1-1-1984

Carbon Monoxide-Driven Reduction of Ferric Heme and Heme Proteins

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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 α and β chains of human hemoglobin become reduced under CO much 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 ($CO + H_2O \rightarrow CO_2 + 2e^- + 2H^+$). 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 a_3. 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 Chanady (Nicholls, P., and Chanady, 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 a_3 to cytochrome a and cytochrome c.
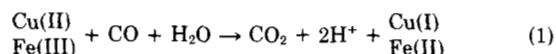
It has been known for years that when cytochrome c oxidase in the ferric, Fe(III), form is stored under an atmosphere of carbon monoxide, the heme iron of cytochrome a_3 will become reduced to Fe(II) and then bind CO (1–5). This reduction, accomplished under a CO atmosphere in the absence of other added reductants, has been referred to as "autoreduction." The reaction as it is usually carried out is very slow; even at

20 °C it takes several hours to reduce cytochrome a_3 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 a_3 and copper a_3 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 CO_2 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 ^{13}CO is oxidized to $^{13}CO_2$, and have suggested the reaction $O_2 + 2CO \rightarrow 2CO_2$. 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:

$$\begin{array}{l} Cu(II) \\ Fe(III) \end{array} + CO + H_2O \rightarrow CO_2 + 2H^+ + \begin{array}{l} Cu(I) \\ Fe(II) \end{array} \quad (1)$$

A CO-driven metal reduction as represented in Equation 1 is thermodynamically favorable ($\Delta G°' = -36$ 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 nonoxidizing 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 CO_2. 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.

MATERIALS AND METHODS

HbA[1] 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 $NaHPO_4$, pH 7.4, with $NaNO_2$ and dialyzing against three changes of 500 volumes of the same buffer. MetHbA with extra oxidant (for CO_2 production determination) was prepared by adding excess $K_3Fe(CN)_6$ 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). 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. Dinicotinic 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 0.10 M $KHPO_4$, 0.1 mM EDTA, pH 7.5. Buffers for assays of the back reaction of Equation 1 contained 0.10 M $NaHCO_3$, 0.10 M $KHPO_4$, and 1 mM EDTA, pH 8.3. Buffers for cytochrome c oxidase also contained 1% Tween 80. Hemin in base was prepared by dissolving hemin HCl in KOH or $(CH_3)_4NOH$ immediately before saturating with CO. When used, dithionite (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 repeatedly evacuating and adding CO (MG Scientific Gases), and were stored under a slight positive CO pressure. All other chemicals were reagent grade or better. Changes in pH during CO-driven reduction were measured with a Leeds & Northrup pH meter on expanded scale with a Radiometer combination electrode. Solutions for these pH determinations consisted of 2.5 ml of 20 μM cytochrome c oxidase in 1 mM HEPES, 200 mM K_2SO_4, 1% Tween, pH 7.9. The solutions were saturated with CO and sealed in the cuvette with a rubber stopper. To allow ion flow from the reference electrode, a 3-cm capillary tube extended through the rubber stopper into the solution. After the reduction was complete, the pH change was quantified by titration with enough 1 mM NaOH solution to return the pH to its initial value. The amount of base added was measured, and the acid equivalent produced during reduction was thus determined. This procedure avoided possible errors associated with the buffering capacity of the buffer and protein solution. Comparative studies of the rates of CO-driven reduction by different proteins were done in sealed cuvettes, thoroughly degassed and saturated with CO in tandem. High pressure experiments with metHbA were performed at 20 °C in a Parr Bomb (Parr Instruments Co.) that was bubbled with argon for 5 min, subjected to 1500 psi of CO, and sampled at regular intervals. Absorption spectra were recorded on an Aminco DW-2A (American Instrument Co.) or a Cary 219 (Varian) spectrophotometer. Near IR spectra were observed with the DW-2A equipped with an IR-sensitive photomultiplier tube.

