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Reaction of the Molybdenum- and Copper-Containing Carbon Monoxide Dehydrogenase from *Oligotropha carboxydovorans* with Quinones[†]

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ABSTRACT: Carbon monoxide dehydrogenase (CODH) from *Oligotropha carboxydovorans* catalyzes the oxidation of carbon monoxide to carbon dioxide, providing the organism both a carbon source and energy for growth. In the oxidative half of the catalytic cycle, electrons gained from CO are ultimately passed to the electron transport chain of the Gram-negative organism, but the proximal acceptor of reducing equivalents from the enzyme has not been established. Here we investigate the reaction of the reduced enzyme with various quinones and find them to be catalytically competent. Benzoquinone has a k_{ox} of 125.1 s^{-1} and a K_d of 48 μM. Ubiquinone-1 has a k_{ox}/K_d value of 2.88×10^5 M^{-1} s^{-1}. 1,4-Naphthoquinone has a k_{ox} of 38 s^{-1} and a K_d of 140 μM. 1,2-Naphthoquinone-4-sulfonic acid has a k_{ox}/K_d of 1.31×10^5 M^{-1} s^{-1}. An extensive effort to identify a cytochrome that could be reduced by CO/CODH was unsuccessful. Steady-state studies with benzoquinone indicate that the rate-limiting step is in the reductive half of the reaction (that is, the reaction of oxidized enzyme with CO). On the basis of the inhibition of CODH by diphenyliodonium chloride, we conclude that quinone substrates interact with CODH at the enzyme's flavin site. Our results strongly suggest that CODH donates reducing equivalents directly to the quinone pool without using a cytochrome as an intermediary.

Molybdenum-containing enzymes are very broadly distributed in biology, and members of the xanthine oxidoreductase (XOR)[1] family comprise a large and important group of these enzymes. Family members generally catalyze the oxidative hydroxylation of aromatic heterocycles and aldehydes, and the reducing equivalents generated in this process pass from the molybdenum center, where catalysis takes place, through two [2Fe-2S] clusters and (in most cases) on to an FAD where the electrons are passed on to an oxidizing substrate such as NAD^+ or O_2 (*1*).

Carbon monoxide dehydrogenase (CODH) from aerobic, chemolithotrophic organisms such as *Oligotropha carboxydovorans* and *Hydrogenophaga pseudoflava* is clearly a member of the xanthine oxidase family on the basis of its overall amino acid sequence and three-dimensional structure (*2*−*8*). The functional enzyme is an $(\alpha\beta\gamma)_2$ hexamer that consists of a small 17.8 kDa subunit (CoxS) containing two [2Fe-2S] clusters, a medium 30.2 kDa subunit (CoxM) containing an FAD cofactor, and a large 88.7 kDa subunit (CoxL) that possesses the molybdenum center. CODH is encoded by megaplasmid pHCG3 in the CoxMSL cluster (*9*, *10*). The overall protein fold notwithstanding, two aspects make CODH unique in the XOR family. (1) The reaction itself is not strictly speaking a hydroxylation and does not involve the cleavage of a C−H bond. (2) The active site consists of a unique binuclear Mo−Cu center rather than a mononuclear molybdenum center such as that seen in all other family members. As shown in Figure 1, the active site is an $LMo^{VI}O_2$-(μS)-Cu^{I}-SCys cluster, where L represents the pyranopterin cofactor found in all molybdenum (and tungsten)-containing enzymes other than nitrogenase (*4*, *5*, *11*). The Mo/Cu-containing CODH from *O. carboxydovorans* and *H. pseudoflava* is structurally and mechanistically distinct from the Fe/Ni-containing CODH of the acetogen *Moorella thermoacetica* or the methanogen *Methanosarcina barkerii* (*12*).

In the reaction conducted by CODH, CO is oxidized to CO_2, yielding two reducing equivalents according to the following stoichiometry:

$$CO + H_2O \rightarrow CO_2 + 2H^+ + 2e^- \quad (E_0' = -558 \text{ mV at pH } 7)$$

The reducing equivalents thus obtained by the enzyme are passed to the electron transport chain to provide energy for cell growth. A portion of the CO_2 generated as a product of the reaction is fixed nonphotosynthetically by the pentose phosphate cycle (*13*, *14*) with an overall stoichiometry of

$$2.19CO + O_2 \rightarrow 1.83CO_2 + 0.36 \text{ cell carbon} + \text{energy}$$

The overall reaction is profoundly environmentally important, because aerobic bacteria such as *O. carboxydovorans* clear an estimated 2×10^8 metric tons of CO from the atmosphere annually (*15*).

It has been suggested that reducing equivalents are removed from CODH by either cyt b_{561} or the quinine pool, although no definitive evidence has been provided in support of either (*13*, *16*). In this study, we have investigated the kinetics involved with the oxidative half-reaction of the enzyme with various quinones and found that they serve very effectively as oxidizing substrates for CODH, reacting with the reduced enzyme with rate constants

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[1]Abbreviations: CODH, carbon monoxide dehydrogenase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; XOR, xanthine oxidoreductase.

