# Organization of Clusters and Internal Electron Pathways in CO Dehydrogenase from *Clostridium thermoaceticum*: Relevance to the Mechanism of Catalysis and Cyanide Inhibition†

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ABSTRACT: Cyanide inhibits the CO oxidation activity of carbon monoxide dehydrogenase from *Clostridium thermoaceticum* by binding tightly to the form of the C-cluster yielding the $g_{av} = 1.82$ signal (the $C_{1.82}$ form). $CN^-$ dissociates and the enzyme reactivates upon addition of CO, $CO_2$ plus dithionite, or $CS_2$ plus dithionite. Dithionite slows the inhibition of the enzyme by $CN^-$, but it cannot reactivate the enzyme. This behavior is explained by assuming that binding of CO, $CO_2$, or $CS_2$ at a modulator site accelerates the dissociation of $CN^-$ from the C-cluster. With $CN^-$ bound at the C-cluster, dithionite, but not CO, can reduce those Fe-S clusters in the enzyme whose redox status can be monitored at 420 nm. The electron pathway used for CO oxidation appears to be as follows: C-cluster → Fe-S Clusters → external electron acceptors. The electron used to reduce the NiFe complex originates predominantly from the C-cluster, and this reduction is inhibited when $CN^-$ is bound at the C-cluster. The NiFe complex is reduced more slowly (in the absence of $CN^-$) than CO is catalytically oxidized, indicating that this reduction is not part of the catalytic mechanism for CO oxidation. The form of the C-cluster yielding the $g_{av} = 1.86$ signal ($C_{1.86}$) is proposed to be two electrons more reduced than $C_{1.82}$ and able to bind and reduce $CO_2$. CO is proposed to be oxidized by $C_{1.82}$. Neither CO or $CN^-$ appears to bind $C_{1.86}$.

Carbon monoxide dehydrogenase from *Clostridium thermoaceticum* ($CODH_{Ct}$)[1] catalyzes two types of reactions: the synthesis of acetyl-CoA from CO, $CH_3$-THF, and CoA, and the reversible oxidation of CO to $CO_2$ (Ragsdale, 1991; Wood & Ljungdahl, 1991). The enzyme has an $(\alpha\beta)_3$ hexameric quaternary subunit structure with molecular mass of ca. 460 kDa (Ragsdale et al., 1983a; Morton et al., 1991). On average, each $\alpha\beta$ dimer contains 2 Ni, 11–13 Fe, and ~14 sulfide ions, organized into approximately three types of autonomous structures (Lindahl et al., 1990a,b), including (i) the NiFe complex, (ii) the C-cluster (Anderson et al., 1993), and (iii) one or two $[Fe_4S_4]^{2+/1+}$ clusters.[2]

The catalytic mechanism of $CODH_{Ct}$ and the functions served by each metal complex/cluster are only beginning to be understood. We and others have identified the NiFe complex to be the active site for acetyl-CoA synthesis (Lu & Ragsdale, 1991; Gorst & Ragsdale, 1991; Shin & Lindahl, 1992a,b) and have provided substantial evidence that the C-cluster is the active site for CO oxidation (Anderson et al., 1993). The $Fe_4S_4$ clusters may serve an electron transfer function, but little is known about the relationships between the different centers.

The NiFe complex consists of a Ni ion chemically linked to a large iron–sulfur cluster (Ragsdale et al., 1985; Bastian et al., 1988; Lindahl et al., 1990a,b; Fan et al., 1991; Shin et al., 1993). The complex has two known redox states: a diamagnetic oxidized state ($NiFe_{ox}$) and a one-electron-reduced ($E^{o\prime} = -540$ mV vs NHE), $S = {}^1/_2$ state that has CO bound and yields the so-called NiFeC EPR signal ($g_1 = 2.080$, $g_2 = 2.074$, $g_3 = 2.028$) (Ragsdale et al., 1983b; Gorst & Ragsdale, 1991). The NiFeC state appears to be the form of the NiFe complex used to catalyze acetyl-CoA synthesis and CO/acetyl-CoA exchange (Lu & Ragsdale, 1991). Curiously, the NiFeC state can only be obtained by exposing $CODH_{Ct}$ to CO (Ragsdale et al., 1982) or by reducing $CODH_{Ct}$ with low-potential, electrochemically reduced viologens in the presence of either $CO_2$ (Lindahl et al., 1990a) or acetyl-CoA (Gorst & Ragsdale, 1991). Low-potential reductants such as sodium dithionite or viologens are unable to reduce $NiFe_{ox}$ in the absence of CO, $CO_2$, or acetyl-CoA. The reason for this selectivity is not understood.

