Suhadolnik, R. J. (1982) in *ADP-Ribosylation Reactions* (Hayaishi, O., & Ueda, K., Eds.) pp 65–75, Academic, New York.
Suhadolnik, R. J., Baur, R., Lichtenwalner, D. M., Uematsu, T., Roberts, J. H., Sudhakar, S., & Smulson, M. (1976) *J. Biol. Chem. 252*, 4134–4144.
Tanaka, Y., Hashida, T., Yoshihara, H., & Yoshihara, K. (1979) *J. Biol. Chem. 254*, 12433–12438.
Tanigawa, Y., Tsuchiya, M., Imai, Y., & Shimoyama, M. (1984) *J. Biol. Chem. 259*, 2022–2029.
Ueda, K., & Hayaishi, O. (1985) *Annu. Rev. Biochem. 54*, 73–100.
Vaughan, M., & Moss, J. (1981) *Curr. Top. Cell. Regul. 20*, 205–246.
West, R. E., Jr., & Moss, J. (1986) *Biochemistry 25*, 8057–8062.
Yost, D. A., & Moss, J. (1983) *J. Biol. Chem. 258*, 4926–4929.
Zahradka, P., & Ebisuzaki, K. (1984) *Eur. J. Biochem. 142*, 503–509.

Carbon Monoxide Oxygenase Activity of Cytochrome cd_1[†]

Russell Timkovich* and Joseph S. Thrasher
Department of Chemistry, University of Alabama, Tuscaloosa, Alabama 35487
Received February 1, 1988; Revised Manuscript Received April 6, 1988

ABSTRACT: Cytochrome cd_1 from the denitrifying bacterium *Pseudomonas aeruginosa* catalyzes the oxygenation of carbon monoxide by dioxygen. A minimum estimate of the turnover number for this activity is 7 mol of carbon dioxide produced per hour per mole of cytochrome subunit at 30 °C and pH 7. The reaction is 98% inhibited by 2.5 mM cyanide, but catalase has no effect. The reaction accounts for the unusual reduction of ferric cytochrome in the presence of carbon monoxide, but no additional reducing agent. The reaction is independent of the steady-state oxidation level of the cytochrome during turnover. Under anaerobic conditions, ferricyanide plus water may substitute for dioxygen as the source of oxidizing equivalents and atomic oxygen.

Cytochrome cd_1 is a dissimilatory nitrite reductase found in chemoautotrophic, denitrifying bacteria. It contains one heme *c* and one heme d_1 per polypeptide of molecular weight 60000. Two subunits form the active dimer (Kuronen et al., 1975). In addition to nitrite reduction, it can catalyze the reduction of oxygen to water (Timkovich & Robinson, 1979), although only nitrite reduction is its physiological function (Yamanaka, 1964). The heme *c* is known to be low spin with strong evidence of six-coordinate bonds in both ferric and ferrous oxidation states. The heme d_1 is unusual in that in the ferric state it is low spin and presumably six coordinated, but in the ferrous state it is high spin and presumably five-coordinated (Gudat et al., 1973; Orii et al., 1977; Vickery et al., 1978; Walsh et al., 1979; Timkovich & Cork, 1982, 1983; Timkovich et al., 1985; Sutherland et al., 1986).

When anaerobic, ferric cyt cd_1[1] is exposed to carbon monoxide, the iron centers become ferrous within minutes to hours, depending upon conditions. This behavior has been reported by independent groups (Barber et al., 1976; Vickery et al., 1978) and has been called "autoreduction", because no common or obvious chemical reductant has been added. CO is not generally recognized as a reducing agent for ferric iron, and in the previous reports no attempt was made to identify the reductant source. Reduction in the presence of CO is a property also demonstrated by purified mammalian cytochrome oxidase, cyt aa_3 (Tzagoloff & Wharton, 1965; Greenwood et al., 1974). Tzagoloff and Wharton (1965) originally demonstrated that CO_2 was a product of the cyt aa_3 reaction, while recently Young and Caughey (1986) investigate the catalytic rate of oxidation of CO to CO_2. The enzymatic activity was called oxygenase activity, because the available data indicated that the added oxygen atom came from O_2. Here we demonstrated that cyt cd_1 also possesses strong CO oxygenase activity.

Materials and Methods

Pseudomonas aeruginosa (ATCC 19428) was cultured and cyt cd_1 purified as described previously (Timkovich & Cork, 1982). It was precipitated by the addition of solid ammonium sulfate to 90% saturation, and the pellet was recovered after centrifugation. Protein was redissolved in 0.1 M potassium phosphate buffer with 5 mM EDTA, pH 7.0, that had been sterilized by passage through a Nalgene sterilizing filter unit. Individual samples of 0.5 mL were frozen in liquid nitrogen until use. Protein concentration was determined with standard extinction coefficients (Silvestrini et al., 1979) and will be reported as the concentration of subunits. ^{13}CO, 99.4 atom % ^{13}C, was purchased from MSD Isotopes. Other gases of research purity were purchased from Matheson.