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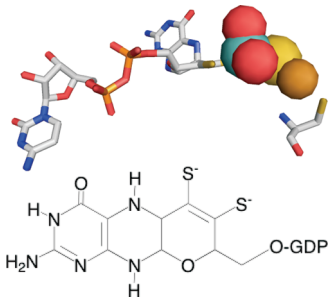

FIGURE 1: Active site of CODH. The top panel shows the active site of the enzyme as rendered using Protein Data Bank entry 1N5W. Atom colors are CPK, with the molybdenum atom colored teal and the copper colored copper. Cys 388, which coordinates the copper, is shown at the far right. The bottom panel shows the structure of the pyranopterin cofactor of the binuclear center, which is present as the dinucleotide of guanine.

sufficiently large to support catalysis. By contrast, cytochrome b_{561} is found not to readily accept reducing equivalents from reduced CODH, and we are unable to identify any other cytochrome from *O. carboxidovorans* capable of doing so. We conclude that quinones are the likely physiological oxidant of CODH.

MATERIALS AND METHODS

Materials. Carbon monoxide gas was obtained from Air, Inc., at a purity of 99.5%. 1,4-Benzoquinone, 1,2-naphthoquinone-4-sufonic acid, 1,4-naphthoquinone, and ubiquinone-1 were purchased from Sigma-Aldrich. Isotopically enriched D_2O was obtained from Cambridge Isotope Lab, Inc. All other chemicals and reagents were obtained at the highest quality and purity commercially available and used without additional purification.

Bacterial Cultivation and Enzyme Purification. *O. carboxydovorans* (ATCC 49405) cells were grown at 30 °C and pH 7 in a 20 L fermentor (BioFlo 415, New Brunswick) containing Minimal Medium and CO as the carbon source (introduced as a mixture of 50% CO and 50% air). Cells were harvested in late log phase (OD_{436} > 5), washed in 50 mM HEPES (pH 7.2), and stored at −80 °C until needed (*17*). CODH was purified according to the procedure described by Zhang et al. (*18*), using a combination of Q-Sepharose and Sephacryl S-300 FPLC.

Cytochrome b_{561} was purified from *O. carboxydovorans* grown as described above; 100 g of thawed cells was resuspended in 50 mM HEPES (pH 7.2) containing 1 mM EDTA, 5 mg of DNase, and 0.2 mM PMSF and broken open with a French press (FA-078A, Thermo Electron Co.). Cell debris were separated by ultracentrifugation at 100000*g* for 2 h. Cell membranes were solubilized in 50 mM HEPES (pH 7.2) containing 1 mM EDTA, 0.2 mM PMSF, and 10% (v/v) Triton X-100. The nonsolubilized membranes were separated by ultracentrifugation at 100000*g* for 2 h. The soluble fraction was loaded onto a CM anion exchange column (11 cm × 1.5 cm) using an AKTA FPLC apparatus (GE Healthcare) at 4 °C; the column was pre-equilibrated with 50 mM HEPES (pH 7.2) containing 0.1 mM EDTA and 0.2% Triton X-100. Elution was conducted over 10 column volumes with a linear gradient from 0 to 500 mM NaCl. Fractions containing cytochrome b_{561} were pooled and concentrated using an Amicon concentrator equipped with a 10 kDa cutoff filter. The identity of cytochrome b_{561} was verified by UV−vis spectroscopy with an absorbance peak at 415 nm in the oxidized cytochrome and peaks at 425, 530, and 561 nm in the reduced cytochrome (Figure 1 of the Supporting Information). Approximately 20−50 μg of cytochrome b_{561} was obtained from 100 g of cells. Fractions containing other cytochromes were also saved and examined for activity with CODH.

Protein Determination and Activity Assay. The quantity of enzyme was determined by the absorbance at 450 nm (ε_{450} = 70 mM^{-1} cm^{-1}) and 550 nm and purity assessed by the ratios of absorbance at 280, 420, 450, and 550 nm; the purified enzyme had the following absorbance ratios: $A_{280}/A_{450} \sim 5.5$, $A_{450}/A_{550} \sim 2.9$, and $A_{450}/A_{420} > 1$ (*18*). Routine activity was determined by the CO-dependent reduction of methylene blue (ε_{615} = 37.11 mM^{-1} cm^{-1}) at 30 °C (*3*, *18*). A second assay utilized 1,4-benzoquinone as an oxidizing substrate, following reduction of, e.g., 50 μM 1,4-benzoquinone at 246 nm using an HP 8452A UV−visible spectrophotometer. The contents of a serum-stoppered cuvette (Starna Cells, Inc.) containing 50 μM 1,4-benzoquinone in 2 mL of 50 μM HEPES (pH 7.2) were bubbled with 100% CO for 10−15 min in the dark, after which 10−20 μL of a 4 μM anaerobic stock CODH solution was added by Hamilton syringe. The specific activity in units per milligram was determined using an extinction change for 1,4-benzoquinone reduction, obtained from an extinction coefficient ε_{247} of 20.6 mM^{-1} cm^{-1} for the oxidized 1,4-benzoquinone (*20*), a reduced coefficient ε_{247} of 0.42 mM^{-1} cm^{-1} determined with the equation $\varepsilon_{red} = (Abs_{red})(\varepsilon_{ox})/(Abs_{ox})$ to give a $\Delta\varepsilon_{247}$ of 20.2 mM^{-1} cm^{-1} for 1,4-benzoquinone, with 1 unit of activity being defined as 1 μmol of CO oxidized/min at 30 °C. The enzyme used in this work exhibited a specific activity of approximately 8.