The C-cluster contains Fe and possibly Ni (Lindahl et al., 1990b; Anderson et al., 1993). Using EPR and $^{13}C$ ENDOR spectroscopy, we found that $CN^-$, a potent inhibitor of CO oxidation activity (Ragsdale et al., 1983a), binds directly to the C-cluster (Anderson et al., 1993), suggesting that this cluster is the active site for CO oxidation. The C-cluster can be stabilized in an oxidized $S = 0$ state (to be called the $C_{S=0}$ state) and a one-electron-reduced $S = {}^1/_2$ state ($E^{o\prime} = -220$ mV) that yields an EPR signal with $g_{av} = 1.82$ ($g_1 = 2.01$, $g_2 = 1.80$, $g_3 = 1.64$) (Lindahl et al., 1990a,b), to be called

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[1] Abbreviations: $CODH_{Ct}$, carbon monoxide dehydrogenase from *Clostridium thermoaceticum*; $CODH_{Rr}$, carbon monoxide dehydrogenase from *Rhodospirillum rubrum*; CoA, coenzyme A; THF, tetrahydrofolate; MV, methyl viologen; EPR, electron paramagnetic resonance; ENDOR, electron nuclear double magnetic resonance; $C_{S=0}$, the diamagnetic, fully oxidized form of the C-cluster; $C_{1.82}$, the form of the C-cluster exhibiting the $g_{av} = 1.82$ EPR signal and presumed to be one electron more reduced than $C_{S=0}$; $C_{1.86}$, the form of the C-cluster exhibiting the $g_{av} = 1.86$ EPR signal and presumed to be three electrons more reduced than $C_{S=0}$; $C_{1.72}$, the form of the C-cluster bound with $CN^-$, exhibiting the $g_{av} = 1.72$ EPR signal, and presumed to be one electron more reduced than $C_{S=0}$; $NiFe_{ox}$, the diamagnetic oxidized form of the NiFe complex; NiFeC, the $S = {}^1/_2$ form of the NiFe complex presumed to be one electron more reduced than $NiFe_{ox}$ and bound by CO; CBP, the competitive binding proposal.

[2] A fourth species called "ferrous component II" has been identified by Mössbauer spectroscopy to be a redox-active, high-spin $Fe^{2+}$ ion possibly ligated to four sulfurs (Lindahl et al., 1990b). No EPR or electronic absorption spectral features have been associated with it. We did not study it here and defer further comment on it pending additional Mössbauer studies.

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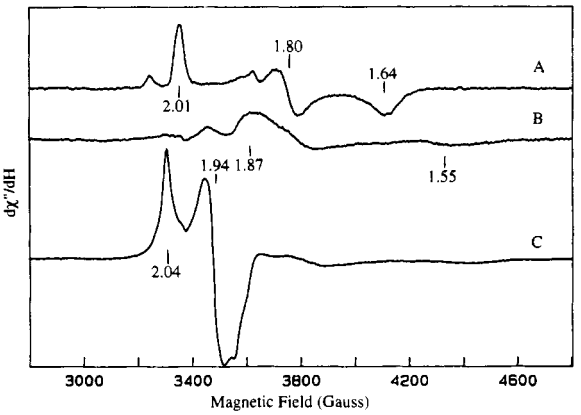



FIGURE 1: EPR of partially oxidized $CODH_{Ct}$ (A), inhibited with $CN^-$ (B), and then reduced with dithionite (C). (A) Dithionite-free $CODH_{Ct}$ (400 μL of 19 μM $\alpha\beta$, batch no. 6, 310 units/mg, in 50 mM Tris, pH 8.0) oxidized with 3.3 equiv/$\alpha\beta$ thionin. (B) Sample in A after adding 5 equiv/$\alpha\beta$ KCN. (C) Sample in B after adding 5 mM $Na_2S_2O_4$. EPR conditions: microwave frequency, 9.43 GHz; microwave power, 20 mW; temperature, 10 K; gain, $2 \times 10^4$ for A and B, $1 \times 10^4$ for C; time constant, 0.327 s.