Special cells were constructed to measure the infrared spectrum of the gas phase above an aqueous enzyme solution. Two types were employed that differed only in the type of seal against the outside atmosphere. In the first type a tube 2.5 cm long with 1-cm i.d. was fused at a right angle to a tube 10 cm long with 2.3-cm i.d. and 2.5-cm o.d.. The smaller side tube was fit with a standard rubber septum where gases and reagents could be added by a needle. The second type was designed to ensure a higher integrity seal. The small side tube

[†] Financial support was provided in part by Grant GM36264 from the National Institutes of Health.
* Address correspondence to this author.

[1] Abbreviations: cyt, cytochrome; p_{O_2}, partial pressure of oxygen; p_{CO}, partial pressure of carbon monoxide; GC/MS, gas chromatography–mass spectrometry.

0006-2960/88/0427-5383$01.50/0

here was 2.5 cm long with 6-mm o.d. and 3-mm i.d. Once the cell was filled with protein reagent solution, the side tube was sealed by attaching a Teflon two-way valve (Model SC 2-24-1A from Fluorocarbon Co., Process Systems Division, Anaheim, CA) by means of compression-type fittings. The other side of the valve could be attached by standard compression-type fittings to a vacuum line for purging and filling with the required gases.

A shallow trough was formed by glass-blowing techniques along the long axis of the main tube and spanning the central third of its length. Enzyme solution pipeted into the cell collected in this depression and was not in contact with the windows. The gas-exposed surface area of a 0.5-mL aliquot of the solution was 3.2 cm^2. Two windows of polished AgCl (disks, 25-mm diameter, 4 mm thick) were attached to the ends of the long tube by a thermally set halocarbon wax (Series 1500, Halocarbon Products Corp., Hackensack, NJ). Before use in enzyme experiments, the cells were tested for leaks by their ability to sustain a vacuum (10–20 μm with no detectable vaccum loss after removing the pump) and to show constant CO absorption when a trace of CO gas was introduced into the cell and the cell monitored for 48 h. The volume of gas inside the cells was measured by pycnometry to be 44.8 mL. An empirical response factor analogous to an extinction coefficient was determined by introducing known quantities of CO_2 gas into the cell and measuring the observed absorption. The partial pressure of CO_2 in units of Torr (p_{CO_2}) was equal to 8.7 times the observation absorption of CO_2, corrected for the cell background as described below. The absorption versus partial pressure calibration curve was linear only up to 0.9 absorption unit because of the cell background as described below. Mole amounts of CO_2 were computed from partial pressures by assuming an ideal gas law.

To begin an experiment, enzyme solution and other reagents as needed were placed into the main tube depression, and the cell was sealed with either the septum arrangement or the Teflon valve. The cell was attached to a vacuum line along with sources of ^{13}CO, O_2, and N_2 so that all could be evacuated up to and including the gas cylinder regulators. The cell was purged by alternate cycles of vacuum pumping and N_2 flushing, taking care not to cause bumping in the protein solution. Purging was then repeated with several cycles of vacuum and CO flushing, ending with vacuum. The cell was then filled with ^{13}CO to the required partial pressure above the residual water vapor pressure (typically 23 Torr). The cell was then filled with O_2 and/or N_2 as required by the experiment to a final total pressure of 760 Torr. Pressures were determined by a Dynisco digital pressure transducer to ±0.5 Torr. The cell was detached from the vacuum line and transfered to the IR spectrometer for following $^{13}CO_2$ production.

Certain experiments will be described as "nominally anaerobic". This means that the cell was purged with vacuum and N_2 before introduction of CO and that no O_2 was added by design. However, it was possible to detect low levels of O_2 in the cell even under these conditions by GC/MS on a Hewlett-Packard Model 5985 equipped with a packed column (Supelco Carbosieve S-II, 100–120 mesh, 1.1-mm i.d. × 1.5 m long, 80 °C). Sources of this O_2 include gas adsorbed on the inner cell walls, cell leakage over prolonged reaction time, and contamination in the reagent CO gas. GC/MS indicated a background of O_2 in the supply of ^{13}CO of 9 parts per thousand. Owing to the expense of this reagent, it was not feasible to scrub the gas before use. Cell leakage was not a source of O_2 for samples that remained in the IR spectrometer for the duration of the experiment, because the cell compartment was maintained under a constant purge of N_2 during the experiment.

