Oxygenation of Carbon Monoxide by Bovine Heart Cytochrome *c* Oxidase†

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ABSTRACT: Cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1), as the terminal enzyme of the mammalian mitochondrial electron transport chain, has long been known to catalyze the reduction of dioxygen to water. We have found that when reductively activated in the presence of dioxygen, the enzyme will also catalyze the oxidation of carbon monoxide to its dioxide. Two moles of carbon dioxide is produced per mole of dioxygen, and similar rates of production are observed for 1- and 2-electron-reduced enzyme. If ^{13}CO and O_2 are used to initiate the reaction, then only $^{13}CO_2$ is detected as a product. With $^{18}O_2$ and ^{12}CO, only unlabeled and singly labeled carbon dioxide are found. No direct evidence was obtained for a water–gas reaction ($CO + H_2O \rightarrow CO_2 + H_2$) of the oxidase with CO. The CO oxygenase activity is inhibited by cyanide, azide, and formate and is not due to the presence of bacteria. Studies with scavengers of partially reduced dioxygen show that catalase decreases the rate of CO oxygenation.

Fenn & Cobb (1932), in their studies on isolated frog sartorius muscle, were the first to detect an oxygen-dependent oxidation of carbon monoxide in mammalian tissue. Later. in vivo studies (Clark, 1950) showed that mice and turtles could oxidize carbon monoxide. Using radioisotopes, Breckenridge (1953) then demonstrated a copurification of oxygenase and oxidase activities from an homogenate of porcine heart. Finally, the observations of Luomanmaki (Luomanmaki & Coburn, 1969) extended the phenomenon to humans.

We here confirm and expand on the work of these investigators by showing that, at least in bovine heart, it is cytochrome *c* oxidase that demonstrates this catalytic capability. The use of sterile enzyme samples and the brisk response of the system to addition of oxygen rule out bacterial contamination as a source of the activity. Inhibition of the turnover of CO by cyanide, azide, and formate shows that indeed it is the oxidase that is catalyzing the reaction. The results of studies with stable isotopes of carbon and oxygen, as monitored by both infrared spectroscopy and GC[1]–mass spectroscopy, firmly establish the oxidation of carbon monoxide as being the consequence of a CO oxygenase activity. The specific effects of dioxygen on the optical spectra of CO-complexed enzyme have also been examined. Although the postoxygenation spectral form seems to vary according to the extent of reduction, it is always characterized by a loss of intensity at 590 nm.

It has been known for some time that the noble metals will catalyze the oxidation of CO with 2 mol of CO_2 produced per mole of O_2 (Langmuir, 1922). This is exactly the same stoichiometry that is found here for the enzyme-catalyzed reaction. In a recent review (Engl & Ertl, 1979) convincing evidence was shown in support of a Langmuir–Hinshelwood model, according to which a molecule of O_2 is dissociatively chemisorbed to the catalyst surface and reacts with two molecules of chemisorbed CO. However, only two metal sites are available in the oxidase as opposed to the minimum of three required for the surface reaction. If analogy is to exist between the chemical and biological systems, it would appear that one of the metal centers in the active site of the enzyme can produce 2 mol of activated oxygen.

Materials and Methods

Bovine heart cytochrome *c* oxidase was isolated according to a standard method (Yoshikawa et al., 1977) and stored as a concentrated solution (0.5–1.0 mM in heme *a*) at −70 °C. Enzyme concentrations were calculated by using an extinction coefficient of 19.9 mM^{-1} cm^{-1} at 605 nm for the dithionite-reduced form.

Oxidase samples were always passed through two 0.22-μm Millipore filters prior to infrared gas spectroscopy. Enzyme cultured immediately after passage through the filters or following a 48-h incubation in a gas cell showed no growth for aerobes, anaerobes, faculatative anaerobes, *Mycoplasma*, and fungi.

Double-difference gaseous infrared spectroscopy was used to monitor both the rate of production and total production of carbon dioxide by the oxidase. Details of the experimental protocol used to prepare samples for the infrared investigation have been outlined elsewhere (Young, 1981). Typically, the sample and reference cells are flushed in tandem with USP nitrogen (99.9%), purified by passage through an oxygen-removal cannister (Diamond Tool and Die Co.), and wetted with either distilled water or Sweetser's solution (Sweetser, 1967). Flush times vary between 1 and 2 h for the gas cell body and between 10 and 30 min for the ports. Following the septum-sealed port flush, the entire system is purged with CO for 2 min, with the gas wetted as above. In order to monitor the reduction of the enzyme under the atmosphere of carbon monoxide and nitrogen, the sample cell has a 2-mm path length quartz cuvette epoxied to the cell body.

Oxidoreductase activities were determined according to a standard method (Smith, 1955). In one series of experiments, sterile samples were incubated at 29.6 °C (the temperature of an oxidase solution in the gas cell in the IR spectrophotometer) under 0.85 atm of air or N_2, and the activity was followed as a function of time.

In experiments to determine the effect of incubation time on CO oxygenase activity, samples were prepared as above except for the solution being under CO. When a level of

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[1] Abbreviations: CcO, cytochrome *c* oxidase; CcO(IV), oxidized cytochrome *c* oxidase; CcO(II), half-reduced cytochrome *c* oxidase; CcO(0), fully reduced cytochrome *c* oxidase; GC, gas chromatography.

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reduction of 0.85–1.0 electrons per oxidase was achieved by autoreduction, 5 mL of O_2 was added and the production of CO_2 followed for 1 h. The extent of reduction was determined by using IR-correlated optical spectra (Yoshikawa & Caughey, 1982).

Oxygenase activity as function of reduction state was measured with a similar technique. In some of the experiments an induction period was observed, but interpretation is qualified subject to the limitations of base-line stability, signal to noise ratio, and the sensitivity of the IR method.

An instrumental extinction coefficient for CO_2 was measured by using injection and flow techniques and is not considered to be "theoretically" rigorous. Data were taken at 30.6 °C (the temperature of the gas phase in the gas cell in the IR spectrophotometer), at 0.85 atm, and in a total gas volume of 0.0884 L.

With respect to the first methodology, 0.05–0.10-mL aliquots of 99.99% CO_2 were injected into a nitrogen-flushed gas cell, and after attainment of thermal equilibrium, the absorbance at 2360 cm^{-1} was recorded against a reference cell containing only nitrogen. Excellent agreement was found between two determinations taken 1 year apart, e.g., 0.129 ± 0.003 mM^{-1} cm^{-1}. The plot of absorbance vs. concentration showed no deviation from the linearity expected when Beer's law is followed. The amounts of CO_2 measured during these instrumental calibrations were selected to encompass the amounts of CO_2 produced during stoichiometry determinations. The flow technique was used solely to demonstrate that even at very high pressures of CO_2 (0.17–0.85 atm) the Beer's law plot was linear. The lower limit of detection of the IR method is 75 nmol of gaseous CO_2, with a reproducibility of 50 nmol.

When the number of moles of gaseous CO_2 is known, its partial pressure can be calculated by using the equation of state, and from Henry's law the molarity of dissolved CO_2 is then found. The value of the Henry's constant used here was corrected (Harned & Davis, 1943) from the value given by Segel (1976). Bicarbonate concentrations were determined by assuming a pK_1 of 6.4, with the experimental pH being 7.4.

The technique employed to measure the total amount of O_2 remaining after the anaerobic protocol uses reduced cytochrome *c* (Brown & Mebine, 1969; Young, 1981) and resting cytochrome *c* oxidase. Oxidase (0.5 mL) and cytochrome *c* (0.5 mL) are placed at opposite ends of the gas cell (c^{2+}:oxidase = 50:1). After a 2-h cell body flush, 30-min port flush, and final 2-min purge (all with highly purified N_2), the solutions are mixed. The extent of oxidation of cytochrome *c* is followed at 622 nm, until after vigorous agitation of the solution no change in absorbance is seen in at least two consecutive spectra taken 10 min apart.