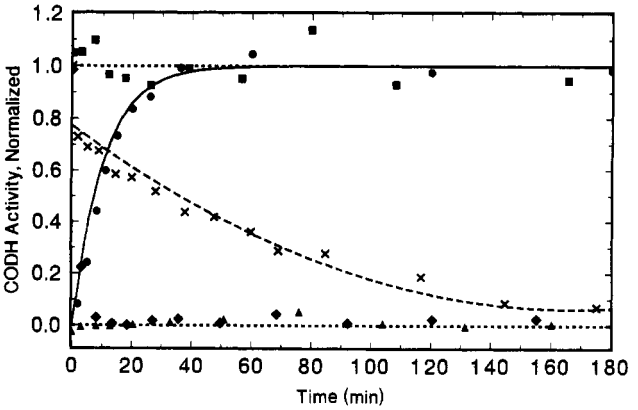



FIGURE 2: CO oxidation activity under various conditions as a function of time. (Triangles) $CODH_{Ct}$ (150 μL of 63 μM $CODH_{Ct}$ $\alpha\beta$, batch no. 1, 200 units/mg, in 50 mM Tris, pH 8.0) oxidized by thionin (3.3 equiv/$\alpha\beta$) and then inhibited by KCN (2.8 equiv/$\alpha\beta$). (Circles) $CODH_{Ct}$ (500 μL of 370 μM $CODH_{Ct}$ $\alpha\beta$, 420 units/mg) inhibited at 1 °C by KCN (2 equiv/$\alpha\beta$) and then exposed after 9 min to 1 atm CO and immediately incubated at 30 °C. (Squares) $CODH_{Ct}$ (100 μL of 63 μM $CODH_{Ct}$ $\alpha\beta$, 2 mM dithionite, 200 units/mg) reduced by 1 atm CO and then mixed with KCN (2.8 equiv/$\alpha\beta$). (Diamonds) $CODH_{Ct}$ (150 μL of 63 μM $CODH_{Ct}$ $\alpha\beta$, 200 units/mg) oxidized by thionin (3 equiv/$\alpha\beta$) and then inhibited by KCN (2 equiv/$\alpha\beta$) and reduced 8 min later by dithionite (2 mM, final concentration). (X's) $CODH_{Ct}$ (500 μL of 3.2 μM $\alpha\beta$, batch no. 6, 260 units/mg) reduced with 5 mM dithionite, cooled to 1 °C, and then inhibited by KCN (5 equiv/$\alpha\beta$). Normalized activities indicated by the squares, triangles, and diamonds were obtained by dividing measured activities by the average of the activities of the squares (180 units/mg). The normalized activities indicated by the circles were obtained by dividing measured activities by the average of the activities of the last four data points (200 units/mg). The normalized activities indicated by the X's were obtained by dividing measured activities by the uninhibited activity (260 units/mg). The solid line is a simulation of the reverse inhibition reaction indicated by the circles, according to the equation $[CODH]_{act}/[CODH]_{tot} = 1 - \exp(-k_{off}t)$, where $[CODH]_{act}/[CODH]_{tot}$ is the proportion of reactivated enzyme at any time $t$, and $k_{off} = 0.0016\ s^{-1}$. The two dashed horizontal lines have ordinate values of 1 and 0 on the normalized activity scale. The dashed line through the X's is a quadratic-fit obtained using the program AXUM 3.0 (Trimetrix Inc.).

the $C_{1.82}$ state. The change in spin suggests that $C_{1.82}$ is one electron more reduced than $C_{S=0}$. $CN^-$ binds to $C_{1.82}$, shifting the $g_{av} = 1.82$ signal to one with $g_{av} = 1.72$ ($g_1 = 1.87$, $g_2 = 1.78$, and $g_3 = 1.55$) (Anderson et al., 1993). The resulting $CN^-$-bound state will be called the $C_{1.72}$ form of the C-cluster. The $g_{av} = 1.82$ signal disappears as solution potentials are lowered (in accordance with $E^{o\prime} = -530 \pm 35$ mV), and another signal, with $g_{av} = 1.86$ ($g_1 = 1.97$, $g_2 = 1.87$, $g_3 = 1.75$), appears simultaneously. The $g_{av} = 1.86$ signal probably arises from the C-cluster as well (and this state will be called $C_{1.86}$), since both signals have similar $g$ values, saturation and relaxation properties, and spin intensities (0.1–0.3 spin/$\alpha\beta$; Lindahl et al., 1990a,b). The behavior of the two signals are related, in that the $g_{av} = 1.86$ signal develops as the $g_{av} = 1.82$ signal disappears, but the $C_{1.82}/C_{1.86}$ conversion is not well understood. The conversion is redox-dependent ($E^{o\prime} = -530 \pm 35$ mV) and is effected by various reductants including CO, dithionite, and electrochemically reduced viologens (Lindahl et al., 1990a). $C_{1.82}$ and $C_{1.86}$ are either isoelectronic or different by an even number of electrons.

