Carbon Monoxide-driven Reduction of Ferric Heme and Heme Proteins*

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Oxidized cytochrome c oxidase in a carbon monoxide atmosphere slowly becomes reduced as shown by changes in its visible spectra and its reactivity toward oxygen. The "autoreduction" of cytochrome c oxidase by this procedure has been used to prepare mixed valence hybrids. We have found that this process is a general phenomenon for oxygen-binding heme proteins, and even for isolated hemin in basic aqueous solution. This reductive reaction may have physiological significance. It also explains why oxygen-binding heme proteins become oxidized much more slowly and appear to be more stable when they are kept under a CO atmosphere. Oxidized a and b chains of human hemoglobin become reduced under CO more slowly than does cytochrome c oxidase, where the CO-binding heme is coupled with another electron accepting metal center. By observing the reaction in both the forward and reverse direction, we have concluded that the heme is reduced by an equivalent of the water-gas shift reaction (CO + H₂O → CO₂ + 2e⁻ + 2H⁺). The reaction does not require molecular oxygen. However, when the CO-driven reduction of cytochrome c oxidase occurs in the presence of oxygen, there is a competition between CO and oxygen for the reduced heme and copper of cytochrome a₃. Under certain conditions when both CO and oxygen are present, a peroxide adduct derived from oxygen reduction can be observed. This "607 nm complex," described in 1981 by Nicholls and Chandy (Nicholls, P., and Chandy, G. (1981) Biochim. Biophys. Acta 634, 256-265), forms and decays with kinetics in accord with the rate constants for CO dissociation, oxygen association and reduction, and dissociation of the peroxide adduct. In the absence of oxygen, if a mixture of cytochrome c and cytochrome c oxidase is incubated under a CO atmosphere, autoreduction of the cytochrome c as well as of the cytochrome c oxidase occurs. By our proposed mechanism this involves a redistribution of electrons from cytochrome a₃ to cytochrome a and cytochrome c.

20 °C it takes several hours to reduce cytochrome a₃ fully. An even slower reduction occurs for cytochrome a, the cytochrome of cytochrome c oxidase which does not bind CO. Both reactions are temperature-dependent, being much faster at 20 °C than at 4 °C (2). Finally, the reaction appears to leave the enzyme spectrally and kinetically indistinguishable from the enzyme which has been reduced by more conventional methods and allowed to react with CO (2). Little else is known about this reaction.

In spite of its undefined nature, the reaction has provided a useful means of reaching difficult to titrate redox states and has been frequently employed in mechanistic studies of cytochrome c oxidase (1, 2, 4, 5). In particular, it has been useful in forming the "mixed valence" form which has cytochrome a₃ and copper a₃ reduced and cytochrome a and copper a oxidized. This form has been indispensable in identifying the spectral components of each cytochrome and in observing their respective kinetics.

The oxidation of CO to CO₂ is known to occur in a few biological systems, such as the nickel-containing enzyme in Clostridium thermoaceticum (6). With cytochrome c oxidase under CO, Young and Caughey (5), have demonstrated that the reaction CO + O₂ → CO₂ has yielded the reaction CO + O₂ → CO₂. However, there has been no accepted mechanism for how autoreduction occurs.

We propose that autoreduction of cytochrome c oxidase is the reduction of copper and ferric heme iron, "driven" by carbon monoxide oxidation:

\[ \text{Cu(II)} + \text{CO} + \text{H}_2\text{O} \rightarrow \text{CO}_2 + 2\text{H}^+ + \text{Cu(I)} \text{Fe(II)} \]  

(1)

A CO-driven metal reduction as represented in Equation 1 is thermodynamically favorable (ΔG' = -36 kcal/mol), but the apparent requirement for ferric heme to bind CO adequately to catalyze this reaction makes it intuitively unattractive. Alternative explanations put forth for autoreduction have been that other sites on the protein or contaminants in cytochrome c oxidase preparations are able to reduce the ferric heme under a nonoxygenizing atmosphere, or that bacterial growth in the solutions has supplied the electrons to reduce the heme. The role of CO would then be only that of stabilizing the reduced heme against reoxidation. We will present evidence that the reduction of ferric heme is directly coupled to the oxidation of CO to CO₂. Moreover, evidence will be presented that this process can occur in the absence of molecular oxygen and that it occurs not only for oxidized cytochrome c oxidase but also for oxidized forms of heme and oxygen-binding heme proteins like hemoglobin and myoglobin.

