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Substitution of Silver for Copper in the Binuclear Mo/Cu Center of Carbon Monoxide Dehydrogenase from *Oligotropha carboxidovorans*

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ABSTRACT: Carbon monoxide dehydrogenase from *Oligotropha carboxidovorans* catalyzes the aerobic oxidation of carbon monoxide to carbon dioxide, providing the organism both a carbon source and energy for growth. The active site of the native enzyme is a unique binuclear molybdenum- and copper-containing center. Here we show that silver can be substituted for copper in the active site to yield a functional enzyme. The characteristic hyperfine coupling of the $I = {}^1/_2$ nucleus of Ag is evident in the EPR signal of the binuclear active site observed upon reduction with CO, indicating both the incorporation of silver into the active site and, remarkably, retention of the catalytic activity. The silver-substituted enzyme is reduced by CO with an observed limiting rate constant of 8.1 s^{-1}, which can be compared with the value of 51 s^{-1} for the wild-type enzyme. Steady-state kinetics for the Ag-substituted enzyme yielded k_{cat} = 8.2 s^{-1} and K_m = 2.95 μM at pH 7.2.

The molybdenum- and copper-containing carbon monoxide dehydrogenase (CODH) of the aerobe *Oligotropha carboxidovorans* catalyzes the oxidation of carbon monoxide to carbon dioxide, yielding two reducing equivalents. The enzyme is an $(\alpha\beta\gamma)_2$ hexamer with a small subunit containing two [2Fe−2S] centers, a medium subunit containing flavin adenine dinucleotide (FAD), and large subunit where CO oxidation takes place.[1−3] As shown in Figure 1, the active site of the large subunit contains an $LMo^{VI}O_2{-}\mu\text{-}S{-}Cu^{I}{-}SCys$ binuclear center (where L is the pterin cofactor found in all molybdenum-containing enzymes save nitrogenase).[1−3] We report here the substitution of silver for copper in the binuclear cluster, yielding a Mo/Ag enzyme that retains the ability to oxidize CO.

Incorporation of silver into CODH was accomplished by a modification of the copper reconstitution developed by Resch et al.[4,6] Approximately 100 μM CODH and 10 mM methyl viologen in 1.0 mL of 50 mM Tris-HCl (pH 8.2) was made anaerobic by alternately evacuating and flushing with O_2-scrubbed argon gas over the course of 1 h. An appropriate volume of an anaerobically prepared stock solution of 100 mM Na_2S was added to give a final concentration of 2.0 mM. Next, a volume of 0.1 M dithionite stock solution sufficient to sustain the blue color of the reduced viologen was added, after which the mixture 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 Ag(I)−thiourea was prepared by dissolving $AgNO_3$ and thiourea in a 1:3 molar ratio in anaerobic water. An appropriate amount of this stock solution

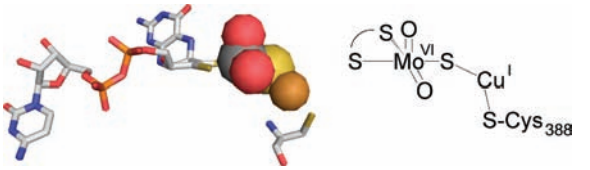

Figure 1. Active site of CODH rendered using Protein Data Bank entry 1N5W. Atom colors are CPK with Mo in gray and Cu in brown coordinated by Cys 388.

was added to the anaerobic enzyme to give a final concentration of 0.2 mM Ag(I), after which the mixture was incubated for 5−10 h at 20 °C. A final G-25 column, equilibrated with 50 mM HEPES (pH 7.2), was used to remove excess Ag(I). The enzyme was assayed for activity,[4] and the degree of functionality was independently determined by comparing the extent of enzyme bleaching (as observed at 450 nm) produced by CO (which reduces only the fully functional enzyme) with that seen using dithionite (which reduces both functional and nonfunctional enzyme).[2] ICP-AES analysis showed that some trace amounts of copper were still present in the enzyme preparations (<10% of the enzyme molybdenum), but on the basis of the observed electron paramagnetic resonance (EPR) spectrum discussed below, these likely were not catalytically relevant. Our preparations of Ag-substituted enzyme typically yielded 30% functional enzyme, which can be compared with ∼50% active protein in the native Cu-reconstituted enzyme.[6] The catalytic velocities reported here were corrected for the amount of nonfunctional protein by adjusting the enzyme concentration used for the concentration of functional enzyme.