Whether $CODH_{Ct}$ contains one (Lindahl et al., 1990a,b) or two (Shin et al., 1992) $[Fe_4S_4]^{2+/1+}$ clusters remains uncertain, but we shall assume there are two for this paper. These clusters have rather unexceptional spectroscopic and redox properties. They are diamagnetic when oxidized and $S = {}^1/_2$ when reduced by one electron ($E^{o\prime} = -440$ mV). The reduced states yield two $g_{av} = 1.94$ signals with the same $g$ values ($g_1 = 2.04$, $g_2 = 1.94$, $g_3 = 1.90$) but different line widths. Both signals together quantify to 0.64 ± 0.14 spin/$\alpha\beta$.

Cyanide is a potent inhibitor of the CO oxidation activity of all CODHs examined so far [see Anderson et al. (1993)]. Through a series of elegant studies by Ludden and co-workers, $CN^-$ has been used to investigate the catalytic mechanism of the CODH from *Rhodospirillum rubrum* ($CODH_{Rr}$). $CODH_{Rr}$ is simpler than $CODH_{Ct}$, in that it catalyzes only the reversible oxidation of CO to $CO_2$, not the synthesis of acetyl-CoA. It is a monomer containing 1 Ni and 7–8 Fe ions (Bonam & Ludden, 1987; Ensign et al., 1989b). The CO oxidation active site is a Ni-Fe-S cluster that exhibits an EPR signal known as signal A ($g_1 = 2.04$, $g_2 = 1.90$, and $g_3 = 1.71$) (Stephens et al., 1989). CO and $CN^-$ bind directly to the Ni (Ensign et al., 1989a,b), which is bridged to an Fe-S cluster (Tan et al., 1992). The Ni also serves to facilitate the transfer of electrons from CO oxidation to the iron–sulfur clusters in the enzyme (Ensign et al., 1989a).

Although $CN^-$ is a potent inhibitor of $CODH_{Ct}$ (Ragsdale et al., 1983a), it is not a very potent inhibitor of the exchange reactions catalyzed by $CODH_{Ct}$ (Lu & Ragsdale, 1991). The exchange reactions are only marginally inhibited at concentrations of $CN^-$ far higher than those required to completely inhibit CO oxidation. Thus, at low concentrations, $CN^-$ *selectively* inhibits the CO oxidation activity of $CODH_{Ct}$.

In this paper, we describe the binding properties of $CN^-$ on the C-cluster, the ability of dithionite and CO to protect the enzyme from $CN^-$ inhibition, and the abilities of CO, $CO_2$, and $CS_2$ to reactivate the enzyme. We propose a model of the redox and $CN^-$-binding properties of the C-cluster. We also utilize the effects of $CN^-$ to uncover electron transfer pathways within the enzyme and determine how the redox sites within the enzyme are interrelated.

## EXPERIMENTAL PROCEDURES

*C. thermoaceticum* was grown, and six batches of $CODH_{Ct}$ were purified and characterized as described (Lundie & Drake, 1984; Ragsdale & Wood, 1985; Raybuck et al., 1988; Ramer et al., 1989; Shin & Lindahl, 1993). Batches 1–6 had CO/acetyl-CoA exchange activities of 0.08, 0.19, 0.56, 0.16, 0.09, and 0.10 units/mg, respectively, and CO oxidation activities of 270, 250, 320, 280, 420, and 410 units/mg. Potassium

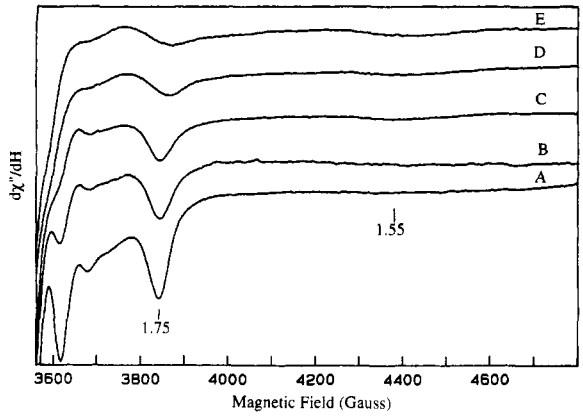



FIGURE 3: Kinetics of the conversion from $C_{1.86}$ to $C_{1.72}$ after adding $CN^-$ to dithionite-reduced $CODH_{Ct}$ at 1 °C. (A) $CODH_{Ct}$ (from a stock of 4.6 mL of 19 $\mu$M $\alpha\beta$, batch no. 6 reduced with 46 $\mu$L of 0.5 M dithionite 360 units/mg). The spectrum exhibits only the $g_2$ = 1.87 and $g_3$ = 1.75 resonances of the $g_{av}$ = 1.86 signal. (B) As in A, except cooled to 1 °C, reacted with 5 equiv/$\alpha\beta$ KCN, and frozen after 10 min (320 units/mg). (C) As in B, except frozen after 56 min (230 units/mg). (D) As in B, except that an additional 5 equiv/$\alpha\beta$ KCN was added after 154 min, and the sample was frozen after 157 min (68 units/mg). (E) As in D, except frozen after 208 min (24 units/mg). The spectrum shows the $g_{av}$ = 1.72 signal. EPR conditions: microwave frequency, 9.43 GHz; microwave power, 20 mW; temperature, 10 K; gain, 1 × $10^4$; time constant, 0.327 s.