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MATERIALS AND METHODS

HbA was prepared by the ammonium sulfate procedure and stripped of ions as described previously (7). Oxidized metHbA was prepared by treating oxy-HbA in 0.1 M NaHPO4, pH 7.4, with NaNO2 and dialyzing three changes of buffer. MethHbA with extra oxidant (for CO2 production determination) was prepared by adding excess K2Fe(CN)6 to HbA solutions and allowing them to stand overnight at room temperature. Cytochrome c oxidase was isolated from bovine hearts by the method of Yonetoni, as described previously in Ref. 8. The purity of each enzyme preparation was determined by spectral criteria (9, 10). Only enzyme preparations with 444 to 424 nm absorbance ratios of greater than 2.25 for the reduced enzyme were used in binding experiments (9).

Cytochrome c oxidase concentrations were expressed in terms of total heme and estimated by using a millimolar difference extinction coefficient of 11 for the reduced minus oxidized state at 605 nm (11). Heme and cytochrome c were used without further purification. Dipyridine heme was prepared by dissolving hemin HCl (Sigma) in a minimum volume of pyridine and mixing the pyridine solution with buffer. Dinitrophenyl acid heme was prepared by dissolving hemin HCl in buffer containing 10 mM nicotinic acid. Buffer solutions were prepared in distilled and deionized water. Buffers for assays of the coefficient of 11 for the reduced minus oxidized state at 605 nm (11) were reagent grade or better. Changes in pH during CO-driven reduction were measured with a Radiometer combination electrode. Solutions for these scale with a Radiometer combination electrode. Solutions for these experiments were made with a millimolar difference extinction coefficient of 11 for the reduced minus oxidized state at 605 nm (11).

Equine crystalline catalase was a gift from Dr. Eraldo Antonini of the University of Rome. Sperm whale metmyoglobin (Sigma) and cytochrome c (Sigma type III) were used without further purification. Dipyridine heme was prepared by dissolving hemin HCl (Sigma) in a minimum volume of pyridine and mixing the pyridine solution with buffer. Dinitrophenyl acid heme was prepared by dissolving hemin HCl in buffer containing 10 mM nicotinic acid. Buffer solutions were prepared in distilled and deionized water. Buffers for assays of the forward reaction of Equation 1 contained 0.1 M KPO4, 0.1 mM EDTA, pH 7.5. Buffers for assays of the back reaction of Equation 1 contained 0.01 M KPO4, 0.1 mM K2SO4, and 1 mM EDTA, pH 8.6. Buffers for cytochrome c oxidase also contained 1% Tween 80. Hemin in base was prepared by dissolving hemin HCl in KOH or (CH3)2NOH immediately before saturating with CO. When used, dithionate (Fisher) was added as a solid. Stock FSA (Aldrich) was prepared shortly before use by mixing 5 ml of deoxygenated, pH 7.5, buffer with excess FSA in a sealed septum bottle and stirring for 1 h at 5 °C. Excess FSA was allowed to settle and the supernatant withdrawn by syringe as needed. CO-saturated solutions with low oxygen content were prepared in a bottle or cuvette with a sealed septum cap by repeated evacuating and adding CO (MG Scientific Gases), and stored over solutions of dithionite and sodium hydroxide for at least 24 h prior to use. The concentrations of O2 and CO2 were measured by mass spectrometry. The mass spectrometer readings were converted to initial solution concentrations by comparing with standards of known 13CO2 concentrations which had been added to the same solution. The concentrations of O2 and CO2 were determined by mass spectrometry and found to be less than 1 mM, assuming a total gas concentration of 44.6 mM (based on a volume of 22.4 liters for 1 mol of gas under standard conditions). Case III: measurements of CO2 formation by oxidized hemoglobin under CO were carried out with 10 ml of deoxygenated, CO-saturated metHbA in a sealed 100-ml septum bottle with 200-ppm positive CO pressure. CO2 production in this case was monitored by removing 5-ml samples of the gas above the CO-saturating gas and measuring the gaseous CO2 concentration with a Beckman Infrared Gas Analyzer.

RESULTS

CO-driven Reduction of Cytochrome c Oxidase—The spectral changes that occur when oxidized cytochrome c oxidase is incubated under 1 atm of CO are shown in Fig. 1. Both cytochrome a2 and cytochrome a become reduced. The spectrum of the reduced and partially reduced species resemble those produced by titration with reductant, except for a slight decrease in total absorbance during the reaction. The reduction under CO takes several hours, the decrease is probably due to some comitant protein denaturation.