IR spectra were obtained on a Fourier transform IR spectrometer (Bio-Rad DigiLab Model FTS40) equipped with a N_2 purging system for both the cell compartment and the optical system. For this single-beam instrument, an interferrogram of an empty cell was used for the background correction and computation of absorption mode spectra. The AgCl windows are resistant to attack by water vapor, but are not inert. In addition to fogging caused by water vapor, they are susceptible to photochemical degradation. The combined effects led to a rapid loss of the optical uniformity of the windows, although they remained largely transparent to the important IR frequencies. The net effect was that the raw absorption mode spectra contained an additional background contribution due to the differences in precise optical path and/or window scattering between experiments. This was subtracted out by a standard algorithm of the Digilab software that can remove a linear absorption background from raw spectra. The effect of this additional correction can best be appreciated by comparing the scattering of absorption data points in Figure 2 with Figure 6. In Figure 2, the cell was removed from the spectrometer in between data points leading to errors as large as 0.03 absorption unit. In Figure 6, the cell remained in the instrument, and the precision error dropped to 0.002. The window fogging problem also led to the limited linear range on the p_{CO_2} calibration curve. At high absorption values, the intensity of the transmitted light is so low that background window effects become proportionately more severe.

Spectra were recorded over the frequency range of 4500–700 cm^{-1} at a resolution of 0.5 cm^{-1}. As few as 32 averaged interferrograms produced spectra with signal-to-noise in excess of 100. For runs with closely spaced absorption points (for example, Figure 3) 1024 interferrograms were averaged to produce one absorption point approximately every 45 min. The computer system records the precise time each point was taken; however, there may be some irregularity in the point spacing as evident in the figures, because of disk file management overhead time. Quantitative absorption values for $^{13}CO_2$ were taken as the absorption of the R(8) rotational line of the 00°1–00°0 vibrational transition at 2294.57 cm^{-1}. Spectra were recorded at a constant 30 °C, and cells were stored at the same temperature if needed in between data points. Plots of absorbance versus time were fit by linear regression to second-order polynomials and the initial slopes computed from the polynomial coefficients. From the calibration curve data, the cell volume, and the ideal gas law, we derived that the initial rate of CO_2 production in micromoles per hour was 20.6 times the initial slope in units of reciprocal hours.

H_2 determinations were performed on a gas chromatograph equipped with a packed column (32-mm i.d. × 2 m, Supelco Carbosieve S-II, 60–80 mesh) and thermal conductivity detector operating at 200 °C. NMR experiments were performed on a 4.7-T Nicolet spectrometer.

Results

Cyt cd_1 catalyzes the production of $^{13}CO_2$ from ^{13}CO at levels that are readily detected by observing the gas phase above an enzyme solution. Figure 1 demonstrates the observed increase in gaseous $^{13}CO_2$. ^{13}CO was used rather than ^{12}CO in order to prove unequivocally that the substrate is externally added CO and to differentiate the product from a low background level of $^{12}CO_2$ that is an unavoidable instrument artifact in our spectrometer. The reaction is catalytic with

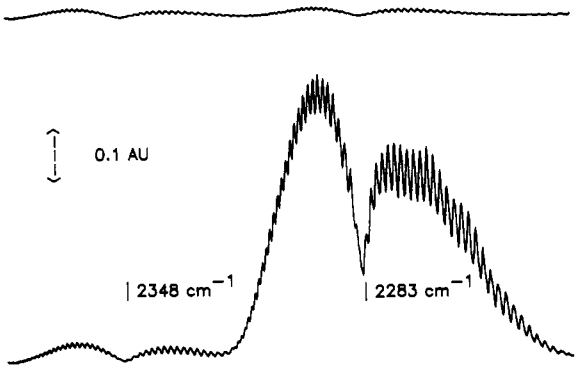

FIGURE 1: Gas-phase IR spectra in the absorption mode showing the production of $^{13}CO_2$ from ^{13}CO catalyzed by cyt cd_1. The top spectrum was recorded 0.6 h after the cell was filled with 730 Torr of ^{13}CO, 9 Torr of O_2, and 0.25 mM cyt cd_1 in the standard assay buffer. The delay from time zero was necessary to remove the cell from the vacuum line, insert it into the spectrometer sample compartment, and purge the spectrometer sample compartment from atmospheric gases which otherwise appear in the final spectrum. The R and P branches of $^{12}CO_2$ appear around 2350 cm^{-1}, and the R and P branches of $^{13}CO_2$ appear around 2280 cm^{-1}. The $^{12}CO_2$ present in an unavoidable background in our spectrometer system. The small $^{13}CO_2$ peak seen near time zero represent a small amount of enzyme-catalyzed reaction plus a contaminating amount of $^{13}CO_2$ present in the commercial ^{13}CO. The bottom spectrum is the same cell after 80 h of $^{13}CO_2$ production.