cyanide (Baker) was prepared in 50 mM NaOH solutions. Sodium dithionite ($Na_2S_2O_4$) and thionin (3,7-diaminophenothiazin-5-ium chloride) were purchased from Aldrich. $CS_2$ (Fisher) was purified as described (Gordon & Ford, 1971). Protein concentrations were determine by the Biuret method (Pelley et al., 1978) using bovine serum albumin as a standard. A molecular weight of 154 700 for each CODH $\alpha\beta$ dimer was used in calculations. Experimental details are given in figure legends.

## RESULTS

*Effect of $CN^-$ on $C_{1.82}$ and $C_{1.86}$ Forms of the C-Cluster.* A sample of $CODH_{Ct}$ was oxidized to the extent required for the C-cluster to be in the $C_{1.82}$ form and the $Fe_4S_4$ clusters to be in the diamagnetic 2+ core oxidation states (Figure 1A). $CN^-$ was added, and the C-cluster shifted to the $C_{1.72}$ form (Figure 1B). Dithionite was then added; the C-cluster remained in the $C_{1.72}$ form, and the $Fe_4S_4$ clusters were reduced to the 1+ core states, yielding the $g_{av}$ = 1.94 signals (Figure 1C). Thionin-oxidized enzyme (most likely containing $C_{1.82}$) was inhibited by $CN^-$ essentially within the time of mixing (Figure 2, triangles). Dithionite-reduced $CODH_{Ct}$ (containing $C_{1.86}$) was inhibited far more slowly (Figure 2, ×'s), and the loss of activity followed the conversion from $C_{1.86}$ to $C_{1.72}$ (Figure 3).

*Effect of CO on $CN^-$-Inhibited $CODH_{Ct}$.* $CN^-$-inhibited $CODH_{Ct}$ was reactivated to essentially its original level by exposure to 1 atm CO for ca. 1 h (Figure 2, circles). This reactivation correlated with the conversion from $C_{1.72}$ to $C_{1.86}$ (Figure 4). The initial effect of $CN^-$ on the enzyme depended on whether samples were first exposed to $CN^-$ and then CO, or to CO and then $CN^-$. If $CN^-$ was added first, the enzyme was initially fully inhibited (Figure 2, circles, at 0 min), but if CO was added first, no inhibition was evident at low concentrations of $CN^-$ (Figure 2, squares). At significantly higher $CN^-$ concentrations, CO was unable to protect the enzyme against $CN^-$ inhibition or to reactivate it once inhibited.

$CN^-$-inhibited $CODH_{Ct}$ did not reactivate by separating it from free $CN^-$ and diluting it 100-fold (Figure 5, 0–80 min). The sample reactivated only after CO was added (Figure 5, >80 min). Reactivation could also be effected by $CO_2$ and $CS_2$, as long as dithionite was present (Figure 6, squares and up-triangles, respectively). $CS_2$ is not a substrate of the enzyme, nor does it appear to be a potent inhibitor of CO oxidation activity at the concentration used in the assay.

Whether the NiFe complex was functional and contained the labile Ni had no effect on the ability of CO to reactivate $CODH_{Ct}$. Phenanthroline-treated $CODH_{Ct}$, containing a Ni-deficient and nonfunctional NiFe complex (Shin & Lindahl, 1992a,b), was inhibited by $CN^-$ and then exposed to CO. The

