Molecular Oxygen Modulates Cytochrome c Oxidase Function*

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This study sought to determine whether molecular oxygen interacts with cytochrome c oxidase to modify its catalytic activity. Such an interaction could explain the observation that mitochondria incubated under low O₂ concentrations exhibit a reversible suppression of State 3 respiration. Oxidized bovine heart cytochrome c oxidase was incubated in oxygen concentrations of <50 μM for 4 h. The enzyme exhibited a reversible decrease in V₉₅ after incubation, compared with control enzyme incubated at higher oxygen concentrations. This change was accompanied by a small increase in the apparent Kₘ of the enzyme for both cytochrome c and oxygen, although the optical absorption spectra of oxidized, cycling, or reduced enzyme were not affected. Spectroscopy studies after 4 h of incubation revealed that heme a₃ was 33% reduced during cycling at [O₂] = 25 μM whereas enzyme at [O₂] = 135 μM was only 18% reduced, suggesting that the site of inhibition occurred at the electron transfer step between heme a₃ and O₂. These results provide a mechanistic explanation for the observation that intact cells or mitochondria exhibit a reversible inactivation of heme a₃ and O₂. These results provide a mechanistic explanation for the observation that intact cells or mitochondria exhibit a reversible inhibition of respiration during prolonged exposure to [O₂] < 25 μM, by demonstrating that the catalytic activity of cytochrome c oxidase function is similarly inhibited, possibly through an allosteric effect of molecular O₂ on the enzyme.

Cytochrome c oxidase is the terminal oxidase of cellular respiration and catalyzes the transfer of electrons from ferro-cytochrome c to molecular oxygen (1, 2). The free energy release associated with this electron transfer is coupled to the translocation of protons from the mitochondrial matrix to the cytosol, generating a proton electrochemical gradient across the inner mitochondrial membrane. This potential is subsequently used to sustain a number of mitochondrial functions including the synthesis of ATP. The enzyme contains four redox-active metal centers, two hemes (a and a₃), and two redox-active copper ions, Cuₐ and Cuₐ₃ (3). Cuₐ is thought to reside in subunit II of the enzyme and to consist of a binuclear center that accepts electrons from reduced cytochrome c. Electrons are transferred to heme a₃ and then to the binuclear oxygen binding site, heme a₃ and Cuₐ₃, in subunit I. Electrons are then transferred to O₂, although the details of this process are incompletely understood.

The kinetic behavior of the oxidase has been studied extensively (5), but we are unaware of any previous reports describing a regulatory effect of either substrate, cytochrome c or oxygen, on its catalytic activity. In a previous study of intact mitochondria, we found that the rate of O₂ consumption with TMPD and ascorbate as substrates was reversibly inhibited at [O₂] = 25 μM compared with 135 μM after incubation under anoxia for 2 h (6). Those results suggested that prolonged exposure of cytochrome c oxidase to low oxygen concentrations [O₂] may cause its activity to become O₂-dependent at [O₂] well above the reported Kₘ of 1 μM (7). The present study was therefore undertaken to determine whether isolated cytochrome c oxidase exhibits (a) a decrease in V₉₅, (b) an increase in the Kₘ for cytochrome c or oxygen, (c) a conformation change of the enzyme as monitored by optical spectroscopy, or (d) a change in the redox state of the enzyme during steady-state turnover (cycling), after incubation under low oxygen conditions.

EXPERIMENTAL PROCEDURES

Chemicals—All biochemicals were obtained from Sigma. The Pdmeso-tetra-(4-carboxyphenyl) porphine dye used for phosphorescence-quenching measurements of oxygen concentration was obtained from Medical Systems, Inc., Greenvay, NY. The isolated cytochrome c oxidase was a gift from Dr. Martin Horvath.

Preparations—Bovine heart cytochrome c oxidase concentrations were based on a value of ε = 1.41 × 10⁸ M⁻¹cm⁻¹ at 421 nm for the oxidized enzyme. The enzyme was prepared by the method of Hartzell and Beinert (8) and was stored at a concentration of 0.6 mM at −80 °C until immediately before use. Cytochrome c was reduced as described previously (9). Briefly, bovine cytochrome c (ε = 2.12 × 10⁸ M⁻¹cm⁻¹ at 550 minus 540 nm) (10) was dissolved in a mixture containing equal amounts of 20 mM boric acid and 0.2 M sodium borohydride (pH = 10.2) and incubated for 1 h. Cytochrome c reduction was stopped using sodium phosphate (pH = 6.5).