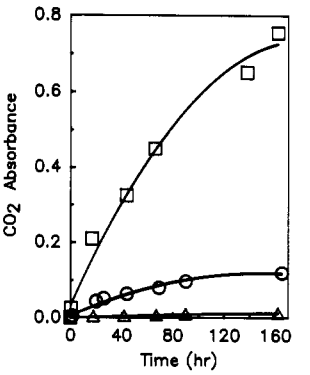

FIGURE 2: Progression curves of absorbance versus time for the production of CO_2 catalyzed by cyt cd_1. The open circles correspond to an experiment in which 0.25 mM cyt cd_1 was under an atmosphere of 487 Torr of CO and 253 Torr of O_2. The open triangles correspond to an experiment in which potassium cyanide was added to a final concentration of 2.5 mM. The open squares correspond to a nominally anaerobic experiment that subsequent analysis showed contained 9 Torr of O_2.

respect to cyt cd_1. The amount of CO_2 produced is several hundred to a thousand times the stoichiometric amount of protein present. This reaction cannot be attributed to the metabolic activity of contaminating microbes for the following reasons: (1) In addition to the sterilization steps described under Materials and Methods, enzyme samples sterile filtered through additional 0.4-μm filters after thawing and just before use showed the same level of activity. (2) At the end of experiments, there was no indication of microbial growth. (3) No experiment showed a lag period before CO_2 production. This could have been indicative of microbial growth at 30 °C and the onset of a CO metabolism. (4) Activity was inhibited by low levels of cyanide and modest levels of reducing agents. Although antimicrobial activity cannot be rigorously ruled out, it is easier to understand these effects as operating on the enzyme level. (5) It is difficult to rationalize the O_2 and ferricyanide experiments to be discussed in terms of whole microorganisms.

Figure 2 shows typical rate profiles for CO_2 production. All experiments were started with the normal storage form, the ferric state, of the enzyme. Within a short time, the enzyme had changed to the ferrous state. This was monitored quantitatively by sampling enzyme through the septum port and transferring to an anaerobic cuvette and by companion experiments performed entirely in anaerobic cuvettes. The spectrum of the ferrous CO complex has been reported [see Figure 3 of Barber et al. (1976)]. Qualitatively, the transformation is easy to follow visually, because of the striking color change in the enzyme from a Kelley green to a brown. Under conditions of very low residual O_2, a high surface area, and shaking to promote gas diffusion, the reduction could be accomplished in less than 20 min.

The kinetic curves showed decreasing rates with long times. We attribute this to a natural degradation of the labile enzyme during the long time period at room temperature. Loss of activity upon storage has been noted by others (Gudat et al., 1973). Enzyme at the end of a CO_2 assay was recovered and converted back to the ferric state by shaking in air. When reassayed for its ability to catalyze electron transport between *Pseudomonas* cyt *c*-551 and O_2, only 20–30% of the activity of fresh enzyme was observed. The CO_2 production curves do not begin at zero absorbance at zero time, because of a small amount of contaminant $^{13}CO_2$ in the commercial ^{13}CO.

As shown in the bottom curve of Figure 2, the presence of 2.5 mM KCN inhibited CO_2 production by greater than 98%. The middle curve corresponded to a gas mixture where $p_{CO_2}:p_{O_2}$ was 2:1, the same as the stoichiometry for reaction 2 below and presumably suitable for facile reaction. The top curve was surprising because the sample was nominally anaerobic (subsequent analysis indicated 9 Torr of residual O_2), yet clearly the CO_2 production was faster. The fact that the rate increased with decrease in p_{O_2} forced us to consider if some alternative source of oxidizing equivalents was being utilized.

Five reactions, numbered 1–5, may be reasonably postulated to provide oxidizing equivalents given the experimental conditions:

$$CO + H_2O + 2Fe^{3+} \rightarrow CO_2 + 2H^+ + 2Fe^{2+} \quad (1)$$
$$\Delta G^\circ = -34.5 \text{ kcal}$$
$$CO + {}^1/_2O_2 \rightarrow CO_2 \quad (2)$$
$$\Delta G^\circ = -61.5 \text{ kcal}$$
$$2CO \rightarrow CO_2 + C \quad (3)$$
$$\Delta G^\circ = -28.7 \text{ kcal}$$
$$CO + H_2O \rightarrow CO_2 + H_2 \quad (4)$$
$$\Delta G^\circ = -4.8 \text{ kcal}$$
$$2CO + H_2O \rightarrow CO_2 + H_2CO(aq) \quad (5)$$
$$\Delta G^\circ = -3.0 \text{ kcal}$$

Standard free energy changes indicate that none may be rejected on simple thermodynamic grounds. Reaction 1 alone cannot account for the total production of CO_2, because the only iron present was the heme iron of the cytochrome and there is not enough. Reaction 3 may be ruled out because in some experiments it would have led to the accumulation of an estimated 200 μg of elemental carbon, and such a solid was never seen. Reaction 4 was ruled out when gas chromatography failed to detect any H_2. The technique described under Materials and Methods was calibrated versus standard H_2 samples and found to be useful for detecting H_2 down to 40 parts per million. In enzyme-related experiments assayed for H_2, the amount present on the basis of the stoichiometry of reaction 4 would have been 5 parts per thousand. Reaction 5 was ruled out when ^{13}C NMR failed to detect any aqueous