1 The abbreviations used are: HbA, human hemoglobin; FSA, formic acid; methHb, methemoglobin; H2PES, 4-[(2-hydroxyethyl)-1-piperazinethanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-glycine.
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Fig. 1. Spectra of cytochrome c oxidase at different times during reduction. Cytochrome c oxidase under argon was added to CO-saturated buffer (0.1 M KHPO₄, 0.1 mM EDTA, 1% Tween, pH 7.5, at 25 °C) in a sealed cuvette purged with CO. The final spectrum, labeled 6, representing complete reduction, was recorded several minutes after adding 15 mg of solid dithionite.

Fig. 2. Time courses of CO-driven reduction of cytochrome c oxidase (10 μM) and cytochrome c (15 μM) as observed under 1 atm of CO in 0.1 M KHPO₄, 0.1 mM EDTA, 1% Tween, pH 7.5, 25 °C. Percent reduction is plotted for the hemes of cytochrome aₐ (△), cytochrome a (○), and cytochrome c (□). See “Materials and Methods” and “Results” for further details.

Relative Rates of CO-driven Reduction—We found that reduction under a CO atmosphere can be demonstrated with several proteins and heme compounds. Table 1 describes relative half-times of reduction for a number of oxidized heme proteins or heme compounds incubated under CO under similar experimental conditions. The rates of reduction are not constant during the course of the reduction for cytochrome c oxidase, hemoglobin, or isolated heme. The rates of reduction are also dependent on the temperature, pH, heme, and CO concentrations. In Table 1, for purposes of comparison, we report the time to 50% reduction for samples at the same temperature, pH, and CO concentration, and at similar protein concentrations. No heme reduction was detectable after 200 or more hours for solutions of catalase, cytochrome c (alone), or horseradish peroxidase, proteins that cannot bind CO even when reduced. As mentioned, blocking the CO-binding site of cytochrome c oxidase with cyanide drastically reduces the rate of the reaction. Isolated ferric heme in a high pH aqueous medium with no additional ligands also becomes reduced when incubated under CO. If the heme is liganded with pyridine or nicotinic acid, the reduction does not occur. Either heme or heme proteins transferred into sterile cuvettes through 5-μ Millipore filters became reduced at the same rate as unsterile solutions. After repeated evacuation and CO saturation, oxygen concentrations in the sample solutions were less than 0.3 μM, the detection limit of our oxygen electrode. Since heme concentrations were routinely greater
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Half-times for reduction of heme compounds under CO

<table>
<thead>
<tr>
<th>Heme compounds</th>
<th>Concentration (heme)</th>
<th>Half-time for reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome c oxidase</td>
<td>µM</td>
<td>h</td>
</tr>
<tr>
<td>Cytochrome a₂</td>
<td>6</td>
<td>0.5</td>
</tr>
<tr>
<td>Cytochrome a</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Human hemoglobin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbA</td>
<td>70</td>
<td>1000</td>
</tr>
<tr>
<td>HbA + 200 µM IHP</td>
<td>70</td>
<td>800</td>
</tr>
<tr>
<td>HbA at 100 atm CO</td>
<td>76</td>
<td>40</td>
</tr>
<tr>
<td>Sperm whale myoglobin</td>
<td>70</td>
<td>1200</td>
</tr>
<tr>
<td>Equine cytochrome c</td>
<td>15</td>
<td>No reduction</td>
</tr>
<tr>
<td>Equine catalase</td>
<td>35</td>
<td>No reduction</td>
</tr>
<tr>
<td>Horseradish peroxidase</td>
<td>35</td>
<td>No reduction</td>
</tr>
<tr>
<td>Hemin in 1 M KOH</td>
<td>35</td>
<td>0.7</td>
</tr>
<tr>
<td>Dipyridine heme</td>
<td>35</td>
<td>No reduction</td>
</tr>
<tr>
<td>Dinicotinic acid heme</td>
<td>35</td>
<td>No reduction</td>
</tr>
</tbody>
</table>

than 20 µM, oxygen cannot be a necessary reactant in heme reduction. It is notable that cytochrome c oxidase, with multiple electron accepting sites, shows a much faster rate of reduction than other proteins studied.

