k_{+1}e} \frac{1}{[O_2]} - \frac{1}{2k_{+3}AC_T} \frac{v}{4k_{+1}e[O_2]} \tag{6}$$

where $A$ = ascorbate concentration [$AH_2$]. Eqn. 6 is equivalent to the empirical rate equation (Eqn. 3) except for the negative term, which will give rise to non-linear concave upward double reciprocal plots. However, this negative term can usually be neglected. At high concentrations of ascorbate and cytochrome *c* the term $1/(2k_{+3}\,AC_T)$ is relatively small, as demonstrated in Fig. 1, where the cytochrome $aa_3$ concentration ($e$) is varied, but the negative term is even smaller since $v/(k_{+1}\,e[O_2])$ equal to the ratio reduced [$aa_3$]/total [$aa_3$] is always less than one. The constants $k_a$, $k_b$ and $k_c$ in the empirical rate equation (Eqn. 3) then correspond to the constant $k_{+3}$, $k_{+2}$, and $k_{+1}$ in Eqns. 4—6. It is thus possible to obtain values for the constants $k_{+1}$, the on-constant for oxygen, and $k_{+2}$, the rate of electron transfer within the cytochrome *c* · cytochrome oxidase complex, as summarized in Table I.

The value of the 'on' constant for oxygen $k_{+1}$, equals $9.5 \cdot 10^7\ M^{-1} \cdot s^{-1}$ under our conditions, very close to the value of $8.5 \cdot 10^7\ M^{-1} \cdot s^{-1}$ determined by the flow-flash method at low oxygen concentrations. The same value was found with enzyme either fully reduced [16] or partially reduced [17] before the flash.

A value of $3.3 \cdot 10^7\ M^{-1} \cdot s^{-1}$ obtained for intact pigeon heart mitochondria using the flash technique [18] is somewhat lower. The values reported by Schindler [5] measured by the bacterial luminescence method, vary from $4.0 \cdot 10^7\ M^{-1} \cdot s^{-1}$ for pigeon heart mitochondria respiring on succinate to $9.0 \cdot 10^7\ M^{-1} \cdot s^{-1}$ for submitochondrial particles respiring on NADH, when recalculated

TABLE I

KINETIC CONSTANTS OBTAINED FOR THE CYTOCHROME *c* OXIDASE REACTION WITH SOLUBILIZED CYTOCHROME $aa_3$

70 mM potassium phosphate 2 mM EDTA, 0.5% Tween-80 at 30° C, pH 7.4

| | |
|---|---|
| $K_m^{O_2}$ * = 0.95 μM | $k_{+1}$ *** = $9.5 \cdot 10^7\ M^{-1} \cdot s^{-1}$ |
| $K_m^{cyt\ c}$ ** = 20 μM | $k_{+2}$ *** = 400 $s^{-1}$ |

* At infinite cytochrome *c* concentration.
** At infinite oxygen concentration.
*** Eqn. 4a.

using the value of 27 for EmM (reduced-oxidized, 605—630 nm) of cytochrome $aa_3$. All these methods, including our own, will underestimate the velocity constant when part of the oxidase is inactivated.

The model proposed in Eqn. 4 seems to explain the empirical rate Eqn. 3 in a relatively simple manner, while models which demand that cytochrome *c* bind to the oxidase before the reaction with oxygen takes place (ordered mechanisms) will require special values of the rate constant in order to give parallel plots of $1/v$ against $1/[O_2]$ at varying fixed concentrations of cytochrome *c*. The results also show that it is not necessary to postulate any back reaction within the oxidase that is sensitive to changes in oxygen concentration nor an apparent reaction order of oxygen with the oxidase different from one.

Both the cytochrome *c* kinetics and the oxygen kinetics seem to indicate a rather simple reaction mechanism for the enzyme not involving cooperativity; while evidence for various types of interactions within the oxidase molecule is accumulating from the measurements of the physico chemical properties of the prostetic groups [19,20]. This might suggest that the interactions are mainly involved to couple the redox reaction to the phosphorylation reaction.

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