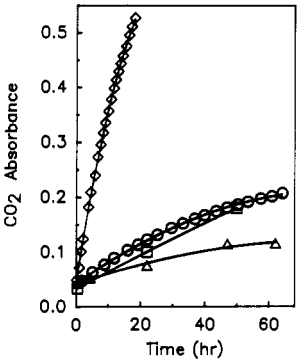

FIGURE 3: Effects of O_2 partial pressure on CO_2 production rate. The open circles correspond to a nominally anaerobic experiment in which 0.25 mM cyt was under an atmosphere of 740 Torr of CO. This was the benchmark experiment for this series, and all others used the same cyt preparation and concentration and the same pressure of CO. The open triangles correspond to an experiment in which sodium sulfite as an O_2 scavenger had been added to a final concentration of 0.1 M. Sulfite is also a reducing agent for the cyt. The open squares correspond to an experiment in which sodium ascorbate had been added to a final concentration of 0.1 M. Ascorbate is also a reducing agent for the protein but is less efficient at O_2 removal. The open diamonds correspond to an experiment in which 13 Torr of O_2 was added.

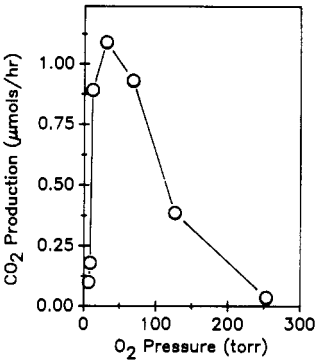

FIGURE 4: Rates of CO_2 production versus partial pressure of O_2 in the assay.

formaldehyde. It should be noted that formaldehyde is extremely soluble in water, where it immediately hydrates to "formalin", $CH_2(OH)_2$. Under the conditions of the present experiments, all the hypothetical product would remain in the aqueous phase. Control experiments indicated that the NMR technique could readily detect the anticipated amount of [^{13}C]formalin.

Young and Caughey (1986) concluded that O_2 was a direct reagent in their reactions with cyt aa_3 on the basis of isotope incorporation from $^{18}O_2$. However, the level of actual isotope incorporation into $C^{18}O^{16}O$ was very small, because of the rapid hydration/dehydration of the acid anhydride CO_2:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \quad (6)$$

On the basis of the reported turnover number of Young and Caughey and that for cyt cd_1, vide infra, the rate of CO_2 production by cyt cd_1 is too slow to compete with solvent exchange. Furthermore, in the case of cyt cd_1, since it can reduce O_2 to water, it would be difficult to distinguish incorporation from reaction 2 from incorporation due to a combination of reactions 6 and 7:

$$4H^+ + {}^{18}O_2 + (c^{2+}d_1{}^{2+})_2 \rightarrow 2H_2{}^{18}O + (c^{3+}d_1{}^{3+})_2 \quad (7)$$

Here the notation $(c^{2+}d_1{}^{2+})_2$ is used to denote the native dimer with each monomer possessing one heme c with ferrous iron, one heme d_1 with ferrous iron, and so on.

Further evidence that O_2 was a direct reactant in the cyt cd_1 catalyzed oxidation of CO came from experiments such as those illustrated in Figure 3. Sulfite is a good scavenger for O_2, and when added to the enzyme solution, it inhibited the rate of CO_2 production by 50%. Sodium ascorbate is not a good scavenger for O_2, although at modest concentrations it does react to some extent. It exhibitied only a slight inhibition of 10%. On the other hand, when a slight aliquot of O_2 was added to a cell that was nominally anaerobic, it stimulated production more than 10-fold.

Figure 4 presents the results on systematically varying p_{O_2} and the effect this had on rates. Rates are greatest when p_{O_2} is between 25 and 65 Torr. At 40 Torr of O_2 the concentration of dissolved O_2 is 66 μM. This is comparable to the reported K_m of the cytochrome for oxygen reduction to water when soluble cytochrome c-551 is used as electron donor (Timkovich et al., 1982). The decrease at lower p_{O_2} is presumably a kinetic