All enzyme preparations were reconstituted with sulfur and copper using a modification of the procedure used by Resch et al. (*19*). Approximately 100 μM CODH in 1.0 mL of 50 mM Tris-HCl (pH 8.2) was made anaerobic by alternately evacuating and flushing with O_2-scrubbed Ar gas over the course of 1 h. Appropriate volumes of stock solutions of 10 mM methyl viologen and 100 mM Na_2S were added to give final concentrations of 0.1 and 2.0 mM, respectively, followed by the addition of a sufficient volume of an ~0.1 M dithionite stock solution to sustain the blue color of the reduced viologen. This was incubated at 20 °C for 12−18 h under an atmosphere of argon gas. The enzyme was then passed through a G-25 chromatography column equilibrated with anaerobic 50 mM Tris-HCl (pH 8.2) to remove excess Na_2S, dithionite, and methyl viologen. A stock solution of 10 mM Cu(I)-thiourea was prepared by dissolving Cu(I)Cl, thiourea, and sodium ascorbate in a 1:3:1 (w/w) ratio in anaerobic water, and the concentration of the enzyme solution was then changed to 0.2 mM in Cu(I) using this solution and the mixture incubated for 5−10 h at 20 °C. A final G-25 column, again equilibrated with anaerobic 50 mM Tris-HCl (pH 8.2), was used to remove excess Cu(I). The enzyme was assayed for activity as described above, and the degree of functionality was independently determined by comparing the extent of enzyme bleaching, as observed at 450 nm, by CO (which reduces only the fully functional enzyme) with that seen using dithionite (which reduces both functional and nonfunctional enzyme) (*5*). Our enzyme was approximately 40% active, comparable to the levels seen previously (*19*). Unless otherwise stated, enzyme concentrations are given as functional enzyme, corrected for the fraction of nonfunctional enzyme present.

Steady-State Kinetics. Steady-state kinetic studies monitoring the reduction of ubiquinone-1 and 1,4-benzoquinone were performed by bubbling anaerobic solutions of each in 50 mM

HEPES (pH 7.2) with CO for 15 min to give 1 mM CO [previous studies having shown that CO concentrations of > 100 μM were saturating in steady-state assays (*18*)]. The concentration ranges used were 11−97 μM for ubiquinone-1 and 8.4−191 μM for 1,4-benzoquinone. The reaction was initiated by the addition of 10 μL of 2 μM CODH, and the reaction was monitored by the spectral change at 275 or 246 nm over 300 s at 25 °C. Activities were obtained from the initial slopes of each assay, calculated using an ε_{278} of 14.7 mM^{-1} cm^{-1} for ubiquinone-1 (*22*) and a $\Delta\varepsilon_{247}$ of 20.2 mM^{-1} cm^{-1} for 1,4-benzoquinone.

A kinetic isotope study was performed under the same conditions described above using 50 mM HEPES in D_2O (pD 7.6) with the addition of varying concentrations of ubiquinone-1 in D_2O. CODH in D_2O was prepared with an anaerobic buffer exchange G-25 column in 50 mM HEPES in D_2O (pD 7.6).

Rapid Reaction Kinetics. The oxidative half-reaction of CODH was monitored by stopped-flow spectroscopy (using an Applied Photophysics, Inc., SX-18MV instrument). Standard reaction conditions were used: 50 mM HEPES, pH 7.2, and 25 °C. Enzyme at a concentration of ~10 μM before mixing was placed in a glass tonometer equipped with a side arm cuvette and made anaerobic by repeated evacuation and flushing with O_2-scrubbed Ar over the course of 1 h. The anaerobic enzyme was then titrated with an anaerobic solution of ~0.1 M sodium dithionite in 50 mM HEPES (pH 7.2), monitoring enzyme reduction spectrophotometrically. The reduced enzyme was then mounted on the stopped-flow apparatus and mixed with varying concentrations of anaerobic, oxidized quinone substrate in 50 mM HEPES (pH 7.2), the reaction being monitored by the absorbance increase observed at 450 or 550 nm. Kinetic transients thus obtained were fit using the ProData Viewer package to obtain the rate constants, which were averaged and plotted as a function substrate concentration. When saturating kinetic behavior for k_{obs} as a function of quinone concentration was observed, values for k_{ox}, the limiting rate constant at high concentrations of S, and dissociation constant, K_d, were obtained from hyperbolic fits to these plots using SigmaPlot (Systat Software, Inc.). When linear behavior was observed, the k_{ox}/K_d ratio was determined directly from the slope.