**CO-driven Hemoglobin Reduction**—The half-time of reduction of metHbA under CO is dependent on CO concentration. The time necessary for half-reduction is decreased 25-fold by an increase in CO pressure from 1 to 100 atm. The rate is not increased by 100 atm of N₂ and 1 atm of CO, implying that the rate enhancement is not an effect of pressure per se. Table I shows that the rate of reduction is different for hemoglobin and myoglobin. To see if the α and β chains of HbA also differed in their rate of reduction, we examined the spectral dependence of the CO recombination after flash photolysis at varying stages of the reductive process. Because the kinetics of recombination differ for the α and β subunits, the contribution to the total absorbance change from each subunit can be determined at different wavelengths. At 437.5 nm, both chains contribute about equally to the total absorbance change (12). Fig. 3 shows a representative time course for the absorbance change at 437.5 nm, upon CO recombination after complete flash photolysis of partially reduced HbA. Since the fast and slow fractions observed for partially reduced samples of HbA are of approximately equal magnitude at this wavelength, we conclude that the α and β subunits are reduced at similar rates.

Polyansions such as IHP can shift the conformational equilibrium of HbA toward its low affinity (T) conformation. We found that under 1 atm of CO, a small excess of IHP over heme will consistently increase the rate of HbA reduction; decreasing the half-time for reduction by about 20%. Under 100 atm of CO, the effect is noticeable only after 75% of the HbA is reduced, at which point the rate of HbA reduction without IHP declines sharply. With IHP present, the rate of reduction decreases less quickly after the first three out of four heme groups are reduced.

**CO-driven Hemin Reduction**—As documented in Table I, hemin, freshly dissolved in basic solution, becomes reduced and binds CO when incubated under an atmosphere of CO. Fig. 4 shows the spectra, recorded at regular intervals, of hemin in a solution of 0.1 M KOH after it was degassed and saturated with CO. Fig. 5 shows the time courses of the reaction at varying concentrations of KOH. The rates for hemin reduction, as for metHbA reduction, vary during the course of reduction, giving distinctly sigmoidal curves when the per cent reduction is plotted as a function of time. For hemin, the rates are symmetrical around the half-time, and average rates were therefore equal to the reciprocal half-times. As further shown in Fig. 5, for a given concentration of hemin, the rate of reduction is base concentration-dependent. The rate at 25 °C, calculated from the plot of the base dependence of the half-time (inset of Fig. 5), is 1.4 M⁻¹ h⁻¹, where KOH is the molarity of interest. Oxidized hemin dissolved in air equilibrated KOH solution for several hours prior to degassing and saturation with CO does not become reduced as quickly as freshly dissolved hemin. After "aging" for 24 h, the hemin solutions do not become reduced under CO at any measurable rate. It is relevant that equilibration reactions of hemin can lead to μ-oxo-bridged dimers and/or other aggregated states. Moreover, no convincing evidence has been reported for any ligand binding trans to μ-oxo-bridged dimers (13). We presume that the failure of "aged" solutions of hemin in aqueous KOH solutions to become reduced under CO is associated with a loss of CO-binding sites.

**CO₂ Generation**—The CO₂ production predicted by Equation 1 was monitored with samples of oxidized forms of cytochrome c oxidase, hemoglobin, and isolated heme under CO (see "Materials and Methods"). In the case of cytochrome c oxidase, the oxidized enzyme was first carefully degassed and saturated with CO. The initial CO and residual CO₂ and O₂ concentrations were then determined by mass spectrometry, and determined again after 24 h. The results are shown in Table II. Note that while the CO₂ concentration increases over 30-fold, there is no decrease in the O₂ concentration. Control experiments carried out in the absence of protein showed no CO₂ production.

From the gas and solution volumes and the pH, the total...
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Fig. 4. Spectral changes associated with CO-driven reduction of hemin in 0.1 M KOH. The hemin solution was prepared as described under “Materials and Methods,” and spectra were recorded from 500 to 695 nm at 0.1 nm/s (33 min/scan) with 33 min between scans. Temperature was maintained at 25 °C. The initial spectrum is that of oxidized hemin which lacks distinctive peaks between 500 and 600 nm.

Fig. 5. Hemin reduction at different KOH concentrations. Curves from right to left are for 0.05 M KOH, 0.1 M KOH, 0.2 M KOH, 0.5 M KOH, and 1.0 M KOH. Solutions were prepared as described under “Materials and Methods,” and reduction monitored by recording the spectrum at regular intervals and measuring the change at 605 nm. Temperature was maintained at 25 °C. Inset, the reciprocals of the half-times for hemin reduction are plotted against KOH concentration.