Cytochrome c Oxidase Incubation—Oxidized bovine cytochrome c oxidase (1–10 nm) (11) was incubated in spinner flasks in 100 mM KH₂PO₄ containing 0.5% dodecyl β-maltoside at pH = 7.4, 25 °C (7). The headspace of the flask was continuously flushed with gas containing oxygen ranging from 2.8 to 14.2%, balance nitrogen. The solution was continuously mixed with a magnetic stirrer (60 rpm), and the dissolved oxygen tension was monitored using a polarographic electrode placed in a side port of the flask (12). The flasks were also equipped with glass tubing extending into the media to allow the anaerobic removal of aliquots for analysis.

Measurements of Oxygen Consumption—The apparent Kₘ of cytochrome c oxidase with respect to cytochrome c was assessed in stirred solutions by measuring the decrease in oxygen partial pressure in a water-jacketed respirometer (1.5 ml) equipped with a calibrated polarographic O₂ electrode. Bovine cytochrome c (5 μM), TMPD,¹ (500 μM), and ascorbate (5 mM) were used as electron donors, and the decrease in oxygen tension over 5–10 min was recorded using a 12-bit analog-to-digital converter. However, this method was not suitable for assessing the apparent Kₘ of cytochrome c oxidase with respect to oxygen because the response speed of polarographic electrodes declines at [O₂] of less than 10 μM. Accordingly, for those studies the oxygen concentrations in solution were measured using the oxygen-dependent phosphorescence quenching of a porphyrine dye (13). Accuracy of the system was confirmed by comparison of test solutions assessed simultaneously by polarographic

¹ The abbreviation used is: TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine.
graphic and optical systems under quasi-steady-state conditions. The time constant for phosphorescence decay under anaerobic conditions was confirmed in solutions using the glucose oxidation reaction to scavenge O$_2$. In both O$_2$ measurement systems, the turnover number (s$^{-1}$) for cytochrome c oxidase was calculated by dividing O$_2$ consumption (nmol O$_2$/s) by the enzyme concentration (nm). In studies requiring the use of TMPD and ascorbate, the contribution of autooxidation of ascorbate (14) was small but was nevertheless measured and subtracted from the overall rate of oxygen consumption.

Spectrophotometric Determination of the $K_m$ of Cytochrome Oxidase for Cytochrome c—The apparent $K_m$ and $V_{max}$ of the enzyme with respect to cytochrome c were determined spectrophotometrically by measuring the oxidation of reduced cytochrome c over time from the absorbance at 550 minus 540 nm. First-order rate constants were calculated from the slopes of the linear plots using polarographic measurements of O$_2$ consumption in these studies. The apparent rate constants and the concentrations of ferrocytochrome c ferricyanide, which completes the oxidation of cytochrome c oxidase incubated at O$_2$ tensions-dependent decrease in turnover number compared with the turnover number was measured during acute (5 min) re-equilibration to O$_2$ tensions ranging from 5 to 135 $\mu$M. Turnover number was then assessed at that oxygen concentration, in the presence of cytochrome c, TMPD, and ascorbate.

**RESULTS**

To examine the oxygen dependence of cytochrome c oxidase turnover number, oxidized bovine cytochrome c oxidase (1–2 $\mu$M) was incubated in KH$_2$PO$_4$ (100 mM), dodecyl $\beta$-D-maltoside (0.1%), pH = 7.4, 25 $^\circ$C for 4 h at oxygen concentrations (O$_2$) ranging from 135 to 25 $\mu$M. Subsequently, and at the same oxygen concentration, cytochrome c (5 $\mu$M), TMPD (400 $\mu$M), and ascorbate (5 $\mu$M) were added as substrates, allowing the reaction to proceed to anaerobicity. The cytochrome c oxidase was incubated at [O$_2$] = 25 $\mu$M for 4 h, followed by acute (<5 min) re-equilibration to oxygen concentrations ranging from 5 to 135 $\mu$M. Turnover number was then assessed at that oxygen concentration, in the presence of cytochrome c oxidase, with TMPD and ascorbate added to reduce ferricytochrome c oxidase.