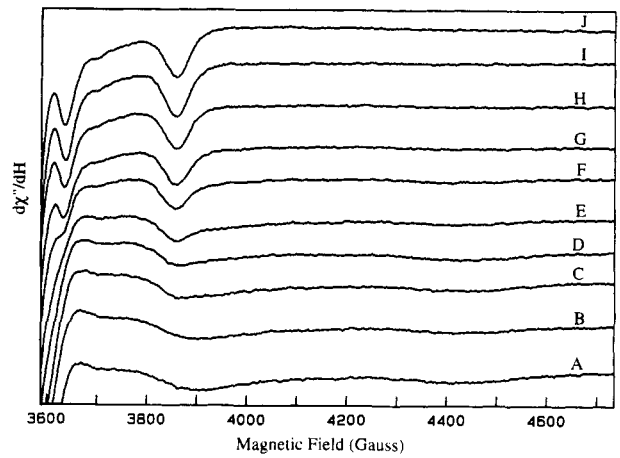



FIGURE 4: Kinetics of the conversion from $C_{1.72}$ to $C_{1.86}$ after adding CO to $CN^-$-inhibited, dithionite-reduced $CODH_{Ct}$ at 27 °C. $CODH_{Ct}$ (2.5 mL of 52 $\mu$M $CODH_{Ct}$ $\alpha\beta$, batch no. 2) was inhibited with 20 $\mu$L of 10 mM KCN and then reduced 5 min later by adding 5 $\mu$L of 1 M dithionite. The sample was cooled to 1 °C, exposed to 1 atm of CO, and then warmed to ca. 27 °C. Aliquots were removed periodically and assayed for activity or analyzed for the NiFeC EPR signal. (A) Dithionite-reduced, $CN^-$-inhibited $CODH_{Ct}$; remaining spectra are of dithionite-reduced, $CN^-$-inhibited $CODH_{Ct}$, obtained from the same stock as sample A, after exposure to 1 atm CO for the following times (respective CO oxidation activities are given in parentheses): (B) 5.5 min (0.8 units/mg); (C) 9 min (11 units/mg); (D) 13 min (29 units/mg); (E) 19 min (73 units/mg); (F) 29 min (150 units/mg); (G) 44 min (240 units/mg); (H) 62 min (280 units/mg); (I) 93 min (300 units/mg); (J) 124 min (330 units/mg). Although there is some overlap of signals, spectra A–E exhibit the $g_{av}$ = 1.72 signal while spectra F–J exhibit the $g_2$ = 1.87 and $g_3$ = 1.75 resonances of the $g_{av}$ = 1.86 signal.

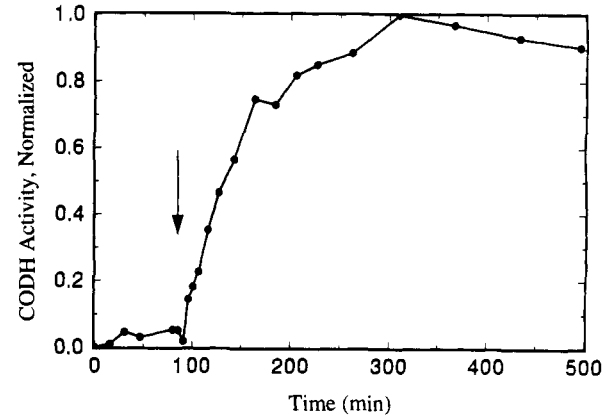



FIGURE 5: Activity of $CN^-$-inhibited $CODH_{Ct}$ after removing free $CN^-$ and diluting 100-fold. Dithionite-free $CODH_{Ct}$ (0.50 mL of 46 $\mu$M $CODH_{Ct}$ $\alpha\beta$, batch no. 3, 290 units/mg in 50 mM Tris, pH 8.0) inhibited by 5.0 equiv/$\alpha\beta$ KCN, then freed of unbound KCN using gel filtration chromatography (Sephadex G-25 equilibrated in 50 mM Tris, pH 8.0), and diluted 100-fold with the same buffer. Aliquots of the diluted sample (290 nM) were removed and assayed at the indicated times. After 82 min, 1 atm of CO was introduced (arrow). Normalized activities were obtained by dividing measured activities by the maximum activity obtained during reactivation (180 units/mg).