Table II

CO₂ formation during CO-driven reduction of cytochrome c oxidase

The gas concentrations above degassed and CO-saturated cytochrome c oxidase solutions were measured by mass spectroscopy (see “Materials and Methods”). The range of values found with repeated measurements of the same sample are indicated.

<table>
<thead>
<tr>
<th></th>
<th>After 30 min</th>
<th>After 21 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO</td>
<td>44.99 mM</td>
<td>44.90 mM</td>
</tr>
<tr>
<td></td>
<td>(99.98%)</td>
<td>(99.78%)</td>
</tr>
<tr>
<td>O₂</td>
<td>4.5 µM ± 2</td>
<td>10.9 µM ± 3</td>
</tr>
<tr>
<td></td>
<td>(0.01%)</td>
<td>(0.024%)</td>
</tr>
<tr>
<td>CO₃</td>
<td>2.6 µM ± 1</td>
<td>87 µM ± 5</td>
</tr>
<tr>
<td></td>
<td>(0.006%)</td>
<td>(0.20%)</td>
</tr>
</tbody>
</table>

amount of CO₂ formed was estimated to be 9 µmol. This is 3 to 4 times the amount predicted by Equation 1, based on knowledge of the number of cytochrome a and a₈ sites reduced. This variance is further examined under “Discussion.”

To test for CO₂ production by oxidized hemoglobin under CO, metHbA with extra oxidant (K₂Fe(CN)₆) was repetitively deoxygenated and saturated with carbon monoxide, and the sealed metHbA sample was left at room temperature under a slight positive CO pressure for several days. The concentration of gaseous CO₂ was monitored at regular intervals (see “Materials and Methods”). By constantly reoxidizing the heme, the CO₂ production was not limited by lack of oxidized heme. The percentage concentration of CO₂ in the gas phase above a CO-saturated solution containing metHbA with K₂Fe(CN)₆ was found to increase steadily, amounting to 1.8% after 24 h, 6.2% after 48 h, and 15% after 72 h. Control solutions under CO containing reduced (ferrous) HbA or K₂Fe(CN)₆ alone showed no CO₂ formation, showing that the production of CO₂ requires oxidized heme and CO.

CO₂ production by isolated heme was assayed by monitoring the formation of ¹³CO₂ from ¹³CO in the presence of heme. The basic heme solutions were degassed and saturated with ¹³CO, and aliquots were removed and acidified during the course of the reaction. The dissolved ¹³CO₂ and ¹³CO concentrations were determined by mass spectroscopy. Upon heme reduction the ¹³CO₂ concentration was found to increase to that predicted by Equation 1. Repeated reoxidations of the heme by O₂ and re-reductions further increased the ¹³CO₂ concentration, in the stoichiometry predicted by Equation 1. These results are shown in Table III.

Acidification by CO-driven Reduction—Equation 1 predicts protons will be released as CO is oxidized to CO₂. A solution of cytochrome c oxidase under 1 atm of CO does indeed become more acidic as the cytochrome c oxidase is reduced. The decrease in pH was, however, greater than that calculated.

Table III

CO₂ formation by CO-driven reduction of hemin

Solution ¹³CO₂ concentrations were determined by mass spectroscopy (see “Materials and Methods”).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Observed [¹³CO₂]</th>
<th>Predicted [¹³CO₂]</th>
</tr>
</thead>
<tbody>
<tr>
<td>No heme</td>
<td>0.9 µM</td>
<td></td>
</tr>
<tr>
<td>800 µM heme prior to reduction</td>
<td>12 µM</td>
<td>0 µM</td>
</tr>
<tr>
<td>800 µM heme after first reduction</td>
<td>400 ± 50 µM</td>
<td>400 ± 50 µM</td>
</tr>
<tr>
<td>800 µM heme after 6th reduction</td>
<td>1900 µM</td>
<td>2200 ± 200 µM</td>
</tr>
</tbody>
</table>

* Predicted ¹³CO₂ concentrations are based on the heme concentrations and the stoichiometry of Equation 1 (i.e., two hemes reduced per CO₂ formed). The range of values in the predicted ¹³CO₂ concentrations reflects the cumulative error in the heme concentration and volume measurements.
on the basis of Equation 1. At pH 7.9, two electrons and two protons should be produced for every CO oxidized. Assuming all the electrons go to cytochrome c oxidase, and one heme and one copper of cytochrome c oxidase are reduced for every heme reduction observed, with equilibration of CO with HCO3, the predicted ratio of protons produced per electron is about 1.75. The observed ratio varied, but, as with CO2 formation, the acidification was always larger than that predicted; sometimes as much as ten times that. This observation is further examined under “Discussion.”