The chemicals used were of reagent grade and unless noted otherwise were not purified further. USP-grade nitrogen (99.9%), oxygen (99.9%), and carbon dioxide (99.99%) were from General Air Products, while CP-grade CO (99.7%) and research-grade CO (99.99%) were from Scientific Gas Products. ^{13}CO (97.0 atom %) and $^{18}O_2$ (99.1 atom %) were purchased from Prochem.

Electronic spectra were taken on a Cary 17 spectrophotometer. GC–mass spectrometry was done with a MM-16VG-LTD mass spectrometry unit interfaced to a PE-Sigma computer system. Infrared spectra were recorded by using a PE-180 spectrophotometer in the linear absorbance, constant energy mode. Scan rates were 5–6 cm^{-1} min^{-1} with a resolution of 1.0 cm^{-1} at 2400 cm^{-1} and a time constant of 4. Spectra were taken on a 20-fold ordinate expansion, with the absorbance being measured from the raw data by using 20 mm = 0.0075 absorbance units.

As an explanatory note, inversion is the act of rotating the gas cell so as to fill the quartz cuvette with the solution of oxidase. With this maneuver, the physical state of the enzyme is changed with respect to its surface to volume ratio.

Table I: Isotope Ratios for CO_2 Produced from $^{12}C^{16}O$ and $^{18}O_2$ or $^{12}C^{16}O$ and $^{16}O_2$

	$^{18}O^{12}C^{16}O$: $^{16}O^{12}C^{16}O$ (%)	$^{18}O^{12}C^{18}O$: $^{16}O^{12}C^{16}O$ (%)
$C^{16}O + {}^{18}O_2$ (run 1)	6.0	nd[a]
$C^{16}O + {}^{18}O_2$ (run 2)	5.1	nd[a]
$C^{16}O + {}^{16}O_2$ (run 1)	0.7	nd[a]
$C^{16}O + {}^{16}O_2$ (run 2)	0.2	0.004

[a] nd = not detected.

RESULTS

That CO_2 can be produced by the oxidase in the presence of CO and O_2 is shown in the data of Figure 1. The top trace reflects the instrumental CO_2 difference spectrum, dominated by the $^{12}CO_2$ contribution from 2380 to 2300 cm^{-1}. After 15 h under a nitrogen atmosphere the absorbance at 2360 cm^{-1} increases by 0.0034. This is thought to be a consequence of the Kelvin effect[2] and is discussed elsewhere (Young, 1981). There has been no change of absorbance in the $^{13}CO_2$ region of the spectrum (2320–2250 cm^{-1}) during this time. However, with addition of ^{13}CO and O_2 plus a 21-h incubation there is a dramatic increase in $^{13}CO_2$ of 0.036, while the change in $^{12}CO_2$ is very small (0.0019). Part of this latter increase is accounted for by oxidation of ^{12}CO to $^{12}CO_2$, leaving a residual of 0.0008, which is below the limit of measurable significance. The data also prove that all the CO_2 produced came from CO and not because of a fortuitous oxidative decarboxylation. This point is furthur supported by the observation that the addition of O_2 to oxidase under N_2 does not produce CO_2. When CO is added to this same solution, there is a rapid increase in CO_2.

In Figure 2 it is seen how a 5-mL bolus of $^{18}O_2$ (99.1 atom %) changes the absorption profile of gaseous CO_2 over a CO-saturated solution of oxidase. The extent of isotope incorporation is best appreciated by comparing spectra c and d with respect to the relative intensities of the 2360- and 2340-cm^{-1} peaks. Also to be noted is an artifactual "step" at 2310 cm^{-1}, apparent in both spectra.

The results of GC–mass spectrophotometry experiments are collected in Table I. Enzyme samples (0.25 mM in heme A) were incubated in IR gas cells under $C^{16}O$, for 26 h prior to addition of 5 mL of either $^{18}O_2$ or $^{16}O_2$. After the dioxygen was added, carbon dioxide production was checked for by using the IR method. The gas phases were sampled for GC–mass spectrophometry 1–2 h later. With a subtractive correction to the level of $^{18}O^{12}C^{16}O$ expected from the natural abundance of ^{18}O, the ratio of singly labeled to unlabeled CO_2 in the $^{18}O_2$ experiment is 26 times that found in the $^{16}O_2$ run. In neither run was any of the doubly labeled species detected.[3]

Proof of the essential role of the oxidase for the oxygenation of carbon monoxide is to be clearly found in the effects of CN^-, N_3^-, and $HCOO^-$ on the production of carbon dioxide (Figure 3). Turnover is initiated by addition of 5 mL of O_2, with anion

[2] The vapor pressure of small droplets is greater than an equivalent volume with a plane surface. Hence the partial pressure of the CO_2 will increase until equilibrium is established with respect to the solvent–gas interface.

[3] Although the final level of incorporation of label was only 5–6%, it should be noted that exchange can occur with the solvent. Thus this extent of incorporation must be considered quite significant in this experiment.

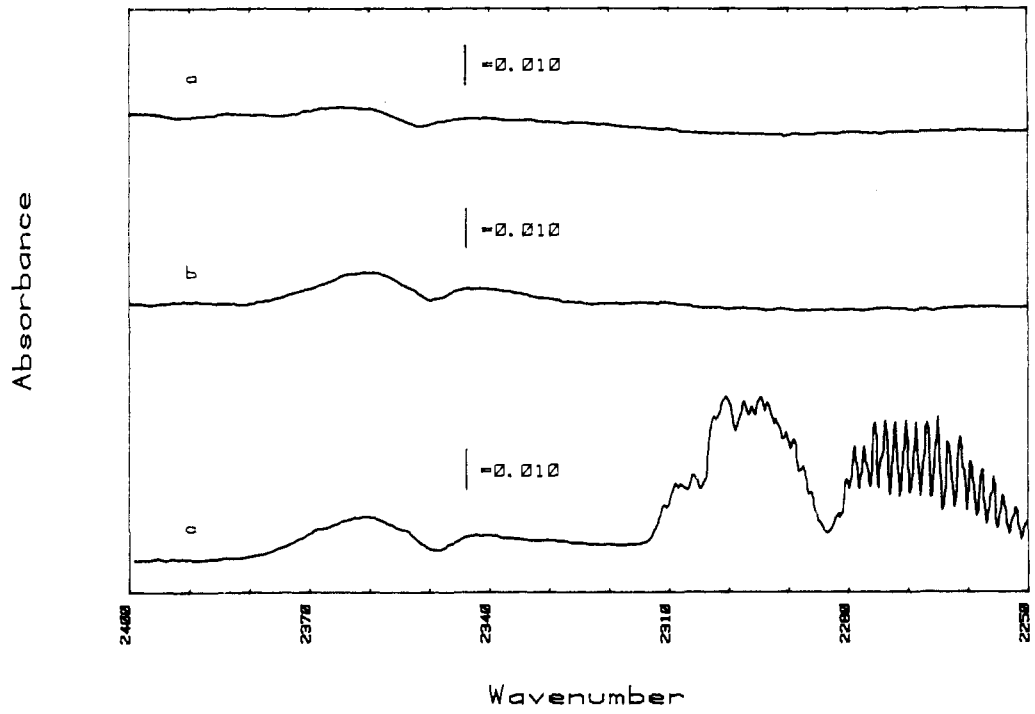


FIGURE 1: Gas-phase spectra showing production of $^{13}CO_2$ from ^{13}CO and O_2. The sample cell contained enzyme at a concentration of 0.9 mM in heme A dissolved in 0.5 mL of 0.01 M sodium phosphate, pH 7.4 (standard buffer). The reference cell contained 0.5 mL of distilled water. Spectrum a was taken just after completion of the nitrogen purge, while spectrum b was recorded 15 h later. Following this, 1.0 mL of O_2 and 4.0 mL of ^{13}CO (97.0 atom %) were added, and 21 h later, spectrum c was obtained. Spectra are manually digitized from the original data and hence smoothed. No loss or gain of information content is intended or implied.