Titrations of CODH with Quinones. Titrations were performed using 4 or 8 μM CODH in 50 mM HEPES (pH 7.2) at 20 °C. Titrations of oxidized enzyme with oxidized quinone were conducted aerobically, following the spectral change observed in the vicinity of 450 nm. Titration of the oxidized enzyme with reduced quinone (prepared by directly bubbling Ar in a cuvette for 15 min to make the solution anaerobic and titrated to reduction with 0.1 M sodium dithionite using a Hamilton syringe, following quinone reduction at 247 nm) was conducted anaerobically, by alternately evacuating and flushing the enzyme solution in an anaerobic cuvette with O_2-scrubbed Ar gas for 1 h. Aliquots of reduced 1,4-benzoquinone were then added with a Hamilton syringe, and the spectral change in the visible region was monitored spectrophotometrically. For 1,4-benzoquinone, plots of absorbance change versus quinone concentration were used to obtain K_d, which was determined by fitting the data to the hyperbolic equation

$$A_{obs} = (\Delta A_{max} x)/(K_d + x)$$

Inhibition of CODH by Diphenyliodonium Chloride. Inactivation of the FAD cofactor of CODH was accomplished by covalent modification of the flavin with diphenyliodonium chloride using a modification of the procedure of Chakraborty and Massey (*21*). CODH (10 μM) in 50 mM HEPES (pH 7.2) was flushed with Ar for 1 h and reduced with an ~2-fold excess of sodium dithionite. Diphenyliodonium chloride was added to a final concentration of 1 mM and incubated at 20 °C for 2 h. Excess diphenyliodonium chloride was removed with a G-25 column and the enzyme assayed for activity. Spectral changes and the specific activity of the enzyme with ubiquinone-1 and methylene blue were used to assess the degree of inhibition, as described above.

Electron Paramagnetic Resonance Spectroscopy. EPR spectra were recorded using a Brüker Instruments ER 300 spectrometer equipped with an ER 035M gaussmeter and HP 5352B microwave frequency counter. The temperature was controlled at 150 K using a Brüker ER 4111VT liquid N_2 cryostat. Samples were prepared by reducing 50 μM anaerobic CODH in 50 mM HEPES (pH 7.2) with a stock solution of 0.1 M dithionite, followed by addition of 0.5 equiv of 1,4-benzoquinone or ubiquinone-1. Samples were immediately frozen in liquid N_2. Controls of 50 μM CODH in 50 mM HEPES (pH 7.2), either fully oxidized or fully reduced by titration with dithionite, were also prepared. A final sample of 50 μM CODH was prepared by first reducing the enzyme with 0.1 M dithionite and reoxidizing with 12 equiv of quinone prior to freezing.

RESULTS

Reactivity of CODH toward Cytochrome b_{561}. Cytochrome b_{561} was isolated from *O. carboxydovorans* as described in Materials and Methods and examined for reactivity toward CODH in both steady-state and rapid-reaction experiments. In the assays performed, cytochrome b_{561} in 50 mM HEPES (pH 7.2) containing 0.2% Triton X-100 or *N*,*N*-dimethyldodecylamine *N*-oxide was made anaerobic by repeated evacuation and flushing with O_2-scrubbed Ar followed by CO bubbling. The activity assay was conducted over 10 min observing the total spectral change with an emphasis near 415 and 561 nm, but no reduction of cytochrome b_{561} was seen in these experiments. Other cytochrome-containing fractions isolated from soluble and membrane cell fractions included multiple cytochrome *c* forms, and cytochrome *a*, and these were also examined for activity toward CODH; in no case was cytochrome reduction observed (Figure 2 of the Supporting Information). Only when 1,4-benzoquinone (at a concentration of 5 μM) was added to the assay with cytochrome *c* was reduction observed, as reflected in the absorbance increase at 550 nm and a shift of the Soret band from 410 to 415 nm. These results suggest that cytochrome reduction was mediated by the quinone, which served as the proximal oxidant for the enzyme.

Steady-State Kinetic Studies. On the basis of the results described above suggesting that 1,4-benzoquinone is an oxidizing substrate of CODH, a steady-state study was performed using 1,4-benzoquinone as the oxidizing substrate. The assay was performed under the standard conditions of 25 °C in 50 mM HEPES (pH 7.2), with solutions of 8.4−191 μM 1,4-benzoquinone placed in a serum-stoppered cuvette and bubbled first with argon and then CO to give a concentration of 400 μM. The reaction was followed by the spectral change at 246 nm associated with reduction of the quinone. Significant catalytic rates were observed. A plot of the observed catalytic velocity versus 1,4-benzoquinone concentration was hyperbolic, and a fit to the data yielded a k_{cat} of 104 s^{-1}, a K_m of 16.4 μM, and a k_{cat}/K_m of

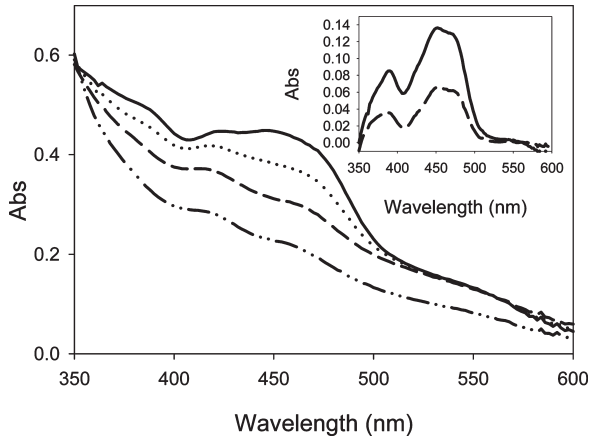

FIGURE 2: Diphenyliodonium chloride inhibition of CODH. (A) Spectral change over the course of inhibition of CODH in 50 mM HEPES (pH 7.2) with diphenyliodonium chloride. Spectra of oxidized CODH (—), dithionite-reduced CODH (−·−·−), air-oxidized CODH following diphenyliodonium chloride inhibition (− − −), and diphenyliodonium chloride-inhibited CODH after 18 h (···). The inset shows the oxidized-minus-inhibited difference spectrum following diphenyliodonium chloride inhibition (—) and after an 18 h incubation (− − −).