Measurements of CO_2 formation by incubation under CO of oxidized cytochrome c oxidase (Case I), hemin (Case II), and metHbA (Case III) were carried out. Case I: CO_2 production by cytochrome c oxidase under CO was measured by mass spectrometry. The oxidized enzyme (7.5 ml, 355 μM heme) in 0.1 M $KHPO_4$, 0.1% Tween, 50 μM EDTA, at pH 7.0 was passed through a sterile Millipore Millex GS filter (0.22 μm) into a sterile flask. The flask had a volume of 65 ml and a single opening, a small bore glass, high vacuum valve. The solution was degassed and saturated with oxygen to oxidize any available sites in the solution. After 45 min of gentle swirling, the

solution was repeatedly degassed and saturated with N_2, and finally swirled gently under low pressure for 30 min. The solution was then again saturated with N_2 and degassed for three more cycles, then degassed and saturated with CO for five cycles. After 30 min of swirling, the gas phase above the solution was assayed. The assay was performed by attaching the vessel to the mass spectrometer intake via a ground glass joint above the glass high vacuum valve and, after evacuating the line, bleeding a small aliquot of gas from the vessel into the mass spectrometer. The gas was assayed for CO, O_2, and CO_2. Case II: for measurement of $^{13}CO_2$ production by hemin under CO by mass spectrometry, 0.8 mM solutions of hemin in 0.5 M $(CH_3)_4NOH$ in a sealed flask were repeatedly degassed and saturated with N_2, then degassed and saturated with ^{13}CO. The ^{13}CO-saturated solution was stored in the dark until the heme was fully reduced (about 3 h). To repeat the cycle, oxygen gas was then added in an amount equal to one-fourth of the heme concentration, to give an equimolar oxidative equivalent of the reduced heme. The added oxygen quickly reoxidized the heme (<1 min), which then slowly rereduced (about 3 h). The cycle brought about by O_2 addition was repeated five times, allowing full reduction before each addition. To assay for $^{13}CO_2$ production, aliquots of heme solution (1 ml) were removed by syringe (1) immediately after ^{13}CO saturation (2) after the first reduction, and (3) after reduction of the 5 electron equivalents of O_2. The aliquots were treated with 0.2 ml of 20 N H_2SO_4 to convert the $^{13}CO_3^-$ to $^{13}CO_2$, and 0.8 ml of N_2 was added as a carrier gas. After vigorous shaking, the carrier gas was assayed for ^{13}CO and $^{13}CO_2$ by mass spectroscopy. The mass spectrometer readings were converted to initial solution concentrations by comparing with standards of known ^{13}CO and $Na_2^{12}CO_3$ concentrations which had been treated in the same fashion. To remove O_2 and CO_2 from the ^{12}CO and N_2 used in the mass spectrometry experiments, these gases were stored over solutions of dithionite and sodium hydroxide for at least 2 days prior to each experiment. The ^{13}CO was shaken with a solution of dithionite in sodium hydroxide for several minutes prior to use. The concentrations of O_2 and CO_2 in the N_2 and CO gases were determined by mass spectrometry and found to be less than 1 μM, 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 CO_2 formation by oxidized hemoglobin under CO were carried out with 10 ml of deoxygenated, CO-saturated metHbA/ $K_3Fe(CN)_6$ solution in a sealed 100-ml septum bottle with 200-torr positive CO pressure. CO_2 production in this case was monitored by removing 5-ml samples of the gas above the CO_2-generating solution and measuring the gaseous CO_2 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 a_3 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. Because the reduction under CO takes several hours, the decrease is probably due to some concomitant protein denaturation. Fig. 1 shows representative absorption spectra at different times during the reduction. In the initial stages of the process cytochrome a_3 (whose reduced form has an absorbance maximum at 589 nm) has larger incremental changes in absorbance than cytochrome a (whose reduced form has an absorbance maximum at 605 nm). This is true in spite of the smaller overall increase in absorbance associated with reduction of cytochrome a_3 (2). This indicates that the net cytochrome a_3 reduction exceeds that of cytochrome a reduction as the reaction proceeds. Cytochrome c also becomes reduced if it is present in the solution. Time courses of reduction for cytochromes a_3, a, and c are shown in Fig. 2. Control experiments, carried out under similar experimental conditions, showed that in the absence of cytochrome c oxidase, or with argon instead of CO, cytochrome c showed no reduction. Moreover, in the presence of cyanide and cytochrome c oxidase, cyto-
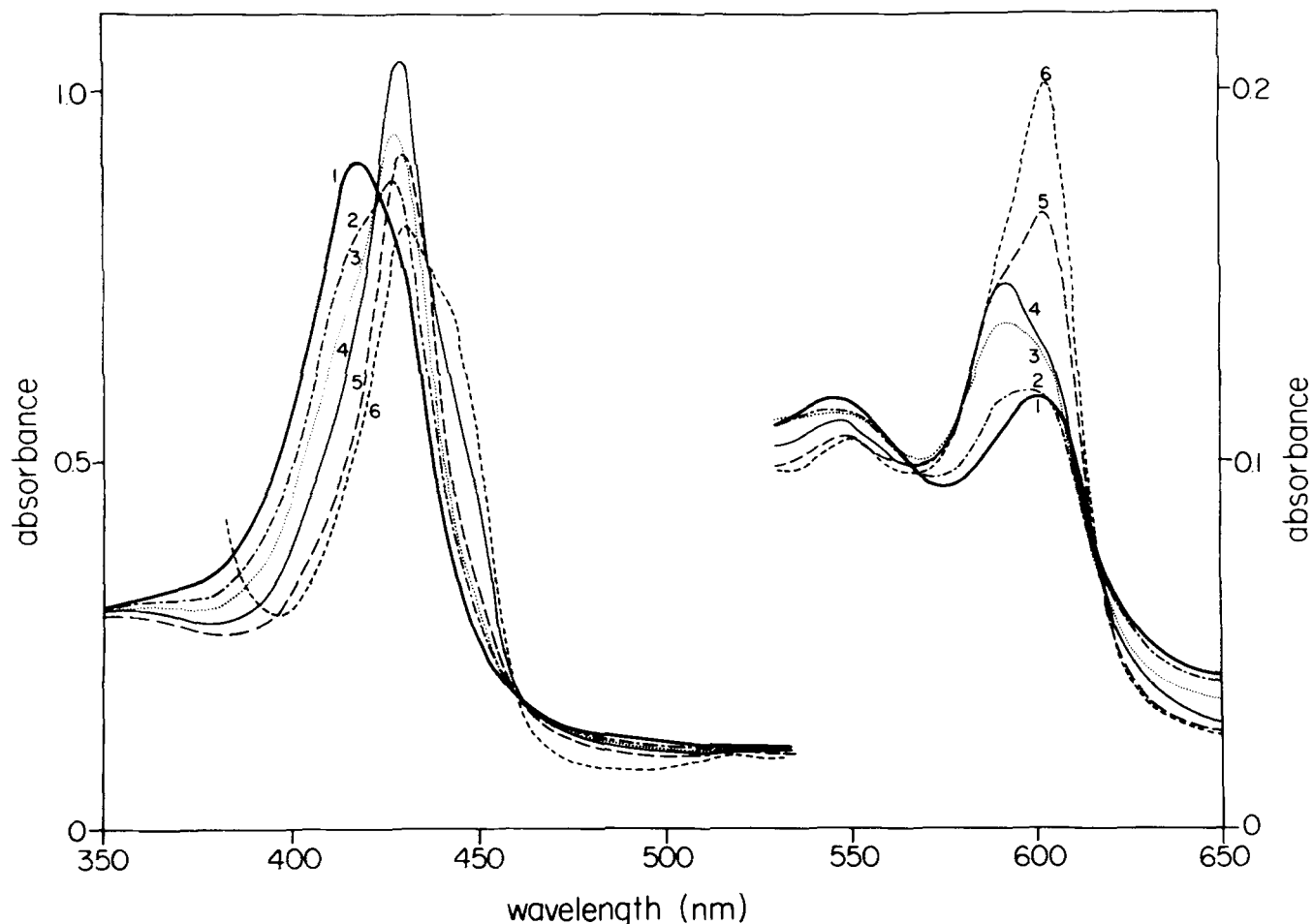
FIG. 1. **Spectra of cytochrome *c* oxidase at different times during reduction.** Cytochrome *c* oxidase under argon was added to CO-saturated buffer (0.1 M KHPO$_4$, 0.1 mM EDTA, 1% Tween, pH 7.5, at 25 °C) in a sealed cuvette purged with CO. The final spectrum, (labled 6) representing complete reduction, was recorded several minutes after adding 15 mg of solid dithionite.
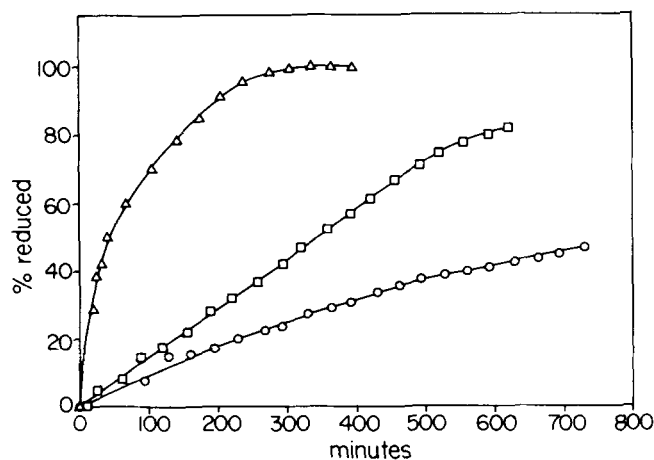