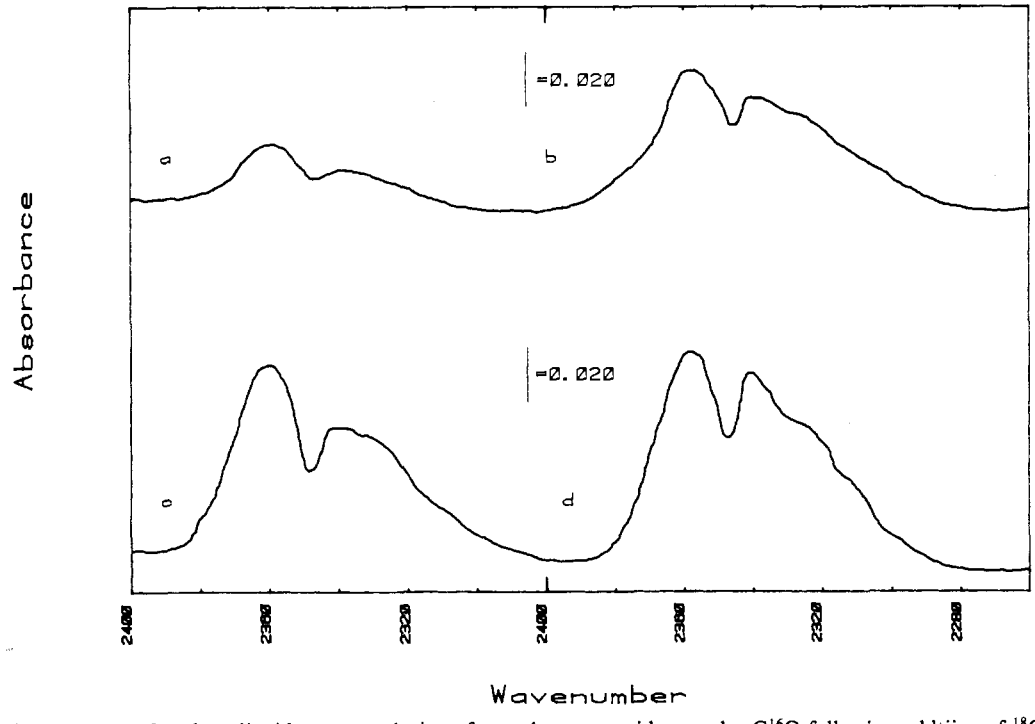


FIGURE 2: Gas-phase spectra of carbon dioxide over a solution of cytochrome *c* oxidase under $C^{16}O$ following addtiion of $^{18}O_2$. The enzyme concentration was 0.45 mM in heme A for spectra a, b, and d and 0.90 mM in heme A for spectrum c. Spectrum a was recorded 12 h after the end of the CO purge. At this time, 5 mL of $^{18}O_2$ (99.1 atom %) was added with spectrum b and d taken 0.15 and 0.80 h later. Spectrum c is from a different experiment in which $^{16}O_2$ was used.

introduced 1–2 h later. In each case there is inhibition, with concomitant collapse of the optical species generated when dioxygen is added to partially reduced, CO-complexes enzyme (the spectra are not shown). The background level of CO_2 is higher in the CN^- run because air had been added prior to the dioxygen. The N_3^- experiment contained bovine catalase at a concentration of 0.07 mM, while 5 mM mannitol was present in the $HCOO^-$ sample. The latter two samples are the same as those used in the experiments outlined in Table III. Suitable control experiments have shown that neither of these additional perturbants has any effect on the observed inhibition. The absorbance of the uninhibited reaction is obtained by extrapolation of the slope of the trace prior to adding inhibitor.

Although the changes are small, there is a good correlation between oxidase and oxygenase activities (data not shown).

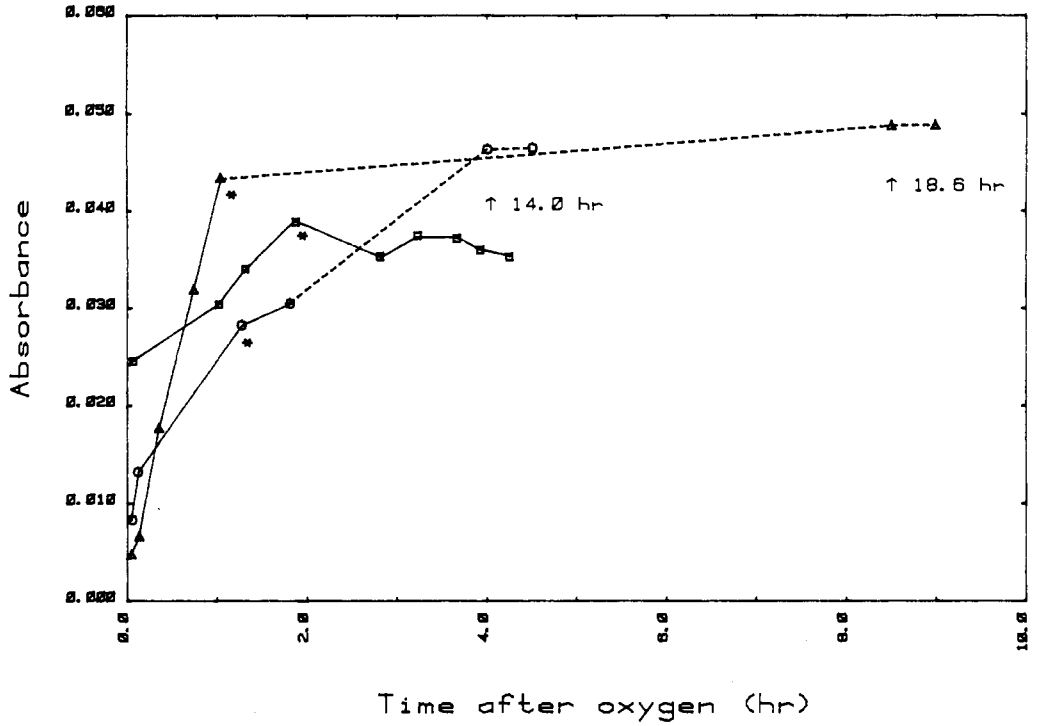


FIGURE 3: Inhibition of CO oxygenase activity by cyanide, azide, and formate. The absorbance of gaseous CO_2 was measured at 2360 cm^{-1}. The final concentrations of oxidase were 0.4, 0.2, and 0.2 mM in heme A and the ratios of inhibitor to heme A were equal to 4:1, 23:1, and 25:1 for the CN^-, N_3^-, and $HCOO^-$ runs, respectively. Bovine liver catalase was present at 0.071 mM in the azide run and mannitol at 5 mM for the formate experiment (△). All samples were dissolved in standard buffer. The test for cyanide inhibition (□) was carried out in the time frame of the abscissa. Dashed lines indicate the addition of 10 h to the abscissa reading. The asterisks indicate the time of addition of inhibitor.