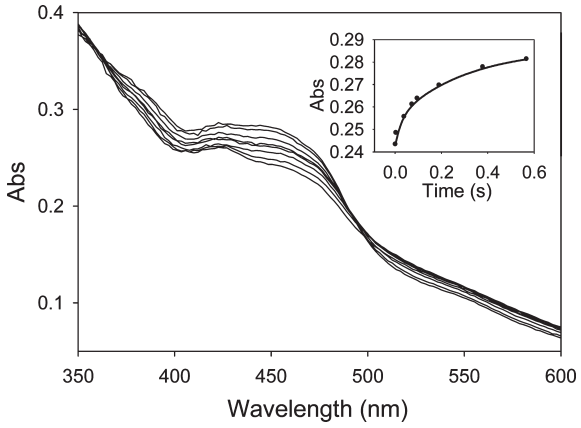

FIGURE 3: Oxidation of reduced CODH by 25 μM 1,4-benzoquinone. Oxidation of dithionite-reduced 5 μM CODH by 1,4-benzoquinone in 50 μM HEPES (pH 7.2) at 25 °C over 0.6 s. The traces shown are those recorded at 0.004, 0.007, 0.015, 0.027, 0.042, 0.076, 0.098, 0.0193, 0.0382, and 0.0571 s, increasing in absorbance from 0.004 to 0.0571 s at 450 nm. The inset shows the time course of the absorbance change at 450 nm over 0.6 s (absorbance measurements shown for 0.004, 0.007, 0.015, 0.027, 0.042, 0.076, 0.098, 0.0193, 0.0382, and 0.0571 s) during the course of CODH oxidation. Fits to the data yield observed rate constants of 37 s^{-1} for the first phase and 3.3 s^{-1} for the second phase (with an R^2 value for the fit of 0.993).

6.37×10^6 M^{-1} s^{-1} (Figure 3 of the Supporting Information). This is essentially identical to the previously observed k_{cat} of 93.3 s^{-1} using methylene blue as the oxidizing substrate, consistent with the previous conclusion that the reductive half-reaction was principally rate-limiting (*18*). Ubiquinone-1 was also examined as a substrate, but in this case, it proved to be impossible to obtain sufficiently high concentrations of substrate to yield saturating kinetics given the limited water solubility of ubiquinone-1. A plot of the observed catalytic velocity versus ubiquinone-1 concentration thus yielded a straight line, and from the slope, a value for the k_{cat}/K_m ratio of 5.81×10^4 M^{-1} s^{-1} was obtained. When the steady-state kinetics with ubiquinone-1 were repeated in D_2O, a solvent isotope effect of 1.4 was obtained from the H(k_{cat}/K_m)/D(k_{cat}/K_m) ratio (Figure 4 of the Supporting Information).

With other enzymes of the XOR family, reducing equivalents enter at the molybdenum center in the reductive half of the catalytic sequence and leave via the FAD (after intramolecular electron transfer involving the iron−sulfur clusters) (*1*). To establish that quinone substrates interacted with CODH at its FAD site, the enzyme was reacted with diphenyliodonium chloride to covalently modify the FAD and render the cofactor redox-inert (*21*). This treatment of the enzyme lowered the steady-state rates of ubiquinone-1 reduction by 83% and methylene blue by 80%; modification of the flavin was confirmed by comparing the absorption spectrum of the reoxidized, modified enzyme with that of the original (Figure 2). The unmodified minus modified difference spectra revealed features at 370 and 450 nm consistent with flavin modification seen in xanthine oxidase (Figure 2, inset) (*21*). With the modified enzyme, reoxidation of the iron−sulfur clusters is observed after exposure to air, while the additional absorbance increase expected for reoxidation of the FAD is not observed. The reaction was conducted over longer time periods to achieve a greater degree of inhibition, but no further inhibition was observed. The incomplete inactivation was most likely due to incomplete covalent modification of the flavin because of the lower solvent accessibility of the FAD of CODH relative to xanthine oxidase. We observed, for example, that CODH inhibited with diphenyliodonium chloride slowly regained activity over time, suggesting that the inhibited flavin−diphenyliodonium chloride enzyme complex was slowly returned back to the functional enzyme. Consistent with this, the absorbance of oxidized FAD is slowly recovered over the next 18 h, reflecting the slow breakdown of the flavin−diphenyliodonium chloride complex to yield the oxidized cofactor (*21*).