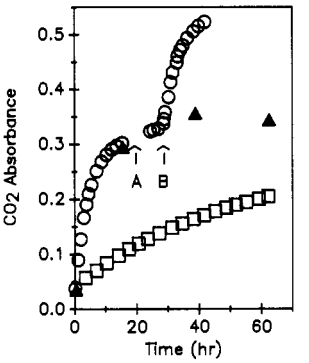

FIGURE 5: Ferricyanide stimulation of CO_2 production by cyt cd_1. The open squares correspond to the control experiment in which 0.25 mM cyt cd_1 was incubated under 740 Torr of CO under nominally anaerobic conditions. The open circles correspond to the same conditions but with potassium ferricyanide added to a final concentration of 20 mM. At point A, O_2 was added to a final partial pressure of 12 Torr. At point B, more ferricyanide was added from a concentrated stock solution to a final concentration of 0.3 M. The triangles correspond to an independent experiment with 20 mM ferricyanide that was allowed to run for a longer time.

effect due to lower concentration of a direct reactant. The effect at high p_{O_2} is not straightforward to explain. It was observed qualitatively by eye and confirmed by visible spectrophotometry that at high p_{O_2} the steady state of the enzyme was a mixture of oxidized and reduced forms. This could suggest that the turnover rate depends upon the steady-state concentration of the ferrous form of the enzyme. The experiments illustrated in Figure 5 indicated that this was not true and that other sources of oxidizing equivalents may replace O_2 under appropriate conditions. The addition of 20 mM ferricyanide to a low-p_{O_2} experiment greatly stimulated the rate of CO_2 production. Control experiments indicated that ferricyanide at this concentration with CO but without enzyme did not produce CO_2 after 24 h. This level of ferricyanide is sufficient to keep the steady state of cyt cd_1 mainly in the oxidized form. At this concentration, spectrophotometery could not detect any reduced form, but a small percentage would not have been detectable. The rate decrease that occurred corresponded to the point where most of the ferricyanide had been converted to ferrocyanide. The addition of 12 Torr of O_2 at point A led to no further reaction for the next 4.5 h. It may be that ferrocyanide or the ferri/ferro ratio inhibited CO_2 production. Addition of a fresh aliquot of ferricyanide from a concentrated stock solution to a final concentration of 0.3 M at point B stimulated the reaction again.

Examination of the initial portion of kinetic profiles such as Figure 6 revealed a minor initial burst phase. In all experiments, this comprised a small portion of the total absorption change and was difficult to quantitate with accuracy

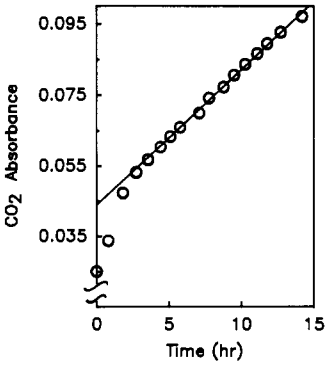

FIGURE 6: Expansion of a progression curve for CO_2 production catalyzed by cyt cd_1 to illustrate the minor initial burst. The straight line corresponds to the regression analysis best initial slope for data points in the entire run. The first four data points that do not fall on this line represent the initial burst. Note that the ordinate has been displaced from the origin.

or precision. The amount of CO_2 produced during this initial burst was approximately the same, at least the same order of magnitude, as the amount of cyt cd_1 in the assay mixture.

Discussion

The turnover number for CO_2 production by cyt cd_1 under conditions of optimum p_{O_2} is 7.0 μmol of CO_2 produced per micromole of cyt cd_1 per hour. The turnover number for the ferricyanide-stimulated reaction was the same. This turnover number is fivefold slower than that reported by Young and Caughey (1986) for the CO oxygenase activity of cyt aa_3. In spite of this apparent difference, the rates of absorbance change reported here per unit time are much larger for lower protein concentrations. Compare Figures 1, 2, 3, and 5 here, with Figures 1–5 of Young and Caughey (1986).

The rate of CO_2 production by cyt cd_1 reported here is only a lower estimate of the actual catalytic rate. The observed rates are limited by mass transfer of CO_2 and possibly CO between the gas and aqueous phases. Under the conditions of the experiments, the majority of the CO_2 formed will be in the gas phase, not in the aqueous phase as soluble CO_2 or bicarbonate. Yet for practical reasons, agitation could not be used to promote mass transfer. On the basis of qualitative comparisons between experiments in 1-cm visible spectrophotometric cuvettes and the IR cells, the rate of reduction of ferricytochrome by CO is highly dependent on surface area and residual O_2. We can concluded that cyt cd_1 has CO oxygenase activity, but an improved quantitative measure of the rate would be justified.