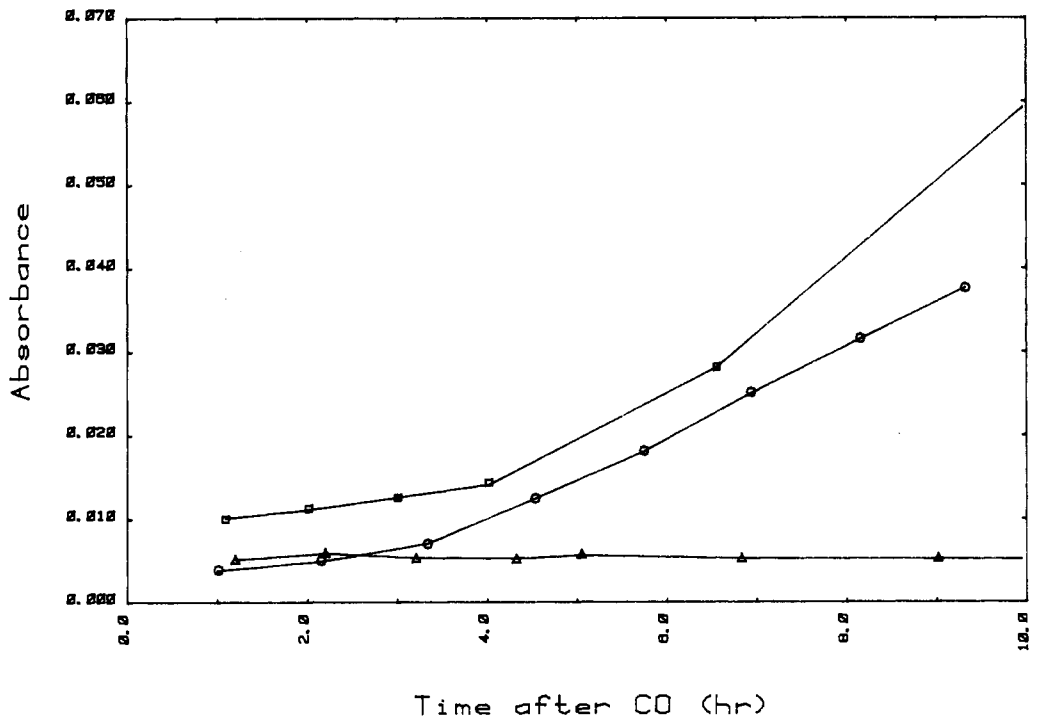


FIGURE 4: Effect of air leaks on $^{12}CO_2$ production by cytochrome *c* oxidase. (□, ○) Absorbance change at 2360 cm^{-1} for two separate samples of filtered oxidase incubated under CO in a gasket-sealed infrared gas cell. The enzyme concentrations were 0.9 and 1.1 mM in heme A with a sample volume of 0.5 mL. (△) Oxidase in an epoxy-sealed gas cell at 0.9 mM in heme A and the same standard buffer. All samples were recorded against a reference cell containing 0.5 mL of distilled water.

When incubated under air, N_2 and or CO each show an initial increase followed by a slight loss (ca. 20%) over the next 2 days.

Great care was taken to be certain that the results which have been presented so far were not the result of bacterial contamination. If the enzyme is not passed through two 0.22-μm Millipore filters, it takes about 10 h of incubation at 29.6 °C, under air, before bacterial production of CO_2 occurs. No CO_2 is detected even after 4 days when the enzyme is filtered. There is no effect of filtration on oxidase activity.

The gas cell must be rigorously airtight. Figure 4 shows that leaks in a gas cell sealed only with rubber gaskets give

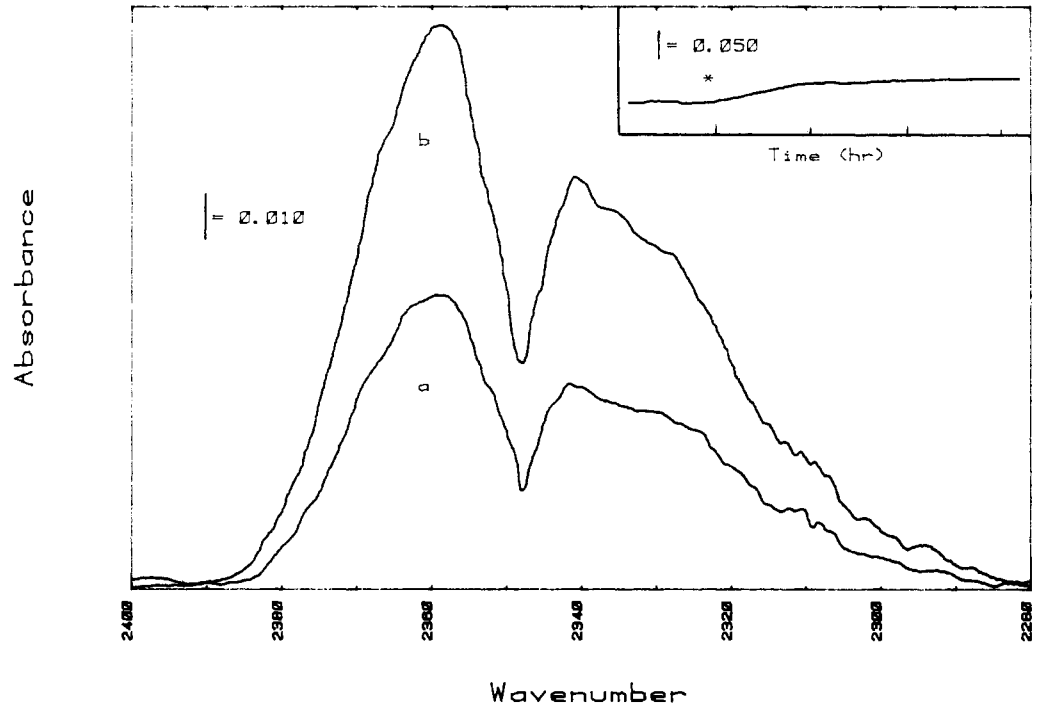


FIGURE 5: Change in gaseous CO_2 following addition of 0.2 mL of air to cytochrome *c* oxidase. The sample and reference cells contained carbon monoxide and nitrogen, respectively, as well as enzyme at 0.23 mM in heme A (in 1.0 mL of standard buffer). The absolute spectra were taken at the start (a) and end (b) of run 7 (Table III), with the kinetic profile (inset) observed at 2360 cm^{-1}. Each division on the time axis is equivalent to 5 h. The asterisk marks the time of addition of air.

rise to an apparent anaerobic production of CO_2 by the filtered enzyme. When epoxy resin is used as the seal, there is no production of CO_2 for 19 h after the end of the CO purge. In later experiments using a cell such as this, no CO_2 was detected for 48 h after the initial exposure to CO.[4]

By use of the extinction coefficient for CO_2 and the data in any of Figures 1–4, it is easily shown that the oxidase is functioning as a catalyst. In each case the number of moles of CO_2 produced is much greater than the number of moles of enzyme present. One can also calculate a turnover number for the enzyme by using the data of Figure 2. Under the conditions of that experiment a value of 0.01 mol of O_2 per mole of enzyme per second is found.

It is important to know if dioxygen is absolutely required for carbon dioxide production by the oxidase or if it could be generated via an anaerobic water–gas reaction. A direct measurement of the amount of "anaerobic" CO_2 was done as follows. Oxidase (0.85 mM in heme A) was incubated under CO until it was reduced to the extent of 2.7 electrons (0.7 electrons beyond the donor capability of a single water–gas reaction). After the data were corrected for overreduction, background oxygen contamination, and the instrumental CO_2 difference spectrum, a value of 0.5 mol of CO_2 per mole of oxidase was found. This is only half of the amount expected from the anaerobic oxidation of 1 mol of carbon monoxide.