Oxidative Half-Reaction of CODH with Quinone Substrates. The rapid reaction kinetics of the reoxidation of reduced CODH by several quinones was next examined by stopped-flow spectrophotometry at 25 °C, following the reoxidation of enzyme at 450 nm. The substrates used were 1,4-benzoquinone, 1,4-naphthoquinone, 1,2-naphthoquinone-4-sulfonic acid, and ubiquinone-1. Figure 3 shows a typical time course for the reaction with 25 μM 1,4-benzoquinone with 5 μM CODH after mixing in the stopped-flow apparatus. At low substrate concentrations, the reaction appeared to be biphasic with apparent rate constants of 37 s^{-1} for the first phase (with an associated absorbance change of 0.01 OD, 24% of the total absorbance change) and 3.33 s^{-1} for the second (with an amplitude of 0.03 OD, 73% of the total absorbance change). (A third phase was sometimes observed at low substrate concentrations, with a rate constant of 0.5 s^{-1} and an absorbance change of ~0.001 OD, 2% of the total absorbance change, that was attributed to oxygen contamination. This phase was neglected on the basis of the very small absorbance change associated with it.) The overall kinetic complexity of the reaction is a reflection of the fact that 3 equiv of quinone must react with the fully reduced enzyme in turn for full reoxidation. We attribute the first phase to the reaction of fully reduced (i.e., six-electron-reduced) CODH with a first equivalent of 1,4-benzoquinone to yield the four-electron-reduced enzyme and 1,4-benzoquinone-H_2, and the second rate constant to the subsequent reaction of the four-electron-reduced enzyme with an additional 2 equiv of 1,4-benzoquinone in turn (which are kinetically unresolved). At higher substrate concentrations, the amplitude of the faster process increases at the expense of the slower, the latter eventually disappearing by 500 μM. Plots of the observed rate

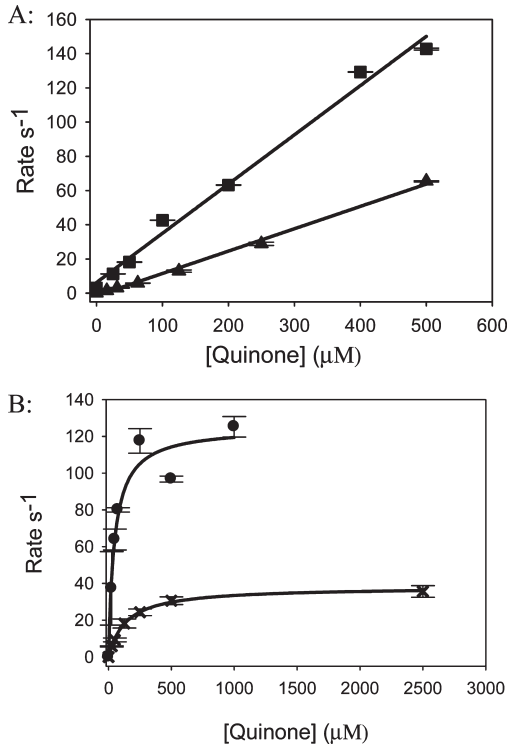

FIGURE 4: Substrate concentration dependence of reoxidation of dithionite-reduced CODH by quinones. (A) Plots of k_{fast} vs quinone concentration for the reaction of 5 μM enzyme with ubiquinone-1 (■) and 1,2-naphthoquinone-4-sulfonic acid (▲) in 50 mM HEPES (pH 7.2) at 25 °C. The solid line is a fit with a linear equation using SigmaPlot (Systat Software, Inc.). The k_{ox}/K_d ratio (corresponding to the second-order reaction of the reduced enzyme with substrate in the low-Q concentration regime) was determined by the slopes of the fitted lines, which for ubiquinone-1 and 1,2-naphthoquinone-4-sulfonic acid were 2.88×10^5 M^{-1} s^{-1} ($R^2 = 0.990$) and 1.31×10^5 M^{-1} s^{-1} ($R^2 = 0.995$), respectively. (B) Plots of k_{fast} vs quinone concentration for the reaction of 5 μM enzyme with 1,4-benzoquinone (●) and 1,4-naphthoquinone (×) in 50 mM HEPES (pH 7.2) at 25 °C. The solid line is a fit using the hyperbolic equation $k_{obs} = k_{ox}[Q]/(K_d + [Q])$ using SigmaPlot, where k_{ox} is the limiting rate constant for reoxidation at high Q concentrations and K_d is the dissociation constant for substrate binding. Kinetic parameters thus determined for 1,4-benzoquinone were as follows: $k_{ox} = 125.1$ s^{-1}, $K_d = 46.7$ μM, and $k_{ox}/K_d = 2.60 \times 10^6$ M^{-1} s^{-1} (line fit $R^2 = 0.955$). Kinetic parameters thus determined for 1,4-naphthoquinone were as follows: $k_{ox} = 38.1$ s^{-1}, $K_d = 140$ μM, and $k_{ox}/K_d = 2.72 \times 10^5$ M^{-1} s^{-1} (line fit $R^2 = 0.999$).

constant versus 1,4-benzoquinone concentration were hyperbolic for both phases of the reaction; the first phase yielded a k_{ox} of 125.1 s^{-1}, a K_d of 48 μM, and a k_{ox}/K_d (i.e., the slope of the plot of k_{obs} vs 1,4-benzoquinone concentration) of 2.60×10^6 M^{-1} s^{-1}, while the second phase yielded a k_{ox} of 32.7 s^{-1} and a K_d of 154 μM. Again, the second phase was only clearly resolved below 500 μM 1,4-benzoquinone when 5 μM enzyme was used.