FIG. 2. **Time courses of CO-driven reduction of cytochrome *c* oxidase (10 μM) and cytochrome *c* (15 μM) as observed under 1 atm of CO in 0.1 M KHPO$_4$, 0.1 mM EDTA, 1% Tween, pH 7.5, 25 °C.** Per cent reduction is plotted for the hemes of cytochrome a_3 (△); cytochrome *a* (○), and cytochrome *c* (□). See "Materials and Methods" and "Results" for further details.

chrome *c* is reduced at less than 1% of the rate observed without cyanide.

Relative Rates of CO-driven Reduction—We found that

reduction under a CO atmosphere can be demonstrated with several proteins and heme compounds. Table I 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 I, 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

Carbon Monoxide-driven Reduction of Heme Proteins

TABLE I

Half-times for reduction of heme compounds under CO

Protein and dipyridine- or dinicotinic acid-liganded heme solutions were in 0.1 M KHPO$_4$, pH 7.5, 0.1 mM EDTA at 25 °C. Samples of cytochrome c oxidase also contained 1% Tween 80. Basic heme solutions were in distilled water with KOH. The samples were injected into sealed sterile cuvettes through a 5-μ Millipore filter and saturated with CO. The rate and degree of reduction under 1 atm of CO were determined by scanning the absorbance from 450 to 700 nm at regular intervals and measuring the change in absorbance at the wavelength with greatest change. The rate of hemin reduction was found to be 1.4 M^{-1} h^{-1} at 25 °C, molarity in terms of KOH concentration (see text). Complete sample reduction was achieved by adding 15 mg of dithionite. No reduction indicates no detectable reduction after 200 or more hours under CO. See text for further details.