The stoichiometry data of Table II give values of 1.90 and 0.09 for the mean and standard deviation of the mean of the number of moles of CO_2 produced per mole of O_2. The measurements were obtained over the course of 2 years under a variety of experimental conditions. Air was always the source of dioxygen and was added slowly into the gas phase in a volume of 0.1–0.3 mL. The reference contained either sterile distilled water (runs 1–4) or enzyme (runs 5–9). Flush times

Table II: Absolute Number of Moles of CO_2 Produced and Molar Ratio of CO_2 Produced to O_2 Added

	CO_2 ($\times 10^7$)			
run	gas	liquid	O_2 ($\times 10^7$), gas	CO_2:O_2
1	41.8	1.5	21.3	2.0
2	44.1	1.6	21.3	2.1
3	31.4	1.2	21.3	1.5
4	39.6	3.2	21.3	2.0
5	27.0	2.3	14.2	2.1
6	28.3	2.4	14.2	2.2
7	26.3	2.2	14.2	2.0
8	33.4	2.8	21.3	1.7
9	29.8	2.5	21.3	1.5

with nitrogen and carbon monoxide for the gas cell body and port, respectively, were for 1 h and then 15 min in the first series and twice these in the second. In the fourth experiment, reduced cytochrome *c* (cytochrome *c*:cytochrome *c* oxidase = 4.5:1) activated the oxidase while in all other runs autoreduction under CO was used. Sequential measurements were done in runs 6 and 7 as well as runs 8 and 9. In all cases initiation or termination of a given stoichiometric measurement was effected only after 1 h of spectral stability. Samples were incubated under CO for 3–18 h prior to addition of dioxygen.

In Figure 5 are shown the gas-phase spectra obtained at the end of run 6 (spectrum a) and the end of run 7 (spectrum b), as well as the kinetic trace for the course of the reaction (inset). The kinetic profile is not rigorously accurate for reasons outlined under Materials and Methods; however, the reaction may be reasonably estimated to be complete 7 h after adding 0.200 mL of air.

It might be expected from what has been seen so far that O_2 would significantly perturb the electronic spectrum of partially reduced, CO-complexed oxidase, and indeed it does (Figures 6 and 7). The most striking change observed following exposure to O_2 and inversion is the loss of intensity at 590 nm, which occurs whether or not the 602-nm shoulder is present. It is also apparent that the spectral form following

[4] As will be discussed later, this observation may be important in understanding the apparent disparity between the results found here and those of Bickar (Bickar et al., 1984).

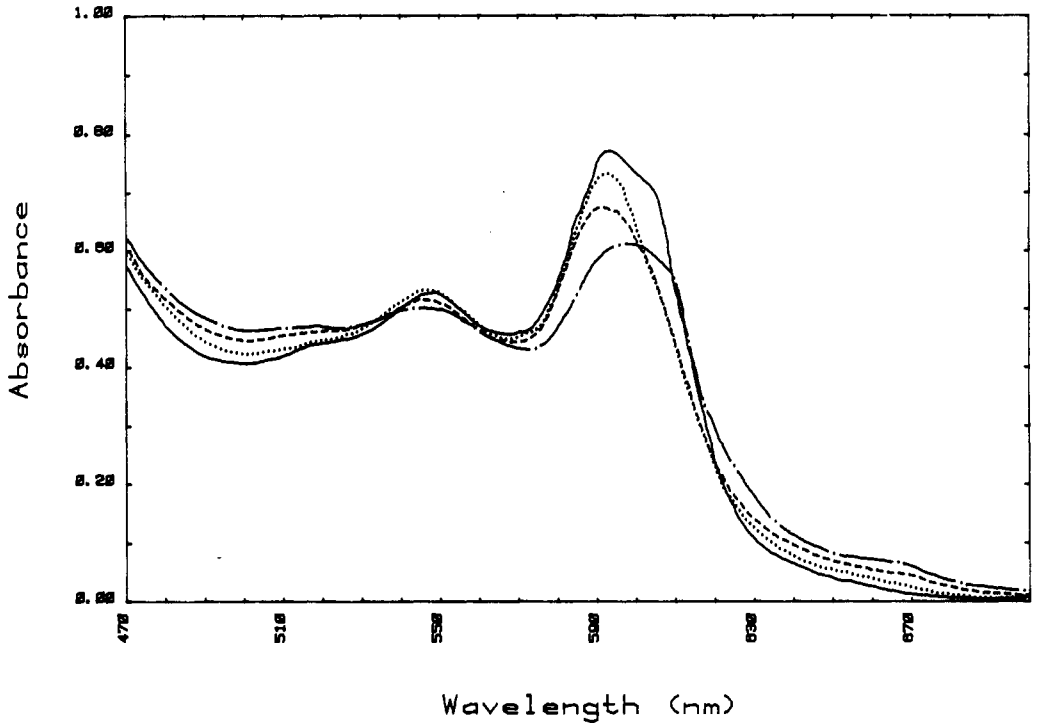


FIGURE 6: Effect of dioxygen, inversion, and CO oxygenation on the optical spectrum of cytochrome *c* oxidase reduced under CO by approximately 1.5 equiv. The spectra are of a single of enzyme at 0.3 mM in heme A in standard buffer. (—) Spectrum of 1.5-equiv-reduced, CO-complexed cytochrome *c* oxidase. (-·-) The latter species plus 5.0 mL of dioxygen, recorded 1 min after addition. (---) The latter species recorded following 9 min of incubation of the sample in an inverted position. (···) The latter species after an additional 49 min of incubation. (-·-) The latter species recorded after emptying the contents of the cuvette into the body of the gas cell, mixing for 1 min, and then refilling the cuvette. Spectra were recorded at 25 °C.

inversion is different depending on the amount of dioxygen present, the reduction state of the enzyme, and the length of time the sample is in the gas cell body after oxygenation.

Considering first the 1.5-electron-reduced species, one notes that if the filled cuvette is left in position in the Cary cell compartment, there is rebinding of CO at cytochrome a_3 (Figure 6). However, although the intensity at 590 nm increases, there is no return of the 602-nm feature, even with a 1-hour incubation. When the contents of the cuvette are emptied into the body of the gas cell, mixed for 1 min, and scanned immediately after inversion, the spectral species is identical with that which was present 1 h earlier.

With respect to the 1.0-electron-reduced form, the postinversion shoulder at 590 nm in Figure 7 is thought to be the result of incomplete displacement of CO from cytochrome a_3, since it could be eliminated by increasing the time of exposure to the gas phase prior to inversion. Although not apparent from these data, the spectra of CO rebinding to cytochrome a_3 do not share an isosbestic point with the spectrum induced by dioxygen. This is also true for the 1.5-electron-reduced enzyme. Both species display an increased absorption between 620 and 700 nm after O_2 exposure.

The experiments with O_2 radical scavengers were done to probe more directly the mechanism by which dioxygen produces the optical and IR spectral changes. The effect of catalase on the rate of the CO oxygenase reaction (Table III) is most significant and is observed when it is present at one-third of the concentration of the oxidase. That this is not a nonspecific consequence of added protein is shown by the lack of inhibition with superoxide dismutase which is present at twice the oxidase concentration. The reaction rates found in the dismutase and mannitol experiments are the same as the control rates. All perturbants were added to the enzyme at the beginning of sample preparation, prior to flushing with nitrogen.

Table III: Effect of O_2 Radical Scavengers and Ferricyanide on CO Oxygenase Activity

sample[a]	CO_2 rate[b] (h^{-1})	% inhibition[c]
mannitol (5 mM)	0.94	NS
superoxide dismutase (0.32 mM)	1.01	NS
catalase (0.071 mM)	0.55	43
ferricyanide (5 mM)	1.17	−22

[a] Enzyme concentration was 0.31 mM in heme A for the ferricyanide run and 0.36 mM for the others. [b] Rate measured as the change in absorbance at 2360 cm^{-1}, immediately after addition of 5 mL of O_2 to a normalized one-electron-reduced sample. [c] NS = not significantly different from the control rate of 0.97 h^{-1}.

Table IV: Effect of Reduction State on CO Oxygenase Activity

sample[a] (no. of electrons)	CO_2 rate[b] (h^{-1})	lag phase[c] (min)	N_2:CO ratio
0.5	0.60	8.5	0.42
0.9	0.83	2.5	0.42
1.45	0.75		0.42
1.6	0.75		0.22
2.0	0.90		0.42

[a] The enzyme concentration was 0.31 mM in heme A. [b] Rates were measured as in Table III. Corrections were made for the effects of incubation time and differences in the ratio of the partial pressures of N_2 and CO. [c] A lag phase is the length of time from the addition of O_2 until the start of CO oxygenase activity.