The $V_{max}$ of enzyme incubated at [O$_2$] = 135 $\mu$M was 103 s$^{-1}$ (Fig. 1A) and 52 s$^{-1}$ after incubation at [O$_2$] = 25 $\mu$M (Fig. 2B). From the slopes of the regression lines, the apparent $K_m$ for cytochrome c oxidase was increased after 4 h of incubation at [O$_2$] = 25 $\mu$M (0.53 $\mu$M) compared with [O$_2$] = 135 $\mu$M (0.11 $\mu$M). The apparent $K_m$, with respect to oxygen was obtained by measuring the enzymatic turnover number as a function of [O$_2$] between 15 and 0 $\mu$M in the presence of excess ferrocyanide (5 $\mu$M) after a 4-h exposure to either [O$_2$] = 135 $\mu$M or [O$_2$] = 25 $\mu$M. Oxygen consumption rates were determined using the phosphorescence quenching method in these studies. The $V_{max}$ of enzyme incubated at [O$_2$] = 135 $\mu$M was 102 s$^{-1}$ (Fig. 2C), while enzyme incubated at [O$_2$] = 25 $\mu$M exhibited a $V_{max}$ of 43 s$^{-1}$ (Fig. 2D). The apparent $K_m$ of the enzyme for oxygen was 0.51 $\mu$M after 4 h of incubation at [O$_2$] = 25 $\mu$M, compared with 0.31 $\mu$M after incubation at 135 $\mu$M.

In its native state, cytochrome c oxidase exhibits first-order kinetics even at high cytochrome c concentrations, and the rate constant decreases with increasing cytochrome c concentration (17). To determine whether this behavior holds true for enzyme incubated under low [O$_2$] conditions, enzymatic turnover (1-10
nm) was measured spectrophotometrically from the change in ferrocytochrome c concentration over time. In these studies, TMPD and ascorbate were not added, so the concentration of ferrocytochrome c decreased with time. As shown in Fig. 3, the oxidase exhibited first-order kinetics, and the first-order rate constant decreased as the initial cytochrome c concentration was increased from 0.8 to 20 μM after 4 h incubation, regardless of whether the [O2] was 135 μM (Fig. 3A) or 25 μM (Fig. 3B). Enzyme incubated at [O2] = 135 μM yielded a Vmax and apparent Km for cytochrome c of 57 s⁻¹ and 1.53 μM, respectively (Fig. 3C), under the conditions of this assay. The Vmax and the apparent Km for cytochrome c of enzyme incubated at [O2] = 25 μM were 38.5 s⁻¹ and 2.8 μM, respectively (Fig. 3D). As expected, these values differed from the corresponding measurements made in the presence of TMPD plus ascorbate (see Fig. 2) due to the ability of TMPD to reduce ferricytochrome c that is still bound to cytochrome oxidase (1). Collectively, these results demonstrate that prolonged exposure to [O2] = 25 μM (Fig. 4D) was 16% reduced during cycling while enzyme incubated at [O2] = 25 μM (Fig. 4B) was 11% reduced. Thus, the enzyme was more reduced during cycling after prolonged exposure to [O2] = 25 μM. To determine whether the redox state of hem a remained unaffected during prolonged exposure to [O2] = 25 μM, absorbance was measured at 605 nm was measured. As shown in Fig. 4, enzyme incubated at [O2] = 135 μM (Fig. 4C) was 20% reduced while enzyme incubated at [O2] = 25 μM (Fig. 4D) was 33% reduced during cycling. These results indicate that hem a remains in a more reduced state during cycling after prolonged exposure to [O2] = 25 μM, compared with oxidase incubated at [O2] = 135 μM.

Experiments were carried out to examine whether the above kinetic changes were accompanied by changes in the absorption spectra in the oxidized, cycling, and reduced states of the enzyme. Spectra were recorded for oxidized cytochrome c oxidase incubated for 4 h at [O2] = 135 μM or [O2] = 25 μM. Cytochrome c, TMPD, and ascorbate then were added to initiate cycling of the enzyme, and spectra were again recorded (18). After the solution reached anaerobicity, the enzyme became fully reduced and spectra were again recorded. Fig. 5 displays the absorption spectra between 350 and 500 nm, the region where hem a and a₃ display a strong π-π* transition. The reduced, cycling, and oxidized forms of the enzyme incubated at [O2] = 135 μM or [O2] = 25 μM display single absorption bands at ~421, 427, and 444 nm, respectively. No apparent changes in the absorption spectrum were detected between the native and conformer states of the enzyme. Thus, hem a and a₃ do not undergo significant changes in their conformation as
assessed by optical spectroscopy.