| Heme compounds	Concentration (heme)	Half-time for reduction
	μM	h
Cytochrome c oxidase		
Cytochrome a_3	6	0.5
Cytochrome a	6	6
Human hemoglobin		
HbA	70	1000
HbA + 200 μM IHP	70	800
HbA at 100 atm CO	70	40
Sperm whale myoglobin	70	1200
Equine cytochrome c	15	No reduction
Equine catalase	35	No reduction
Horseradish peroxidase	35	No reduction
Hemin in 1 M KOH	35	0.7
Dipyridine heme	35	No reduction
Dinicotinic acid heme	35	No reduction
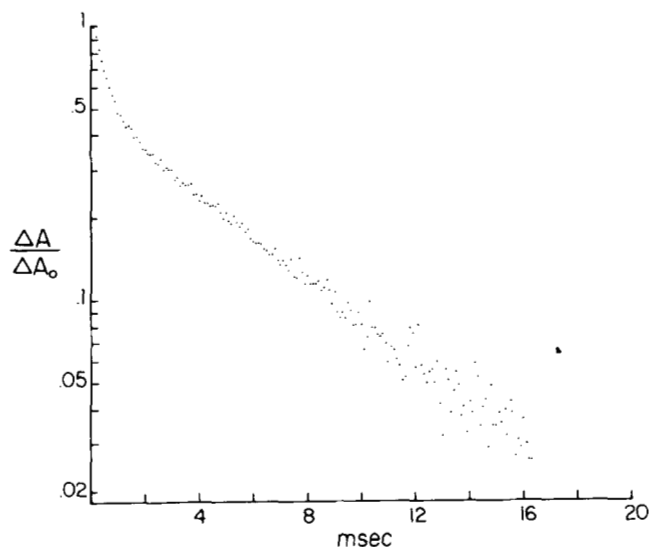
FIG. 3. **Kinetics of CO association with HbA after metHbA was incubated for 14 days in CO-saturated, 0.1 M Tricine, pH 8.0, 0.1 mM EDTA at 20 °C.** The sample was 20% reduced (80% metHbA) at this time. Reassociation after complete flash photolysis was monitored at 437.5 nm.

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 N$_2$ 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.

Polyanions 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 aquation 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.

CO$_2$ Generation—The CO$_2$ 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 CO$_2$ and O$_2$ concentrations were then determined by mass spectroscopy, and determined again after 24 h. The results are shown in Table II. Note that while the CO$_2$ concentration increases over 30-fold, there is no decrease in the O$_2$ concentration. Control experiments carried out in the absence of protein showed no CO$_2$ production.

From the gas and solution volumes and the pH, the total

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.
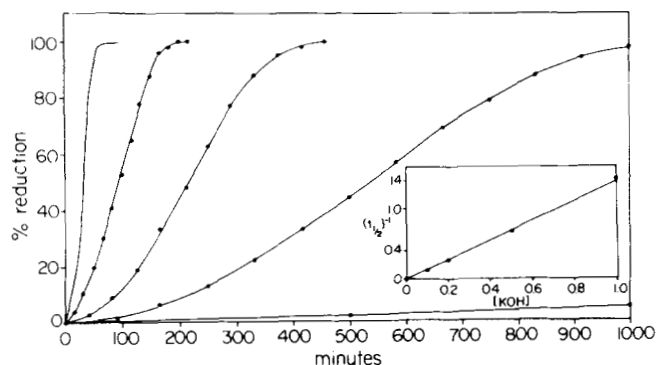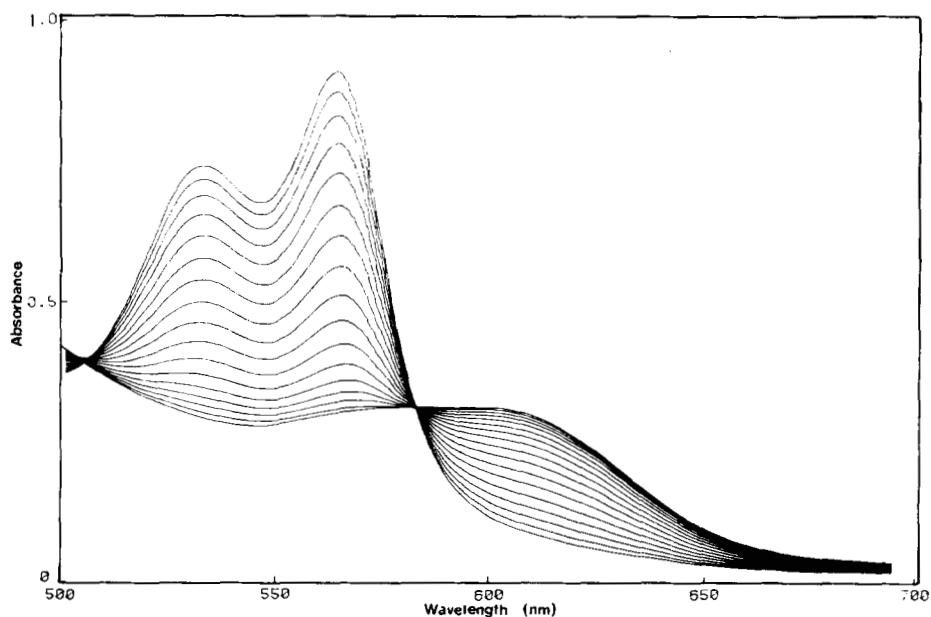