Incorporation of silver into the active site was assessed by EPR spectroscopy.[8] The EPR spectrum of CO-reduced native CODH is shown in Figure 2A. The spectrum shows strong superhyperfine coupling to the $I = {}^3/_2$ ^{63,65}Cu nuclei, with g_1, g_2, g_3 = 2.0010, 1.9604, 1.9549 and A_1, A_2, A_3 = 117, 164, 132 MHz, as reported in previous work.[5] By comparison, as shown in Figure 2B, treatment of the silver-substituted enzyme with CO yielded an EPR spectrum exhibiting strong superhyperfine coupling to the ^{107,109}Ag nuclei ($I = {}^1/_2$). Simulation of the EPR signal for the Ag-substituted enzyme (Figure 2C) yielded g_1, g_2, g_3 = 2.0043, 1.9595, 1.9540 and A_1, A_2, A_3 = 81.98, 78.85, 81.89 MHz.[9] The g_2 and g_3 values for the silver-substituted enzyme are comparable to those found for the copper enzyme, but g_1 differs to a rather greater degree from the value of 2.0010 for the native copper-containing enzyme.[5] Samples prepared in D_2O HEPES (pD 7.6) showed only very slight line-width narrowing, with values of 0.051 mT and 0.048 mT for the H_2O

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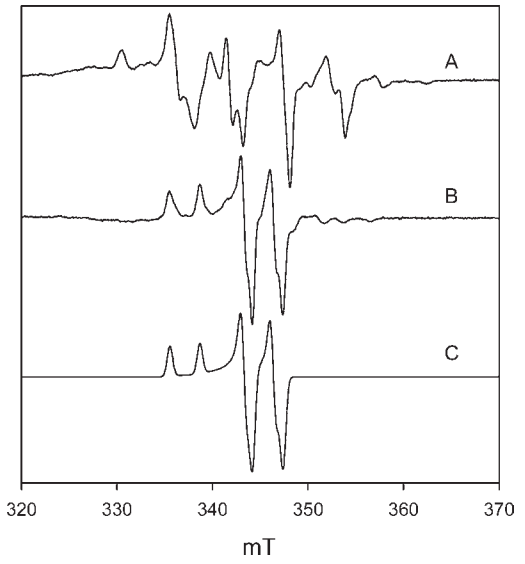

Figure 2. EPR spectra of CO-reduced CODH: (A) 100 μM native Cu-containing CODH reduced with excess CO; (B) Ag-substituted CODH reduced with excess CO; (C) simulation of (B) with parameters g_1, g_2, g_3 = 2.0043, 1.9595, 1.9540 and A_1, A_2, A_3 = 81.98, 78.85, 81.89 MHz. The samples in (A) and (B) were prepared in 50 mM HEPES (pH 7.2). The EPR instrument settings were as follows: microwave frequency, 9.456 GHz; microwave power, 10 mW for the Cu-containing sample and 4 mW for the Ag-containing sample; modulation amplitude, 5 G for the Cu-containing sample and 2 G for the Ag containing sample; temperature, 150 K.

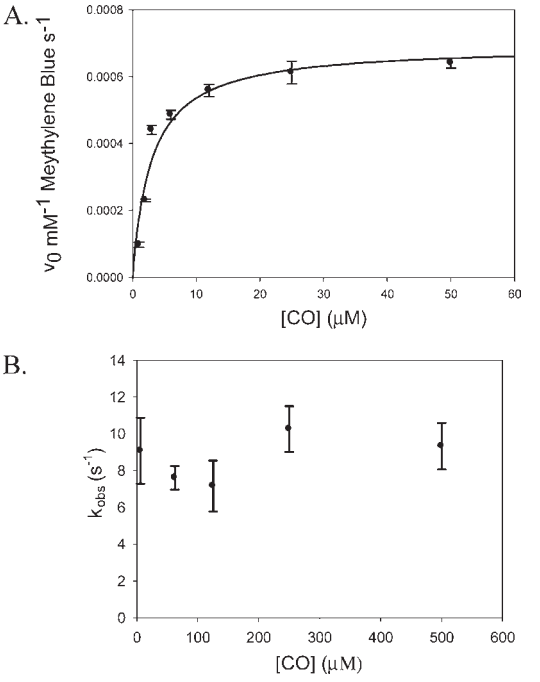

Figure 3. Kinetic data for Ag-substituted CODH. (A) Steady-state CO concentration dependence of the kinetics for Ag-substituted CODH using methylene blue as an electron acceptor in 50 mM potassium phosphate buffer (pH 7.2) at 25 °C. A plot of the initial rate (determined by following the bleaching at 615 nm due to methylene blue reduction after mixing a final concentration of 82.0 nM CODH with CO dissolved in methylene blue) vs [CO] (1−500 μM) is shown. The data were fit with the Michaelis−Menten equation using Sigma Plot (R^2 = 0.95), which gave k_{cat} = 8.2 s^{-1} and K_m = 2.95 μM. (B) Rapid-reaction kinetic plots of k_{obs} (determined by following bleaching at 450 nm due to the reduction of 5 μM CODH) vs [CO] (6−500 μM) in 50 mM HEPES (pH 7.2) at 25 °C. Since the observed data had no concentration dependence, saturating substrate conditions were assumed, and the apparent value of k_{red} was estimated to be 8.1 s^{-1}.

and D_2O samples, respectively (data not shown). We specifically note that there was no evidence of any EPR signal arising from the native, copper-containing enzyme in the EPR spectrum of the silver-substituted form (the high-field features in the spectra of both the native and silver-substituted enzymes are due to hyperfine coupling to the $I = {}^5/_2$ ^{95,97}Mo nuclei, which have a combined natural abundance of 25%).