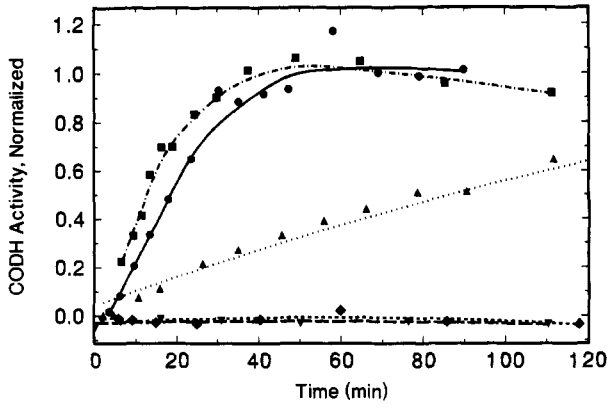



FIGURE 6: Reactivation of $CN^-$-inhibited $CODH_{Ct}$ by $CO_2$ and $CS_2$, and of $CN^-$-inhibited phenanthroline-treated $CODH_{Ct}$ by CO. (Squares) $CODH_{Ct}$ (500 $\mu$L of 3.2 $\mu$M $\alpha\beta$, batch no. 5, 500 units/mg, in 50 mM Tris, pH 8.0) inhibited with KCN (1.9 equiv/$\alpha\beta$), reduced by dithionite (1 mM final concentration), cooled to 1 °C, and then exposed to 1 atm $CO_2$. After the first data point, sample was warmed to 31 °C. (Up-Triangles) $CODH_{Ct}$ (500 $\mu$L of 3.2 $\mu$M $\alpha\beta$, batch no. 5, 530 units/mg, thionin-oxidized in 50 mM Tris, pH 8.0) inhibited with KCN (3.0 equiv/$\alpha\beta$), reduced by dithionite (1 mM final concentration), and then reacted with ca. 40 mM $CS_2$. After the first data point, sample was warmed to 31 °C. (Diamonds) $CODH_{Ct}$ (500 $\mu$L of 3.2 $\mu$M $\alpha\beta$, batch no. 5, 500 units/mg, in 50 mM Tris, pH 8.0) oxidized by thionin (1 $\mu$L of 5 mN), inhibited by KCN (3 equiv/$\alpha\beta$), cooled to 1 °C, and then exposed to 1 atm $CO_2$. After the first data point, sample was warmed to 31 °C. (Down-Triangles) Same as up-triangles except no dithionite was added. The normalized activities of the triangles, circles, and squares data were obtained by dividing the observed activities by the average activity of the fully reactivated control using CO (480 units/mg). (Circles) Phenanthroline-treated $CODH_{Ct}$, prepared as described by Shin and Lindahl (1992b) (500 $\mu$L of 54 $\mu$M $\alpha\beta$, batch no. 5, 310 units/mg, in 50 mM Tris, pH 8.0), oxidized by thionin (1 $\mu$L of 5 mN), inhibited by KCN (4.1 equiv/$\alpha\beta$), and then exposed to 1 atm CO. Activities were normalized by dividing by the average of the fully reactivated control using CO (360 units/mg).

sample reactivated in a manner indistinguishable from native enzyme (Figure 6, circles).

*Dithionite, Not CO, Reduced $A_{420}$-Sensitive Fe-S Clusters in $CN^-$-Inhibited $CODH_{Ct}$.* The electronic absorption spectrum of $CODH_{Ct}$ contains broad S → Fe charge-transfer transitions in the 400-nm region, arising from at least two Fe-S clusters, including the $[Fe_4S_4]^{2+/1+}$ cluster that yields the $g_{av} = 1.94$ signals (Shin et al., 1992). When these clusters are reduced, the intensity at 420 nm ($A_{420}$) declines substantially. The overall redox status of these "$A_{420}$-sensitive" Fe-S clusters can thus be ascertained from changes in $A_{420}$.

$CN^-$-inhibited enzyme was monitored at $A_{420}$ before and after exposure to CO. $A_{420}$ of active, oxidized $CODH_{Ct}$ declined rapidly upon exposure (Figure 7A), while that of $CN^-$-inhibited, oxidized $CODH_{Ct}$ declined far more slowly (Figure 7B), at approximately the rate that $CN^-$-inhibited samples reactivated after being exposed to CO. The situation was quite different when $CN^-$-inhibited $CODH_{Ct}$ was reduced by dithionite. In this case, $A_{420}$ declined (Figure 7C) at essentially the same rapid rate as *active* $CODH_{Ct}$ was reduced by dithionite (Figure 7D).

*The NiFeC Signal Developed Slower Than CO Was Oxidized, and Its Development Was Inhibited by $CN^-$.* The turnover number of CO oxidation at 1 °C was measured to be $k_{cat} = 22\ s^{-1}$ (data not shown), while the NiFeC signal developed under similar conditions in accordance with $k = 0.01\ s^{-1}$ (Figure 8). This rate, ca. 2000 times slower than the rate of CO oxidation, was not noticeably affected by different concentrations of dithionite (50 $\mu$M to 1 mM) and CO (27–