FIG. 5. **Hemin reduction at different KOH concentrations.** Curves from right to left are for 0.05 M KOH, 0.1 M KOH, 0.2 M KOH, 0.5 M KOH, and 1.0 M KOH. Solutions were prepared as described under "Materials and Methods," and reduction monitored by recording the spectrum at regular intervals and measuring 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

CO_2 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.

	After 30 min	After 21 h
CO	44.99 mM	44.90 mM
	(99.984%)	(99.78%)
O_2	4.5 μM $\pm$ 2	10.9 μM $\pm$ 3
	(0.01%)	(0.024%)
CO_2	2.6 μM $\pm$ 1	87 μM $\pm$ 5
	(0.006%)	(0.20%)

amount of CO_2 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 a_3 sites reduced. This variance is further examined under "Discussion."

To test for CO_2 production by oxidized hemoglobin under CO, metHbA with extra oxidant ($K_3Fe(CN)_6$) 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 CO_2 was monitored at regular intervals (see "Materials and Methods"). By constantly reoxidizing the heme, the CO_2 production was not limited by lack of oxidized heme. The percentage concentration of CO_2 in the gas phase above a CO-saturated solution containing metHbA with $K_3Fe(CN)_6$ 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 $K_3Fe(CN)_6$ alone showed no CO_2 formation, showing that the production of CO_2 requires oxidized heme and CO.

CO_2 production by isolated heme was assayed by monitoring the formation of $^{13}CO_2$ from ^{13}CO in the presence of heme. The basic heme solutions were degassed and saturated with ^{13}CO, and aliquots were removed and acidified during the course of the reaction. The dissolved $^{13}CO_2$ and ^{13}CO concentrations were determined by mass spectroscopy. Upon heme reduction the $^{13}CO_2$ concentration was found to increase to that predicted by Equation 1. Repeated reoxidations of the heme by O_2 and re-reductions further increased the $^{13}CO_2$ 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 CO_2. 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

CO_2 formation by CO-driven reduction of hemin

Solution $^{13}CO_2$ concentrations were determined by mass spectroscopy (see "Materials and Methods").

Sample	Observed [$^{13}CO_2$]	Predicted[a] [$^{13}CO_2$]
	μM	
No heme	0.9	
800 μM heme prior to reduction	12	0
800 μM heme after first reduction	400	400 $\pm$ 50
800 μM heme after 6th reduction	1900	2200 $\pm$ 200

[a] Predicted $^{13}CO_2$ concentrations are based on the heme concentrations and the stoichiometry of Equation 1 (*i.e.* two hemes reduced per CO_2 formed). The range of values in the predicted $^{13}CO_2$ 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_2 with HCO_3^-, the predicted ratio of protons produced per electron is about 1.75. The observed ratio varied, but, as with CO_2 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.