Generation of CO by the Reversal of Equation 1—The back reaction of Equation 1 can be demonstrated with FSA as an added reductant. If FSA, ferrous HbA (either oxy or deoxy), and sodium carbonate are incubated together in buffered solution, within a few hours the HbA becomes a mixture of ferrous CO-HbA and ferric HbA, demonstrating the production of CO and oxidation of heme. As predicted by Equation 1, the reaction does not proceed if carbonate is left out of the medium. Because FSA only very slowly reduces oxidized heme, it is possible to observe simultaneously both the oxidation of heme and the production of CO associated with the back reaction.

A modification of the experimental conditions makes it possible to quantify the rate of CO production. If a reductant like dithionite that can reduce HbA is added, then any HbA oxidized by the reaction will be reduced and the formation of CO will still occur. The sequential spectral changes associated with formation of the CO adduct of HbA as observed under these experimental conditions are shown in Fig. 6. The rate of CO production can be measured by observing the rate of HbA-CO formation. As shown in Fig. 7, the rate is dependent on the concentration of HbA, as predicted for the back reaction of Equation 1.

DISCUSSION

The preceding results have shown that not only cytochrome c oxidase but other oxygen-binding heme proteins, and even isolated heme are capable of being reduced when kept under CO and that CO2 and protons are produced. The CO is not merely stabilizing the reduced form of heme. As demonstrated, CO2 production also occurs when oxidized hemoglobin is incubated under CO in the presence of extra oxidant (K3Fe(CN)6).

The CO-driven reduction of heme and heme proteins as documented in Table I requires a site where oxygen (or CO) can bind, but does not require molecular oxygen. However, since oxygen and CO can both interact with reduced heme, it is not surprising to find that the presence of oxygen introduces complexities. Cytochrome c oxidase, in the presence of oxygen, has previously been shown to be able to bring about the oxidation of CO (5, 14–16). The possible physiological significance of this oxidation is discussed elsewhere (17). The net reaction is

$$2\text{CO} + \text{O}_2 \rightarrow 2\text{CO}_2$$

Young and Caughey (5) demonstrated that 13CO is oxidized to 13CO2 and suggested that the reaction is a concerted process occurring with oxygen and two molecules of CO in the cytochrome a3 site of cytochrome c oxidase. It seems possible in light of the mechanism of CO-driven reduction described in Equation 1 that the process depicted by Equation 2 is instead a two-step reaction, with an initial reduction of cytochrome c oxidase by CO and the release of CO2, followed by the reduction of O2 by the reduced cytochrome a3.

Nicholls and Chanady (3) have shown that cytochrome c oxidase reduced by incubation under CO will form a species with a difference spectrum absorbance maximum at 607 nm when exposed to oxygen. Spectrally, the species formed is identical with the peroxide adduct of cytochrome c oxidase that we have described elsewhere (8). Kinetically, the 607-nm species forms at the dissociation rate of CO from cytochrome a3, 0.07 s⁻¹ (3). Because the rate constant of oxygen association (5 × 10⁷ M⁻¹ s⁻¹) is faster than that for CO association (7 × 10⁶ M⁻¹ s⁻¹) (18), upon the dissociation of CO from cytochrome a3 oxygen rapidly binds to the cytochrome and forms a peroxide adduct. The peroxide adduct dissociates relatively slowly, 0.01 s⁻¹ (19), a rate compatible with the observed lifetime of the 607-nm species observed by Nicholls and Chanady (3) of “tens of seconds.”