Collectively, the above studies indicated that the kinetics of the enzyme were modulated by incubation at $[O_2] = 50 \mu M$. To determine whether this shift occurs more rapidly when incubated at lower $[O_2]$, isolated enzyme (1–10 nM) was incubated at different $O_2$ concentrations ranging from 0 to 135 $\mu M$. Turnover number was measured polarographically in aliquots sampled hourly, using identical methodology described for Fig. 1. Turnover number in enzyme incubated at $[O_2] = 50 \mu M$ was determined after acute re-equilibration to $[O_2] = 25 \mu M$. As shown in Fig. 6, the decrease in turnover number occurred more rapidly during incubation at $[O_2] = 50 \mu M$, compared with 25 or 50 $\mu M$ concentrations. However, similar turnover numbers were reached after 4 h incubation in 0, 25, or 50 $\mu M$ $O_2$ concentrations.

**DISCUSSION**

Collectively, our data suggest that molecular oxygen directly modulates the $V_{max}$ and the apparent $K_{m}$ of cytochrome c oxidase, leading to a reversible inhibition of the enzyme after it is exposed to low oxygen concentrations ($<50 \mu M$) for several hours. To interpret our data we employed Minnaert’s mechanism IV, which applies first-order kinetics to explain cytochrome c oxidase kinetics (19). Typically, the first-order rate constants of a simple bimolecular collisional mechanism remain constant with increasing substrate concentrations until $V_{max}$ is reached at finite substrate concentration (20). However, although cytochrome c oxidase kinetics show that the enzyme remains strictly first-order even at high cytochrome c concentrations, the rate constant decreases with increasing cytochrome c concentration (17). Minnaert explained this phenomenon by proposing that ferro- and ferricytochromes have equal binding capacity, with ferrocytochrome forming a productive complex while ferricytochrome can act as a competitive inhibitor. Subsequently, Yonetani and Ray (21) demonstrated that under conditions where first-order kinetics are observed, the $K_{m}$ for ferrocytochrome $c$ was the same as the dissociation constant ($K_i$) for ferricytochrome $c$ (21).

Minnaert mechanism IV:

$$a^3^+ + [S] \rightarrow a^3^+[S] \rightarrow a^3^+ + [S] \rightarrow a^3^+ + [P] \rightarrow a^3^+ + [P] \rightarrow a^3^+$$

where $[S] = \text{ferrocytochrome c}$, $[P] = \text{ferricytochrome c}$; $K_m = K_i$, and $k_1, k_2, k_3 = \text{electron transfer rates}$. To apply this model to the interpretation of kinetic data obtained after incubation at low oxygen concentrations, it is necessary to demonstrate that these conditions hold true during both the native and the conformed states of the enzyme. Fig. 3 shows that cytochrome c oxidase exhibits first-order kinetics after incubation at $[O_2] = 135 \mu M$ (Fig. 3A) or at $[O_2] = 25 \mu M$ (Fig. 3B), thus confirming this assumption. Furthermore, the first-order rate constant was observed to decrease with increasing cytochrome c concen-
tion under both incubation conditions. Thus, the assumption for mechanism IV regarding the cytochrome c dependence of enzyme kinetics described by Minnaert remain valid for the enzyme in its conformed and native states.

How might the oxidase $V_{\text{max}}$ decrease and the apparent $K_m$ for cytochrome c and oxygen increase during prolonged exposure to [O2] < 50 μM? Based on Minnaert's mechanism IV, the observed changes could have resulted either from changes in $k_1$, $k_2$, or $k_3$. The rate constant $k_3$ represents the electron transfer step from $a_3$ to O2. To determine which rate constants are affected, we examined the overall redox state ($A_{444\text{nm}}$) during cycling of the enzyme. If $k_3$ were inhibited after prolonged exposure to [O2] = 25 μM, then the enzyme would cycle in a more oxidized state. By contrast, if $k_2$ or $k_3$ were inhibited, then the enzyme would should have remained in a more reduced state during cycling. Our current data show that the enzyme incubated at [O2] = 25 μM was more reduced during cycling compared with the enzyme incubated at [O2] = 135 μM, suggesting that $k_2$ and/or $k_3$ were inhibited. To determine which rate constant was affected, the redox state of heme $a_3$ was obtained from measurements of absorbance at 605 nm after incubation for 4 h under [O2] = 135 μM (C) or [O2] = 25 μM (D).