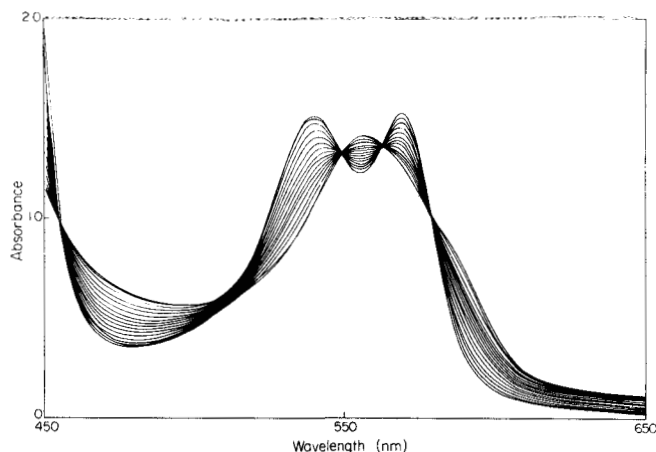A modification of the experimental conditions makes it

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, 3% of saturation, in 0.1 M $KHPO_4$, 0.1 M $NaCO_3$, 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.
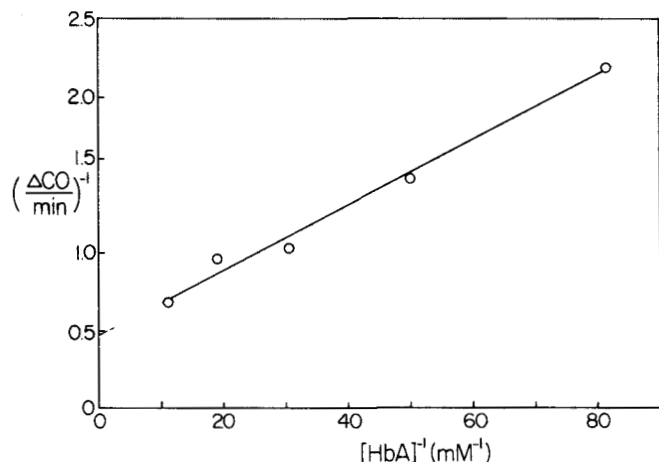

FIG. 7. **Rate of CO formation by the back reaction of CO-driven reduction at different concentrations of HbA.** Solutions were as described in the legend to Fig. 6.

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 CO_2 and protons are produced. The CO is not merely stabilizing the reduced form of heme. As demonstrated, CO_2 production also occurs when oxidized hemoglobin is incubated under CO in the presence of extra oxidant $(K_3Fe(CN)_6)$.

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

$$2CO + O_2 \rightarrow 2CO_2 \qquad (2)$$

Young and Caughey (5) demonstrated that ^{13}CO is oxidized to $^{13}CO_2$ and suggested that the reaction is a concerted process occurring with oxygen and two molecules of CO in the cytochrome a_3 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 CO_2, followed by the reduction of O_2 by the reduced cytochrome a_3.

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 a_3, 0.07 s^{-1} (3). Because the rate constant of oxygen association $(5 \times 10^7$ M^{-1} s$^{-1})$ is faster than that for CO association $(7 \times 10^4$ M^{-1} s$^{-1})$ (18), upon the dissociation of CO from cytochrome a_3 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 CO_2, 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 CO_2 released, but the changes in cytochrome a and a_3 absorbance spectra indicate only how many cytochromes

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 betweeen 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_2 production match those predicted by Equation 1.

It is generally recognized that HbA 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 a_3 became reduced under an atmosphere of NO. If CO-driven reduction has an analog in NO-driven reduction, NO_2 would be an expected reaction product, potentially observable by mass spectroscopy.