The final results of this work are to be found in Tables III and IV and are the consequences of experiments directed toward answering the question of how reduced must the oxidase be so that oxygenation of CO occur. Table III shows that ferricyanide, an oxidant of the fully reduced, CO-complexed enzyme, does not inhibit and perhaps slightly accelerates the reaction rate. The effect of the extent of enzymic reduction on the rate of evolution of CO_2 is seen in Table IV. Rates were corrected for the effect of incubation time on CO oxygenase activity. Multiplicative factors of 1.0, 0.9, 1.0, 1.2, and 1.2

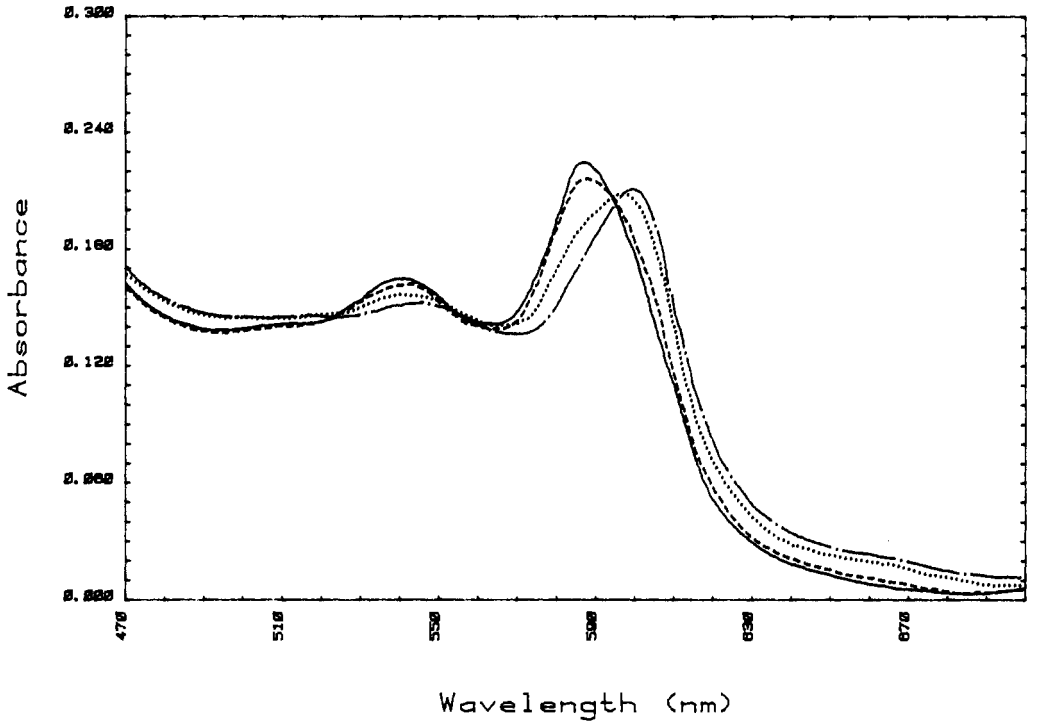


FIGURE 7: Effect of dioxygen, inversion, and CO oxygenation on the optical spectrum of cytochrome *c* oxidase reduced under CO by approximately 1.0 equiv. The spectra are of a single sample of enzyme at 0.13 mM in heme A in standard buffer. (—) The 1.0-equiv-reduced, CO-complexed cytochrome *c* oxidase. (---) The latter species recorded 1 min after the addition of 0.3 mL of air. (—) The latter species 1 h later. (···) The latter species recorded 1 min after the addition of a further 5.0 mL of air. (—) The latter species 1 h later. (-·-) The latter species recorded 2 min after emptying the contents of the cuvette into the body of the gas cell and then refilling the cuvette. Spectra were taken at 25 °C.

were found for the 0.5-, 0.9-, 1.45-, 1.6-, and 2.0-electron runs, respectively.[5] A lag phase appears to be present in the first two runs. If one normalizes the rates to the same partial pressure of CO relative to that of N_2, one obtains values of 0.60, 0.825, 0.75, 0.75, and 0.90 h^{-1} (exclusive of the lag phase) in the runs as listed above.

Discussion

The results presented here prove conclusively that sterile bovine heart cytochrome *c* oxidase can oxygenate CO and in doing so produces 2 mol of CO_2 per mole of dioxygen. This CO oxygenase activity is well correlated to the enzyme's O_2 reductase activity, and both are inhibited by CN^-, N_3^-, and $HCOO^-$. The oxidase must be reductively activated for oxidation of CO to occur.

Much of the early work on CO oxidation was criticized on the point of failure to control bacterial artifact. The experimental protocol used here eliminates bacteria as the source of CO oxygenase activity.

Since all of the CO_2 that is produced comes from CO and not as the result of an oxidative decarboxylation, the question then arises as to what the role of dioxygen is. A striking demonstration of the effect of dioxygen can be seen in Figures 2 and 3, where a burst of CO_2 follows the addition of O_2 to the activated oxidase. The burst phenomenon has been observed many times with a large number of preparations of this protein. Oxygen-dependent production of carbon dioxide is precisely what was found by the original workers in this field (Fenn & Cobb, 1932; Breckenridge, 1953; Clark, 1950). A simple calculation will show that it is not possible to obtain the extent of incorporation of ^{18}O into CO_2 observed in both IR and GC–mass spectrophotometry experiments, if the role of O_2 were only to reoxidize the enzyme. Thus CO and O_2 must be present simultaneously in the active site of the enzyme.

The stoichiometry of 2 mol of carbon dioxide produced per mole of dioxygen, combined with the direct evidence against anaerobic oxidation of carbon monoxide, suggests a di-CO dioxygenase activity as being responsible for CO_2 production. We proffer, however, that the possibility still exists for a water–gas plus monoxygenase activity as the mechanism for CO oxidation, when activated cytochrome *c* oxidase is exposed to CO and O_2. This is so primarily because the background O_2 correction applied to the raw data on the "anaerobic–CO_2" experiment is based on the level of dioxygen present in the gas cell when nitrogen alone is used as the flushing gas. Thus if there was absolutely no dioxygen present in the system after the CO flush (a highly improbable occurrence), the stoichiometric ratio of CO_2 produced per oxidase would be 1.13. To obtain the required ratio of unity, only slightly more than 1 ppm of dioxygen is permitted. This is almost 1 order of magnitude less than any level achieved with the anaerobic protocol used here.

It is important to outline several pertinent technical considerations that support the assumption that there are at least 9–10 ppm of O_2 in the gas cell after the introduction of CO. Only flush (cf. flush–evacuation) techniques were used to rid the system of adventitious dioxygen. The background dioxygen of 9.4 ppm (Young, 1981) most probably is the result of adsorption of the gas to the walls of the gas cell particularly in the side-arm cuvette. Realistically, one cannot expect this situation to be significantly improved by a 2-min CO purge, no matter how pure the CO is. Furthermore, the research-grade CO (99.99%) was scrubbed with the same solution used when the background level of dioxygen was determined with

[5] In the absence of O_2, CO will bind only to cytochrome a_3 and thereby greatly increase the midpoint potential of this center. Thus when there is less than 1 reducing equiv present, the pool of enzyme species is composed of oxidized and one-electron-reduced, CO-complexed oxidase. Similar reasoning applies to states of fractional reduction between 1 and 2 reducing equiv except that now the pool is composed of one- and two-electron reduced, CO-complexed oxidase.

highly purified N_2 (99.9999%). The assay method used is sufficiently sensitive and reproducible so as to make the measurement of contaminant dioxygen highly reliable.

The inhibition with formate can also be used as a point against a water–gas reaction of the enzyme. Since formic acid is the hydrate of carbon monoxide, one could conceive of a water-catalyzed generation of carbon dioxide occurring with formate. The data of Figure 3 plus the observation of collapse of the optical steady-state species to an oxidized formate complex are compelling evidence that such a reaction does not occur with the oxidase.