The CO-driven reduction of cytochrome c oxidase caused a larger change in pH, and generated more CO2, than that predicted from Equation 1, based on the number of cytochromes and associated coppers observed to be reduced. We suspect that more electrons are generated by CO-driven reduction than are measured by the net cytochrome reduction. The pH titration provides a good estimate of the number of protons released, and mass spectrometry determines the amount of CO2 released, but the changes in cytochrome a and a3 absorbance spectra indicate only how many cytochromes

![Fig. 6. CO production, as evidenced by formation of CO-HbA, brought about by the back reaction of CO-driven reduction. A sealed cuvette contained HbA, 114 μM, with dithionite, 5 mg/ml, and FSA, 5% of saturation, in 0.1 M KH2PO4, 0.1 M Na2CO3, 10 mM EDTA, pH 8.3, at 25 °C. Spectra were recorded at 0.5 nm/s, (400 s/scan) with 400 s between scans. The initial spectrum is that of deoxy-HbA with absorption maximum at 555 nm.](image)

![Fig. 7. Rate of CO formation by the back reaction of CO-driven reduction at different concentrations of HbA. Solutions were as described in the legend to Fig. 6.](image)
were reduced rather than how many electrons were generated.

The ability of cytochrome c oxidase to be reduced rapidly by CO-driven reduction has made it particularly difficult to determine the least reduced form which will bind CO. Attempts to titrate the minimum number of reduced sites capable of CO binding (2, 15-17, 20-24) have been hindered by the continuous generation of electrons by CO-driven reduction, which tends to inflate the estimate of the number of electrons which can be removed from cytochrome c oxidase and keep CO bound.

Explanations previously put forth to account for autoreduction are (1) a shift of electrons from some site on the protein to the oxidized heme (2) an endogenous reductant present as a contaminant in the enzyme preparation, and (3) reduction by contaminating bacteria. None of these hypotheses fit all of our experimental data. Electron donation by other sites on the protein is strongly questionable since a number of Millipore-filtered oxygen heme proteins and even hemin in basic aqueous solution will become reduced in a CO atmosphere and therefore must have the postulated electron source. Of course, one possible electron source common to these compounds is heme itself. Shifting an electron from the aromatic porphyrin conjugate to the iron center would produce a π-cation radical and a reduced iron atom (25). This shift could be expected to give a different absorbance spectrum from Fe(II) in a reduced porphyrin, especially around 700 nm, where π-cation porphyrins have been shown to absorb (25). We tested this hypothesis and found no difference in absorbance between dithionite-reduced and CO-reduced cytochrome c oxidase, even at 700 nm. Additionally, the kinetic properties of cytochrome c oxidase after reduction under CO appear identical to those of the conventionally reduced protein (2). It would be unlikely that this would be the case after electron redistribution between the metal and the porphyrin.

The detailed mechanism underlying the reductive process may be clarified through further studies of the CO-driven reduction of hemin. We found that the rate of reduction has a first order dependence on the concentration of base, suggesting an involvement of hydroxyl ions coordinated to the iron of the heme. This is known to occur at pH 12 to 13 (26). In addition, the stoichiometry of heme reduction and CO production match those predicted by Equation 1.

It is generally recognized that Hb is more stable as the CO derivative than as either the oxy or deoxy form (27). A reason for this that has probably not been previously considered is that CO can bring about the reduction of methemoglobin. Since reduced hemoglobin is more stable than the oxidized form (26), the reductive process would tend to improve the stability of the protein.

A reaction equivalent to that described in Equation 1 may occur when oxidized hemoglobin or cytochrome c oxidase solutions are exposed to nitric oxide. Keilin and Hartree (28) observed that methemoglobin can bind NO and become slowly reduced. Brudvig et al. (29) observed that cytochrome c became reduced under an atmosphere of NO. If CO-driven reduction has an analog in NO-driven reduction, NO₂ would provide a reaction product, potentially observable by mass spectrometry.

In summary, three products are predicted by the forward reaction of Equation 1: reduced heme (and in cytochrome c oxidase, reduced copper), CO₂, and protons. Formation of all three has been demonstrated. The formation of Fe(II), Cu(I), and CO₂ requires CO and oxidized heme, features predicted by the proposed mechanism expressed in Equation 1. The forward reaction does not require oxygen or, apparently, any reductant other than CO. Three products are predicted for the back reaction of Equation 1: Fe(III), CO, and H₂O. The formation of two of these, Fe(III) and CO, has been demonstrated. The formation of the products of the back reaction requires a strong reductant, heme, and CO₂ again in keeping with the proposed mechanism. These considerations lead to the conclusion that autoreduction of the oxidized forms of cytochrome c oxidase, hemoglobin, myoglobin, and free heme is due to the oxidation of CO to CO₂, with a concomitant reduction of the heme.

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REFERENCES