The reaction of reduced CODH with 1,2-naphthoquinone-4-sulfonic acid was also biphasic, with amplitudes of 0.02 OD (28% of the total observed spectral change) seen for the first phase and 0.04 OD (57% of the total spectral change) for the second when 5 μM CODH was used. A third very slow phase (0.01 OD, 14% of the total spectral change) was observed that was attributed to photosensitivity of the quinone, as confirmed by monitoring the spectral change of 1,2-naphthoquinone-4-sulfonic acid alone in the observation cell of the stopped-flow apparatus. This phase became negligible at substrate concentrations of >125 μM because of the short time scale of the reaction. Because of the high extinction coefficient of 1,2-naphthoquinone-4-sulfonic acid, concentrations of <1 mM had to be used, and it was not possible to reach saturating concentrations; only the k_{ox}/K_d ratio could be obtained from the linear plot of k_{obs} versus 1,2-naphthoquinone-4-sulfonic acid concentration, with values of 1.31×10^5 and 3.90×10^4 M^{-1} s^{-1} for the faster and slower phases, respectively.

1,4-Naphthoquinone also exhibited biphasic behavior, with amplitudes of 0.04 OD (40% of the total observed spectral change) and 0.06 OD (60% of the observed spectral change) for the first and second phases, respectively, with 5 μM enzyme. 1,4-Naphthoquinone (which is structurally similar to menaquinone) yielded kinetics somewhat slower than those seen with 1,4-benzoquinone, with a k_{ox} of 38.1 s^{-1}, a K_d of 140 μM, and a k_{ox}/K_d of 2.72×10^5 M^{-1} s^{-1} for the faster phase of the reaction; the slower phase gave a k_{ox} of 6.0 s^{-1}, a K_d of 213 μM, and a k_{ox}/K_d of 2.82×10^4 M^{-1} s^{-1}.

Ubiquinone-1 also exhibited biphasic behavior with amplitudes of 0.02 OD (14% of the total observed spectral change) and 0.12 OD (86% of the total spectral change) for the first and second phases, respectively, with 5 μM CODH. Because of the limited solubility of ubiquinone-1, it was again not possible to approach saturating conditions, and only a k_{ox}/K_d ratio could be determined from the slope of the linear plot of k_{obs} versus ubiquinone-1 concentration, with values of 2.88×10^5 and 1.99×10^4 M^{-1} s^{-1} obtained for the faster and slower phases, respectively. Plots of observed rate constant versus quinone concentration for each substrate are shown in Figure 4, with the kinetic parameters thus determined summarized in Table 1. For the purposes of comparison, attention was focused on the faster phases of the overall reaction that reflected the reaction of the fully reduced enzyme with the first equivalent of quinone in the course of reoxidation and thus represented the intrinsic reactivity of the fully reduced flavin site with quinone. It was evident that of the three quinones examined, 1,4-benzoquinone (which is structurally similar to ubiquinone) was the most effective substrate having a k_{ox}/K_d ratio 1 order of magnitude higher than the other two.

Titration of CODH with Oxidized and Reduced Quinone. To establish the affinity of CODH for 1,4-benzoquinone, titrations of CODH with oxidized and reduced 1,4-benzoquinone were conducted. Figure 5 shows the titration of oxidized 1,4-benzoquinone with 8 μM CODH, with the greatest spectral change seen at 424 nm. The inset of Figure 5 shows a plot of the change in absorbance at 424 nm versus 1,4-benzoquinone concentration from which a K_d of 100 μM can be determined. A comparison with the kinetically determined K_d for binding of oxidized 1,4-benzoquinone with reduced CODH of 48 μM indicates that oxidation of the enzyme flavin reduces the affinity for the quinone by a factor of 2. Titration of oxidized CODH with prereduced 1,4-benzoquinone under anaerobic conditions yielded an oxidized-minus-reduced difference spectrum indicating that the FAD and Fe−S centers became fully reduced, as reflected by bleaching at 450 and 550 nm (Figure 5 of the Supporting Information).