The results in Figure 2 show that by the time the first IR spectrum was taken approximately equal amounts of singly labeled and unlabeled CO_2 have been produced. This is not what might be expected for a di-CO dioxygenase reaction but is exactly what is predicted by a water–gas plus monoxygenase mechanism. For this to be true one must propose that essentially no exchange of label has occurred between CO_2 and the solvent in the time (0.15 h) prior to taking the first IR spectrum.

The ^{18}O exchange rate between carbon dioxide and water has been studied (Mills & Urey, 1940), and it has been found to be equal to the rate of hydration of carbon dioxide. With the pH, temperature, and ionic strength normalized to those of the CO oxygenation reaction, a value for the hydration rate constant would be 0.054 s^{-1}. The dehydration rate constant is 3 orders of magnitude greater, while the deprotonation to form bicarbonate is considered to be "instantaneous". The experiments of Mills and Urey were done in a closed system with no gas phase, and thus extrapolation to the situation inside the gas cell must be tempered. This is so because the rate constants for equilibration of newly formed CO_2 with both the gas phase and the preoxygenation pool are not known. The amount of carbon dioxide present before addition of dioxygen is very small and would be completely eliminated from consideration after a single turnover of dioxygen at the enzyme concentrations used in Figure 2. The turnover number is 0.01 s^{-1}, and thus, depending on what the rate constants are for gas-phase equilibration, competition could arise betwen gas–liquid exchange of CO_2 and its hydration.

If the rate-limiting step is not ^{18}O exchange but rather the rate constant for transfer of dissolved CO_2 to the gas phase, almost 3.0 exchanges per mole of CO_2 could have occurred prior to measurement. It is not possible as yet to put a lower limit on the number of exchanges. It is conceivable that an apparatus such as that used by Forster and Itada in their studies with erythrocyte carbonic anhydrase (Forster & Itada, 1977) might allow one to detect the labeling pattern prior to solvent exchange.

The optical spectra of partially reduced, CO-complexed oxidase are significantly altered by the addition of dioxygen (Figures 6 and 7). These differences in spectral form are probably due to the different oxidation state of the enzyme, even though the O_2:oxidase ratios are not exactly the same (a factor of 2 is required for normalization).

The spectrum obtained immediately after inversion cannot rigorously be equated with the true steady-state spectrum. This is because of the time lapse between inverting the sample and recording the spectrum as well as the different physical state of the sample. It may well represent a composite of several enzyme species, but if the intensity at 590 nm is any indicator of ligation of CO to cytochrome a_3, it seems correct to infer that O_2 has displaced it. The data also show no evidence that sustained oxidation of the enzyme has occurred as a result of exposure to O_2; e.g., although the oxidase has been in the presence of high levels of O_2 (0.05 atm), when CO_2 is made the enzyme does not revert to the oxidized form, despite the fact that CO has been displaced from cytochrome a_3. It might be said that the postoxygenation spectra show evidence of immediate and possibly continuing reoxidation of the enzyme. Experiments have been done under conditions identical with those of Figure 6, except that the sample was exposed to the gas phase for 3 min and then for 88 min. In each case spectra identical with the postinversion spectrum of Figure 6 were found. It is not being asserted that no O_2 reductase activity is present under the conditions of these experiments but rather that it is very much inhibited and is not a necessary reaction for the oxygenation of CO. In fact a drift toward oxidized enzyme forms is seen only after prolonged (18–24-h) incubation. When low levels of O_2 are used, this is not observed, and for this reason stoichiometric experiments were done at low O_2 tensions.

One interpretation of the dioxygen-induced optical perturbation is that there are two types of CO-complexed oxidase, but only one oxygenates CO. The other is reoxidized by O_2 to give H_2O_2 and then reautoreduced during prolonged inversion. Depending on the amount of the latter form, the reaction stoichiometry will be reduced from 2:1 to, for example, 1.2:1 if there were a 60:40 conformer mix. Since the measured stoichiometry is 1.9:1, it appears that there cannot be such a distribution. This is true only if the CO oxygenase activity of the two conformers is the same at low and high O_2 partial pressures and if peroxide cannot activate the enzyme for oxidation of CO.

The inhibition of the CO oxygenase activity by catalase is of consequence with respect to these questions. This is so since the inhibition occurs in both the stoichiometry and CO_2 rate studies. The former were done at very low O_2 tensions and the latter at high tensions, but both the stoichiometry and rate were only 60% of the expected values. The implications are that hydrogen peroxide can be released from an oxidase conformer and then activate another conformer for oxygenation of CO at any pressure of O_2. Future experiments will be designed to test this hypothesis.

Before briefly discussing possible reaction mechanisms, it is necessary to elaborate on the results found concerning how the enzyme is activated for oxygenation. That the CO oxygenase activities of the 1.0- and 2.0-electron-reduced enzyme are the same is of consequence in so far as any detailed reaction mechanism is concerned. Although this conclusion is in part the result of data manipulation, if correct it suggests two other varibles that may be of significance in determining the rate of carbon monoxide oxidation, e.g., the presence of appreciable amounts of resting enzyme prior to addition of dioxygen and the relative partial pressures of the gas-phase components. With respect to the former, it has been mentioned earlier that although there appears to be a lag phase when reduction is less than 1 equiv per oxidase, it is difficult to assess. It may represent an effect of O_2. In order to improve the signal to noise ratio, studies using very high concentrations of enzyme are planned.

It is not surprising that the oxygenase activity of the oxidase can be modulated by the composition of the gas phase. It is however unexpected that the measured rate for the 1.6-electron-reduced oxidase can be significantly corrected by normalization with the $pCO:pN_2$ ratio. A similarly effective correction can be achieved with the $pO_2:pN_2$ ratio, which gives a value for the rate of 0.90 h^{-1} (cf. 0.75 h^{-1}). At present it is not known which, if either, of these corrections is applicable. Nonetheless, unless there is a variable as yet not appreciated

(a definite possiblity in this complex system), it would seem that nitrogen can affect the rate of carbon dioxide evolution.

It is evident that reductive activation of the oxidase must occur, since there is no proven precedent for binding of CO or O_2 to trivalent iron or divalent copper. It was necessary to prove that autoreduction was not the only way to accomplish this. The stoichiometry experiment with cytochrome *c* shows this to be so. Furthurmore, we have found that the NADH–phenazine methosulfate reducing system is similarly effective.

The effect of ferricyanide on oxygenase activity can be rationalized with several lines of logic. It has been convincingly demonstrated (Anderson et al., 1976) that, under rigorously anaerobic conditions, ferricyanide will remove three electrons from fully reduced, CO-complexed oxidase. The optical spectrum of the species so oxidized is remarkably similar to the one-electron-reduced, CO-complexed enzyme produced by reduction with NADH–phenazine methosulfate (Yoshikawa & Caughey, 1982). If, in the presence of significant amounts of dioxygen, ferricyanide behaves as it does under conditions of anaerobiosis, the implication is that oxidase reduced by 1 equiv can oxygenate CO. On the other hand, it is entirely conceivable that the presence of both CO and O_2 prevents ferricyanide from oxidizing fully reduced enzyme by more than 2 equiv and a half-reduced enzyme at all. If ferricyanide reacts just with cytochrome *a*, this can be accomplished by ligation of CO or O_2 at the active site copper.