In summary, three products are predicted by the forward reaction of Equation 1: reduced heme (and in cytochrome *c* oxidase, reduced copper), CO_2, and protons. Formation of all three has been demonstrated. The formation of Fe(II), Cu(I),

and CO_2 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 H_2O. 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 CO_2, 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 CO_2, with a concomitant reduction of the heme.

REFERENCES

1. Wharton, D., and Gibson, Q. (1968) *J. Biol. Chem.* **243**, 702–706
2. Greenwood, C., Wilson, M., and Brunori, M. (1974) *Biochem. J.* **137**, 205–215
3. Nicholls, P., and Chanady, G. (1981) *Biochim. Biophys. Acta* **634**, 256–265
4. Brittain, T., and Greenwood, C. (1976) *Biochem. J.* **155**, 453–455
5. Young, L., and Caughey, W. (1980) *Fed. Proc.* **39**, 2090 (Abstr. 2562)
6. Drake, H. L., Hu, S., and Wood, H. G. (1980) *J. Biol. Chem.* **255**, 7174–7180
7. Antonini, E., and Brunori, M. (1971) *Front. Biol.* **21**, 27
8. Bickar, D., Bonaventura, J., and Bonaventura, C. (1982) *Biochemistry* **21**, 2661–2666
9. Gibson, Q., Palmer, G., and Wharton, D. (1965) *J. Biol. Chem.* **240**, 915–920
10. Kuboyama, M., Yong, F., and King, T. (1972) *J. Biol. Chem.* **247**, 6375–6383
11. Brunori, M., Colosimo, A., Rainoni, G., Wilson, M., and Antonini, E. (1979) *J. Biol. Chem.* **254**, 10769–10775
12. Olson, J., and Gibson, Q. (1972) *J. Biol. Chem.* **247**, 3662–3670
13. O'Keeffe, D., Barlow, C., Smythe, G., Fuchsman, W., Moss, T., Lilienthal, R., and Caughey, W. (1975) *Bioinorg. Chem.* **5**, 125–147
14. Young, L., Choc, M., and Caughey, W. (1979) in *Biochemical and Clinical Aspects of Oxygen* (Caughey, W., ed) pp. 355–361, Academic Press, New York
15. Wilson, D., and Miyata, Y. (1977) *Biochim. Biophys. Acta* **461**, 218–230
16. Lindsey, J., Owen, C., and Wilson, D. (1975) *Arch. Biochem. Biophys.* **169**, 492–505
17. Babcock, G., Vickery, L., and Palmer, G. (1978) *J. Biol. Chem.* **253**, 2400–2411
18. Gibson, Q., Greenwood, C., Wharton, D., and Palmer, G. (1965) *J. Biol. Chem.* **240**, 888–894
19. Bickar, D. (1982) Ph.D. dissertation, Duke University
20. Wever, R., Van Drooge, J., Muijsers, A., Bakker, E., and van Gelder, B. (1977) *Eur. J. Biochem.* **73**, 149–154
21. Anderson, J., Kuwana, T., and Hartzell, C. (1976) *Biochemistry* **15**, 3847–3855
22. Schroedl, N., and Hartzell, C. (1977) *Biochemistry* **16**, 1327–1333
23. Schroedl, N., and Hartzell, C. (1977) *Biochemistry* **16**, 4961–4965
24. Schroedl, N., and Hartzell, C. (1977) *Biochemistry* **16**, 4966–4971
25. Felton, R. (1978) in *The Porphyrins* (Dolphin, D., ed) Vol 5, pp. 53–125, Academic Press, New York
26. White, W. (1978) in *The Porphyrins* (Dolphin, D., ed) Vol. 5, pp. 303–339, Academic Press, New York
27. Antonini, E., and Brunori, M. (1971) *Front. Biol.* **21**, 2; 309–314
28. Keilin, D., and Hartree, E. (1937) *Nature (Lond.)* **139**, 548
29. Brudvig, B., Stevens, T., and Chan, S. (1980) *Biochemistry* **19**, 5275–5285