DISCUSSION

Here we have examined the reaction of reduced CODH with several cytochromes and quinones in an effort to determine the oxidizing substrate for the enzyme. Having failed in an exhaustive effort to identify a cytochrome capable of being effectively

Table 1: Kinetic Parameters of CODH Oxidation by Various Quinones

	K_d (μM)	k_{ox} (s^{-1})	k_{ox}/K_d (M^{-1} s^{-1})	R^2 value for oxidative half-fit data	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	R^2 value for steady-state fit data	KIE
1,4-benzoquinone	47.6	125.1	2.60×10^6	0.955	16.4	104.5	6.37×10^6	0.995	
1,4-naphthoquinone	140	38.1	2.72×10^5	0.999					
1,2-naphthoquinone-4-sufonic acid			1.31×10^5	0.995					
ubiquinone-1 H_2O			2.88×10^5	0.990			5.81×10^4	0.980	1.41
ubiquinone-1 D_2O							4.13×10^4	0.996	

reduced by CODH, we find that several quinones are very effective substrates, rapidly oxidizing reduced CODH under anaerobic conditions. 1,4-Benzoquinone is found to be the most effective oxidizing substrate, with a k_{ox} of 125.1 s^{-1} at pH 7.2 and 25 °C for the fastest phase of enzyme reoxidation. The multiple phases observed in the oxidative half-reaction kinetics seen here are attributed to the necessarily sequential nature of the reaction of the fully (six-electron) reduced enzyme with three successive equivalents of quinone.

Although 1,4-benzoquinone was the most effective (and also most soluble) of the quinones tested, ubiquinone-1 was also a fairly effective substrate, but because of its low solubility in water, only a k_{ox}/K_d of 2.88×10^5 M^{-1} s^{-1} and a k_{cat}/K_m of 5.81×10^4 M^{-1} s^{-1} could be determined experimentally; these were approximately 1 order of magnitude slower than those seen with 1,4-benzoquinone (k_{ox}/K_d of 2.60×10^6 M^{-1} s^{-1}). A similar situation has been seen in chromate reductase, a soluble quinone-reducing protein that best utilizes 1,4-benzoquinone over ubiquinone-1 (*23*). Both 1,4-naphthoquinone and 1,2 naphthoquinone-4-sulfonate were poorer oxidizing substrates for CODH, suggesting that the structurally related menaquinone is a less likely physiological substrate for the enzyme than ubiquinone. No semiquinone EPR signal was observed in EPR experiments monitoring catalytic enzyme turnover, and it appears that the principal oxidizing event is an effective two-electron process (Figure 6 of the Supporting Information). Finally, given the overall effectiveness of quinones as a substrate, we conclude that one component of the quinone pool of *O. carboxidovorans* or another constitutes the proximal oxidizing substrate for CODH, and that cytochromes become reduced only subsequent to the introduction of reducing equivalents into the quinone pool.

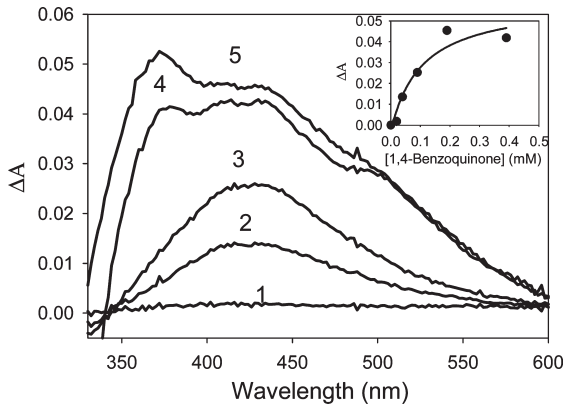

FIGURE 5: Titration of oxidized CODH with oxidized 1,4-benzoquinone. Difference spectra of CODH with bound 1,4-benzoquinone in 50 mM HEPES (pH 7.2) minus oxidized CODH when CODH is titrated with oxidized 1,4-benzoquinone. Additions of 1,4-benzoquinone yielded final concentrations of 20 μM (1), 40 μM (2), 90 μM (3), 190 μM (4), and 390 μM (5). The inset shows the concentration dependence of the spectral change at 424 nm produced by titration of 1,4-benzoquinone with CODH. K_d was found to be 104 μM, determined by a fit using the hyperbolic equation $A_{obs} = (\Delta A_{max} x)/(K_d + x)$ ($R^2 = 0.9359$).

SUPPORTING INFORMATION AVAILABLE

Absorption spectra of oxidized and dithionite-reduced cytochrome b_{561} in 50 mM HEPES (pH 7.2) and 0.2% Triton X-100 (Figure 1), absorption spectra recorded at 1, 250, and 500 s over the course of the reaction of anaerobic reduced CODH with anaerobic cytochrome b_{561} in 50 mM HEPES (pH 7.2) and 0.2% Triton X-100 (Figure 2), a plot of the steady-state catalytic velocity as a function of 1,4-benzoquinone concentration for CODH in 50 mM HEPES (pH 7.2, 25 °C, 1 mM CO) (Figure 3), steady-state catalytic velocity as a function of ubiquinone-1 concentration (in H_2O and D_2O) for CODH in 50 mM HEPES (pH 7.2 or pD 7.6, 25 °C, 1 mM CO) (Figure 4), anaerobic titration of 3.5 μM CODH with reduced 1,4-benzoquinone in 50 mM HEPES (pH 7.2, 25 °C) (Figure 5), and electron paramagnetic resonance spectra of oxidized CODH in 50 mM HEPES (pH 7.2) and enzyme that has been fully reduced with dithionite and then reoxidized with either 0.5 or 12 equiv of ubiquinone-1 (spectra recorded at 150 K, with a 9.5 GHz microwave frequency, a 10 mW microwave power, and a 5 G modulation amplitude) (Figure 6). This material is available free of charge via the Internet at http://pubs.acs.org.

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