On the basis of the data that have been presented in this paper, the following series of reactions is proposed for a di-CO dioxygenase activity of cytochrome *c* oxidase.

$$CcO(II)\cdot CO + O_2 \rightleftarrows CcO(II)\cdot CO\cdot O_2 \quad (1)$$

$$CcO(II)\cdot CO\cdot O_2 \rightleftarrows CcO(II)\cdot O_2\cdot CO \quad (2)$$

$$CcO(II)\cdot O_2\cdot CO \rightleftarrows CcO(II)\cdot O + CO_2 \quad (3)$$

$$CcO(II)\cdot O + CO \rightleftarrows CcO(II)\cdot O\cdot CO \quad (4)$$

$$CcO(II)\cdot O\cdot CO \rightleftarrows CcO(II) + CO_2 \quad (5)$$

$$CcO(II) + CO \rightleftarrows CcO(II)\cdot CO \quad (6)$$

In order to explain the similar reactivity of the one- and two-electron-reduced enzyme within the context of this particular mechanistic scheme, it is proposed that in the presence of dioxygen the one-electron form is converted to a functional two-electron form

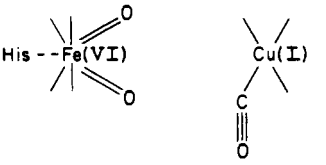
$$R\text{–}S\text{–}Cu\text{-}a_3(II) + O_2 \rightleftarrows R\text{–}S\cdot\text{–}Cu\text{-}a_3(I)\cdot O_2$$

where RS^- may or may not be the bridging cysteinate (Powers at al., 1981).

The slow step could be attributed to the forward reaction in step 2, since the k_{off} for CO in a partially reduced oxidase is on the order of 0.01 s^{-1} (Greenwood et al., 1974) and thus near to the turnover number of the enzyme. Equally as important is the K_d for binding of CO to the active site copper (Chance et al., 1975; Clore et al., 1980; Fiamingo et al., 1981; Nicholls & Chanady, 1981). In fact, if the spectrum seen immediately after inversion is the true steady-state species of CO oxygenation, it would seem that $CcO(II)\cdot O_2\cdot CO$ is a more appropriate choice than $CcO(II)\cdot CO\cdot O_2$. The resolution of the question may be found by examining the effect of surface area on the rate of the reaction; i.e., the role of solvent viscosity in the binding of gaseous ligands to the oxidase is expected to be significantly diminished when the gas–liquid interface is increased. With this variable attenuated, ligand solubilities should be of greater importance, (DeFonseka & Chance, 1980).

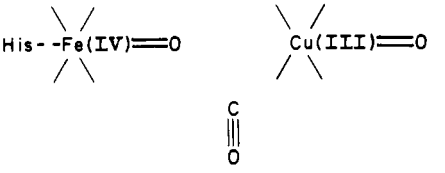
In any case the proposed mechanism accounts for the measured stoichiometry and the dioxygen-induced spectral perturbations and can easily accommodate the ferricyanide and catalase effects. Additionally, it permits a plausible explanation of the oxygen isotope experiments by postulating an active site species (step 3) capable of exchanging its oxygen with solvent. Finally, it shows how oxidation of CO can occur without repeated oxidation of the enzyme. What follows next is a brief outline of some possible chemical structures for the active site intermediates suggested by the kinetic scheme and in particular those for steps 3–5. The nature of the probes used in this study forces these proposals to be speculative.

If a Langmuir–Hinshelwood model is rigorously adhered to, a species of the form:

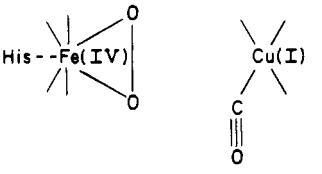


must be predicted for the left-hand side of step 3. After reaction with the molecule of CO, a ferryl iron–oxo species would remain to react with a second molecule of CO and would regenerate the starting species on recombination with a third molecule. As yet, there is no known biochemical precedent for a heme iron participating in a reaction like this. The Eley–Rideal formalism (Engl & Ertl, 1979) can be applied to the above scheme, with the distinction between it and the former being that the CO is in a "physisorbed" or weakly bound state not a "chemisorbed" state. In so far as the enzymic catalysis is concerned, this amounts to there being no requirement for the reduction of the active site copper. Two molecules of CO merely diffuse into the pocket and sequentially remove two atoms of oxygen from the iron.

There are at least two other ways by which oxygen could be activated in the context of a di-CO dioxygenase mechanism. The first of these requires half-reduction and an Eley–Rideal pathway and would give



Here the dioxygen is first bound between the reduced iron and copper. Following rupture of the dioxygen double bond, two molecules of CO sequentially remove two atoms of oxygen, one from each metal. The favored chemism also requires half-reduction and because of steric considerations may have to proceed via a formal Langmuir–Hinschelwood pathway:



Either of the equivalent oxygen atoms reacts first with the carbon of the bound CO to give a bridging percarbonato species. On elimination of CO_2, a ferryl iron–oxo intermediate would be produced with a reactivity toward CO like that described above.

There is one further mechanistic model that in light of the present data cannot be absolutely discounted. As discussed earlier, an assumption was made concerning the amount of contaminant dioxygen in the gas cell following introduction of carbon monoxide. If this assessment was not correct, then anaerobic production of CO_2 by the oxidase, CO, and H_2O has not been ruled out. By itself this type of reaction does not

explain the stoichiometry, the effects of O_2 on the rate of CO_2 production, the ^{18}O isotope results, and the overreduction beyond the level of half-reduction and requires that an oxide, bridging or otherwise, be present at the active site. Even with a concomitant monoxygenase activity of the P-450 type, it must still be explained how CO can react with ferric iron and/or cupric copper [but see Bickar et al. (1984)].

In conclusion then, although the exact details of the reaction mechanism have yet to be unraveled, the catalytic oxygenation of CO by activated bovine left ventricular cytochrome *c* oxidase cannot be disputed.

Registry No. CcO, 9001-16-5; CO_2, 124-38-9; O_2, 7782-44-7; CO, 630-08-0; mannitol, 69-65-8; superoxide dismutase, 9054-89-1; catalase, 9001-05-2; ferricyanide, 13408-62-3.

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Spectroelectrochemical Study of the Cytochrome *a* Site in Carbon Monoxide Inhibited Cytochrome *c* Oxidase†

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ABSTRACT: The reduction potential of the cytochrome *a* site in the carbon monoxide derivative of beef heart cytochrome *c* oxidase has been studied under a variety of conditions by thin-layer spectroelectrochemistry. The reduction potential exhibits no ionic strength dependence and only a 9 mV/pH unit dependence between pH 6.5 and 8.5. The weak pH dependence indicates that protonation of the protein is not stoichiometrically linked to oxidoreduction over the pH range examined. The temperature dependence of the reduction potential implies a relatively large standard entropy of reduction of cytochrome *a*. The measured thermodynamic parameters for reduction of cyctochrome *a* are (all relative to the normal hydrogen electrode) $\Delta G°'(25\ °C) = -6.37$ kcal mol^{-1}, $\Delta H°' = -21.5$ kcal mol^{-1}, and $\Delta S°' = -50.8$ eu. When cytochrome *c* is bound to the oxidase, the reduction potential of cytochrome *a* and its temperature dependence are not measurably affected. Under all conditions studied, the cytochrome *a* site did not exhibit simple Nernstian $n = 1$ behavior. The titration behavior of the site is consistent with a moderately strong anticooperative interaction between cytochrome *a* and Cu_A [Wang, H., Blair, D. F., Ellis, W. R., Jr., Gray, H. B., & Chan, S. I. (1985) *Biochemistry* (following paper in this issue)].

Cytochrome *c* oxidase is the terminal enzyme of the electron transport chain in mitochondria. Spanning the mitochondrial inner membrane, this enzyme catalyzes the reduction of dioxygen to water, using protons derived from the matrix side of the membrane and reducing equivalents provided by ferrocytochrome *c* on the cytosol side. During electron transport, cytochrome *c* diffuses from the membrane-bound cytochrome bc_1 complex to a binding site (Michel & Bosshard, 1984; Antalis & Palmer, 1982) or sites (Wilms et al., 1981) on cytochrome *c* oxidase. Electron transfer from bound cytochrome *c* to cytochrome *a* is rapid (the apparent first-order rate constant is greater than 1000 s^{-1} under some conditions;

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