In order to characterize the kinetic properties of the silver-substituted enzyme, both steady-state and rapid-reaction kinetics were performed. Steady-state kinetics was carried out under anaerobic conditions in 50 mM potassium phosphate buffer (pH 7.2) at 25 °C using CO concentrations of 1−500 μM. Methylene blue bleaching at 615 nm was followed, and the initial linear portions of the time courses were used to determine the observed catalytic velocities. Figure 3A shows averaged rates plotted against CO concentration. Hyperbolic fits to the data yielded k_{cat} = 8.2 s^{-1} per active site and K_m = 2.95 μM, which give $k_{cat}/K_m = 2.78 \times 10^6$ M^{-1} s^{-1}. Comparison of these values with those obtained for the native enzyme (93.3 s^{-1}, 10.7 μM, and 8.7 $\times$ 10^6 M^{-1} s^{-1}, respectively, for the hexamer with two active sites) indicates that the silver-substituted CODH indeed retains significant catalytic activity.[5]

The reduction of CODH by CO under anaerobic conditions was next examined using stopped-flow spectrophotometry[10] by following the bleaching of CODH at 450 nm upon reduction of the Fe/S and FAD centers of the enzyme.[11] At all CO concentrations used over the range 6−500 μM, well-behaved single-exponential transients were observed, and k_{obs} values were obtained from exponential fits to the data. Figure 3B shows a plot of k_{obs} versus [CO] for the reaction carried out in 50 mM HEPES (pH 7.2), where it can be seen that, as with the native enzyme, the rate constant for enzyme reduction was independent of [CO] (presumably because of a prior rapid-equilibrium binding step). The intrinsic k_{red} value of 8.1 s^{-1} is in good agreement with the k_{cat} value of 8.2 s^{-1} obtained from the steady-state kinetics. These results indicate that the reductive half-reaction is overall rate-limiting in catalysis, as is seen with the native copper-containing enzyme.[5]

In the present work, we have developed a protocol for incorporating silver into the active site of CODH in place of the naturally occurring copper and demonstrated that the silver-substituted enzyme thus obtained can also be rapidly reduced by CO and subsequently exhibit an EPR signal that, apart from doublets due to ^{107,109}Ag rather than quartets due to ^{63,65}Cu, is strongly reminiscent of the EPR signal seen with native enzyme upon reduction by CO. There was a modest shift in the g_1 value upon silver substitution (2.0043 vs 2.0010 for the copper-containing enzyme), but no substantial changes in the g_2 and g_3 values were observed. Overall, the EPR results indicate a coordination environment in the silver-substituted enzyme similar to that seen in the native form. Steady-state kinetics studies showed a 5-fold reduction in both k_{cat} and K_m relative to the native system. No dissociation constant (K_d) could be determined through rapid-reaction kinetics because of the rapid formation of complexed CO prior to catalysis. The intrinsic k_{red}

value of 8.1 s^{-1} correlates well with the k_{cat} value of 8.2 s^{-1} obtained using steady-state kinetics, indicating that, as with the native enzyme, the rate-limiting step is present in the reductive half-reaction.[5]

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(4) CODH from *O. carboxydovorans* (ATCC 49405) was purified as described by Zhang et al.[5] and stored at 77 K until needed. In order to incorporate silver into the enzyme, the enzyme was first inactivated by removal of the copper and the bridging sulfur by treatment with potassium cyanide, which was carried out by incubation of 100 μM CODH with 5 mM KCN for 10 h under an Ar atmosphere at 20 °C.[6] The activity was determined using the reduction of methylene blue (ε_{615} = 37.11 mM^{-1} cm^{-1}) in 50 mM potassium phosphate at 30 °C.[7] The fully inactive enzyme displayed no reduction of methylene blue.

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(8) EPR spectra were recorded using a Bruker Instruments ER 300 spectrometer equipped with an ER 035 M gaussmeter and an HP 5352B microwave frequency counter. A temperature of 150 K was maintained using a Bruker ER 4111VT liquid N_2 cryostat. EPR samples of approximately 100 μM Ag-containing CODH were prepared anaerobically by cyclic Ar flushing and vacuum for 1 h. Reduction of molybdenum was achieved by CO incubation for 30 s prior to freezing in a dry ice/acetone bath and stored in liquid N_2.

(9) EPR spectra were simulated using the EasySpin software package. See: Stoll, S.; Schweiger, A. *J. Magn. Reson.* **2006**, *178*, 42–55.

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