Reactions 8–12 summarize a proposed sequence to account for the observed activity. They are not meant to imply a detailed mechanistic or kinetic scheme but are intended to balance the final reaction observed with minimal reference to the number of prosthetic groups in the enzyme. It is offered in the spirt of showing that the experimentally observed activity can be accounted for by a series of reasonable chemical steps, none of which are in contradiction with known properties of the cytochrome. The sequence is formed by consideration of only one subunit of the enzyme, as a simpler starting point. For further brevity, the bicarbonate form of CO_2 is ignored, with the understanding that aqueous CO_2 exists under the equilibrium of reaction 6. The start of the sequence could be the "autoreduction" of ferric cyt cd_1 by CO. As indicated by reaction 8, this initiation step may not directly require O_2, but CO is still the reducing agent and the ferric hemes are the ultimate oxidizing agent. The "autoreduction" previously reported in spectrophotometric curvettes (Barber et al., 1976) may represent one turnover of the cyt, but subsequent production of CO_2 requires additional oxidizing equivalents. The initial burst seen at the reaction start in the IR experiments as in Figure 6 could be due to this step, but this requires further confirmation. In reaction 9, the ferrous heme d_1 produced would combine rapidly with the high concentration of CO. There is ample precedent for this reaction (Parr et al., 1975; Wharton & Gibson, 1976). Attack by O_2 in reaction 10 could produce CO_2 and regenerate ferrous cytochrome which then repeats reaction 9 and so on. No mechanistic information is intended in reaction 10. Certainly, some interesting steps must be occurring, because *dioxygen* is the molecular form present. The present data simply allow no further speculation. Stopped-flow kinetic studies and equilibrium binding studies have indicated that CO and O_2 bind with approximately equal association constants of 10^4 M^{-1} (Parr et al., 1975; Greenwood et al., 1978). An independent study indicated a higher association constant of 4×10^5 M^{-1} for CO (Wharton & Gibson, 1976). Because CO is present in such an excess, the sequence above postulates first CO binding to the enzyme and then O_2 attack. The inhibition by high pO_2 could be due to reactions 11 and 12. These are highly plausible because of the known reduction of O_2 to water by cyt cd_1. Oxygen is known to be capable of displacing CO bound to the ferrous cytochrome and oxidizing it to the ferric form (Parr et al., 1975), although in that study no attempt was made to find or identify other gaseous or aqueous products. The symbol {O} just represents one formal equivalent of an oxygen atom and is not meant to imply any actual intermediate or transient. The reduction of a single dioxygen molecule may require concerted action by two subunits. Young and Caughey (1986) reported that catalase inhibited the CO oxygenase activity of cyt aa_3. In the case of cyt cd_1, catalase up to 0.14 mM had no effect on the reaction.

The ferricyanide experiments illustrated in Figure 5 indicated that cyt cd_1 can catalyze CO_2 production even when the steady state lies predominantly toward the ferric form. It may be that ferricyanide can act as a source of oxidizing equivalents per

initiation

$$c^{3+}d_1{}^{3+} + CO + H_2O \rightarrow c^{2+}d_1{}^{2+} + CO_2 + 2H^+ \quad (8)$$

turnover

$$c^{2+}d_1{}^{2+} + CO \rightarrow c^{2+}d_1{}^{2+}\text{–}CO \quad (9)$$

$$c^{2+}d_1{}^{2+}\text{–}CO + {}^1/_2O_2 \rightarrow c^{2+}d_1{}^{2+} + CO_2 \quad (10)$$

(then 9, 10, 9, 10, ...)

inhibition

$$c^{2+}d_1{}^{2+} + O_2 \rightarrow c^{2+}d_1{}^{2+}\text{–}O_2 \quad (11)$$

$$c^{2+}d_1{}^{2+}\text{–}O_2 + 2H^+ \rightarrow c^{3+}d_1{}^{3+} + H_2O + \{O\} \quad (12a)$$

$$c^{2+}d_1{}^{2+} + \{O\} + 2H^+ \rightarrow c^{3+}d_1{}^{3+} + H_2O \quad (12b)$$

$$c^{2+}d_1{}^{2+}\text{–}CO + H_2O + 2Fe(CN)_6{}^{3+} \rightarrow c^{2+}d_1{}^{2+} + CO_2 + 2H^+ + 2Fe(CN)_6{}^{2+} \quad (13)$$

In a stopped-flow kinetic study (Barber et al., 1978), ferricyanide was shown to oxidize ferrous heme c and heme d_1 in the cytochrome rapidly, but the ferrous CO complex of heme d_1 in the cytochrome was reoxidized in a slower reaction (slow on the stopped-flow time scale of milliseconds). In this case the experimental techniques would not allow one to analyze for the concurrent production of CO_2 or bicarbonate. It is possible that reaction 13 was occurring.