These observations are consistent with the hypothesis that molecular oxygen interacts with cytochrome c oxidase to modify its kinetic behavior. The need for prolonged (>1 h) incubation at low oxygen concentrations to induce conformance, and the rapid reversibility of the inhibition when the enzyme is exposed even briefly to oxygen concentrations greater than 50 μM, suggests that a second binding site for oxygen exists on the enzyme, which is distinct from the catalytic binuclear site. When oxygen is bound to this regulatory site the enzyme would assume its normal catalytic activity. However, when incubated at lower oxygen concentrations, O2 would gradually dissociate from this putative site, causing the enzyme to revert to its inhibited (conformed) state. Exposure to higher oxygen concentrations would result in the rapid reassociation of O2, returning the enzyme to its native state. While supportive of this hypothesis, the data of our study do not directly demonstrate the existence of a regulatory site capable of interacting with oxygen. However, the enzyme did appear to revert to its conformed state more quickly when incubated at lower O2 concentrations, consistent with the notion that oxygen must dissociate from the enzyme to revert to the conformed state.

Cytochrome c oxidase has been the focus of extensive analysis over the past few decades, and a large number of different factors are known to influence its kinetic behavior (2, 3, 5). However, a regulatory effect of oxygen on the catalytic function of the enzyme has not been identified previously. The reason for this may relate to the necessity for prolonged exposure to low oxygen concentrations, and the rapid reversibility of the
adjusted their respiratory rates in accordance with the ambient oxygen concentration. A regulatory effect of molecular oxygen on cytochrome oxidase would provide a mechanism allowing cells to respond to changing oxygen concentrations and could explain how specialized cells within intact tissues are able to respond to changing oxygen concentrations over the physiological range (25). Further elucidation of the significance of this response will require additional studies.

**Fig. 5.** Absorption spectra of cytochrome c oxidase (1−2 μM) at various stages of reduction by cytochrome c/TMPD/ascorbate. Conditions were identical to those in Fig. 4. Representative samples were taken from the three phases of the enzyme. Cytochrome c oxidase was incubated at [O2] = 135 μM (A) or [O2] = 25 μM (B) for 4 h.

Conformed state upon exposure to higher O2 concentrations. Classical studies have shown that cellular respiration remains independent of oxygen concentration above a critically low [O2] of 3−5 μM (13, 22, 23). However, because of technical considerations those studies were carried out using hypoxia of short duration, which would not be expected to reveal effects that require more prolonged exposure to elicit. Indeed, the present study represents an extension of our previous studies of prolonged hypoxia in isolated hepatocytes (12) and cultured cardiomyocytes (24). Those studies demonstrated that prolonged exposure to lowered oxygen concentration was associated with a reversible inhibition of cellular respiration, an effect that was similarly evident during TMPD respiration in isolated mitochondria incubated at low oxygen concentrations (6). The previous observation that intact cells of diverse phenotype demonstrate an O2-dependent regulation of respiration, combined with the present observation that an inhibition of cytochrome c oxidase activity occurs during prolonged exposure to lowered oxygen concentrations, suggests that this response may represent a component of an adaptive response to lowered oxygen availability. A regulatory effect of molecular oxygen on cytochrome c oxidase would provide a mechanism allowing cells to adjust their respiratory rates in accordance with the ambient oxygen concentrations and could explain how specialized cells within intact tissues are able to respond to changing oxygen concentrations over the physiological range (25). Further elucidation of the significance of this response will require additional studies.

**Fig. 6.** Turnover number was measured polarographically in aliquots of cytochrome c oxidase incubated at different [O2] for up to 4 h. Enzyme incubated at [O2] = 0 μM was assayed at [O2] = 25 μM. Conditions were otherwise identical to those in Fig. 1. Incubation at lower O2 concentration was associated with a more rapid decrease in turnover number. Enzyme incubated at 135 μM showed no decrease in turnover number.

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