The physiological function of cyt cd_1 is nitrite reduction under anaerobic conditions. Hence, there is no O_2 present,

and there is no reason to assume any appreciable CO is present. The CO oxygenase activity of cyt cd_1 therefore represents interesting, but nonphysiological, chemistry. However, like the acetylene reduction catalyzed by nitrogenase, the reactivity provides a certain insight into the active site capabilities of the enzyme. Although nitrite reduction is the accepted function of cyt cd_1, controversy has arisen recently about the physiological product of that reduction. Yamanaka and coworkers (Yamanaka et al., 1961; Yamanaka & Okunuki, 1963) demonstrated years ago that in vitro the product was nitric oxide, NO. Wharton and Weintraub (1980) confirmed by GC/MS that NO was the *major* product of in vitro reduction but found that significant levels of N_2O were also formed. Averill and Tiedje (Averill & Tiedje, 1982; Aerssens et al., 1986) addressed a central question of denitrification: on the route from NO_2^- to N_2, where is the first N–N bond formed? They postulated that in vivo it might be formed at the active site of cyt cd_1 by attack of a second NO_2^- upon some enzyme-bound form of NO:

$$(cd_1)\text{–}NO^x + NO_2^- \rightarrow (cd_1)\text{–}N_2O_3^{x-1} \rightarrow \rightarrow \rightarrow cd_1 + N_2O + H_2O \quad (14)$$

We have oversimplified their detailed proposal by not specifying the oxidation state of the prosthetic groups or of the enzyme-bound NO (formal oxidation state "x"). The multiple arrows represent subsequent electron-transfer steps and hydrolyses that ultimately produced N_2O from some species $N_2O_3^{x-1}$. The key feature for the present discussion is that an iron-bound ligand is activated toward formation of a new covalent bond by an incoming reactant. This is the same broad class of reaction as oxygen addition to enzyme-bound CO, the CO oxygenase activity of cyt cd_1.

Acknowledgments

We thank Shayne Green and Darryl Roberts for assistance in the gas chromatography and GC/MS experiments.

References

Aerssens, E., Tiedje, J. M., & Averill, B. A. (1986) *J. Biol. Chem. 261*, 9652–9656.

Averill, B. A., & Tiedje, J. M. (1982) *FEBS Lett. 138*, 8–12.

Barber, D., Parr, S. R., & Greenwood, C. (1976) *Biochem. J. 157*, 431–438.

Barber, D., Parr, S. R., & Greenwood, C. (1978) *Biochem. J. 173*, 681–690.

Greenwood, C., Wilson, M. T., & Brunori, M. (1974) *Biochem. J. 137*, 205–215.

Greenwood, C., Barber, D., Parr, S. R., Antonini, E., Brunor, M., & Colosimo, A. (1978) *Biochem. J. 173*, 11–17.

Gudat, J. C., Singh, J., & Wharton, D. C. (1973) *Biochim. Biophys. Acta 292*, 376–390.

Kuronen, T., Saraste, M., & Ellfolk, N. (1975) *Biochim. Biophys. Acta 393*, 48–54.

Orii, Y., Shimada, H., Nozawa, T., & Hatano, M. (1977) *Biochem. Biophys. Res. Commun. 76*, 983–988.

Parr, S. R., Wilson, M. T., & Greenwood, C. (1975) *Biochem. J. 151*, 51–59.

Silvestrini, M. C., Colosimo, A., Brunori, M., Walsh, T. A., Barber, D., & Greenwood, C. (1979) *Biochem. J. 183*, 701–709.

Sutherland, J., Greenwood, C., Peterson, J., & Thomson, A. J. (1986) *Biochem. J. 233*, 893–898.

Timkovich, R., & Robinson, M. K. (1979) *Biochem. Biophys. Res. Commun. 88*, 649–655.

Timkovich, R., & Cork, M. S. (1982) *Biochemistry 21*, 5119–5123.

Timkovich, R., & Cork, M. S. (1983) *Biochim. Biophys. Acta 742*, 162–168.

Timkovich, R., Dhesi, R., Martinkus, K. J., Robinson, M. K., & Rea, T. M. (1982) *Arch. Biochem. Biophys. 215*, 47–58.

Timkovich, R., Cork, M. S., & Taylor, P. V. (1985) *Arch. Biochem. Biophys. 240*, 689–697.

Tzagoloff, A., & Wharton, D. C. (1965) *J. Biol. Chem. 240*, 2628–2633.

Vickery, L. E., Palmer, G., & Wharton, D. C. (1978) *Biochem. Biophys. Res. Commun. 80*, 458–463.

Walsh, T. A., Johnson, M. K., Greenwood, C., Barber, D., Springall, J. P., Thomson, A. J. (1979) *Biochem. J. 177*, 29–39.

Wharton, D. C., & Gibson, Q. H. (1976) *Biochim. Biophys. Acta 430*, 445–453.

Wharton, D. C., & Weintraub, S. T. (1980) *Biochem. Biophys. Res. Commun. 97*, 236–242.

Yamanaka, T. (1964) *Nature (London) 204*, 253–255.

Yamanaka, T., & Okunuki, K. (1963) *Biochim. Biophys. Acta 67*, 379–393.

Yamanaka, T., Ota, A., & Okunuki, K. (1961) *Biochim. Biophys. Acta 53*, 294–308.

Young, L. J., & Caughey, W. S. (1986) *Biochemistry 25*, 152–161.