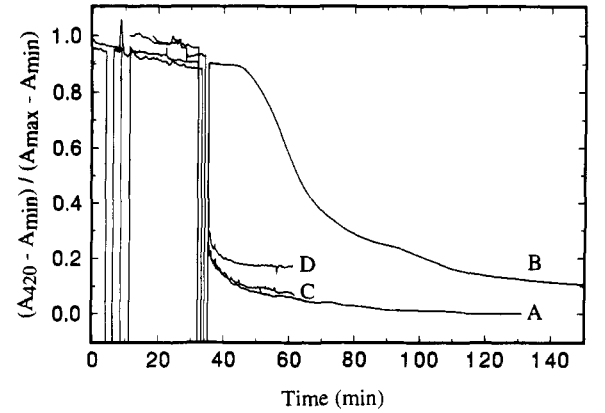



FIGURE 7: UV–vis monitoring of $CODH_{Ct}$ at 420 nm as a function of time after different treatments. (A) Thionin-oxidized $CODH_{Ct}$ (24 $\mu$M $CODH_{Ct}$ $\alpha\beta$, batch no. 3 in 50 mM Tris, pH 8.0, $A_{420}$ = 0.406) before and after exposure to 1 atm CO. CO was added during the region of the $A_{420}$ curve bounded by the sharp decline in $A_{420}$ near the 32 min mark, and the sharp increase a few min later, to be called a "gap" region. Gaps occurred when samples were removed from and reinserted into the spectrophotometer. Final $A_{420} = 0.260$. (B) Thionin-oxidized $CODH_{Ct}$ (24 $\mu$M $CODH_{Ct}$ $\alpha\beta$, $A_{420} = 0.404$) to which 3 equiv/$\alpha\beta$ KCN was added (first gap) followed by 1 atm CO (second gap). Final $A_{420} = 0.275$. (C) Thionin-oxidized $CODH_{Ct}$ (24 $\mu$M $CODH_{Ct}$ $\alpha\beta$, $A_{420} = 0.405$) to which 3 equiv/$\alpha\beta$ KCN was added (first gap), followed by 10 equiv/$\alpha\beta$ dithionite (second gap). Final $A_{420} = 0.286$. (D) Thionin-oxidized $CODH_{Ct}$ (24 $\mu$M $CODH_{Ct}$ $\alpha\beta$, $A_{420} = 0.404$) to which 10 equiv/$\alpha\beta$ dithionite (gap) was added. Final $A_{420} = 0.270$. Samples were in a 0.5-cm pathlength quartz optical cuvette with a double-septum seal. Data are plotted as $(A_{420} - A_{min})/(A_{max} - A_{min})$, where $A_{max} = 0.406$ and $A_{min} = 0.260$.

900 $\mu$M) and is similar to that measured previously by Gorst and Ragsdale (1991).

Development of the NiFeC signal was inhibited by $CN^-$. $CN^-$-inhibited, dithionite-reduced $CODH_{Ct}$ was incubated in 1 atm CO at 1 °C, and aliquots were frozen for EPR analysis at various times. An aliquot frozen after 5 min (Figure 9A) exhibited the NiFeC signal with an intensity only 10% of the fully developed signal (Figure 9C). Active enzyme incubated in CO would have exhibited a fully developed NiFeC signal well within 5 min. The rate of NiFeC development in $CN^-$-inhibited samples at 1 °C ($k_{obs} = 1.2 \times 10^{-4}\ s^{-1}$) was almost 5 times *faster* than the rate of reactivation ($k_{obs} = 2.5 \times 10^{-5}\ s^{-1}$) (Figure 10). An even greater difference in rates was obtained at 27 °C (Figure 11).

## DISCUSSION

*The Three Forms of the C-Cluster.* During *in vitro* catalytic oxidation of CO, $CODH_{Ct}$ is reduced by CO and oxidized by methyl viologen ($MV^{2+}$). The redox sites in the enzyme that function during catalysis probably have redox potentials between that of the $CO/CO_2$ couple (–520 mV at pH 7) and the $MV^{2+/1+}$ couple (–440 mV). $C_{S=0}$ is probably not part of the catalytic cycle because $E^{o\prime}$ for the $C_{S=0}/C_{1.82}$ couple (–220 mV) is too high. $C_{1.82}$ and $C_{1.86}$ may be involved because they are present within the relevant potential region.

If the C-cluster is the active-site for CO oxidation/$CO_2$ reduction, it must bind both CO and $CO_2$. The C-cluster in some state $C^{n+}$ oxidizes CO and, as a consequence, is reduced by two electrons, to a state $C^{(n-2)+}$. It also reduces $CO_2$ in some state and is oxidized by two electrons as a result. The simplest possibility is that $CO_2$ reacts with $C^{(n-2)+}$, yielding $C^{n+}$. These relationships are summarized in reaction 1.

$$C^{n+} + CO + OH^- \rightleftharpoons C^{(n-2)+} + CO_2 + H^+ \qquad (1)$$

Since CO oxidation/$CO_2$ reduction occurs reversibly in

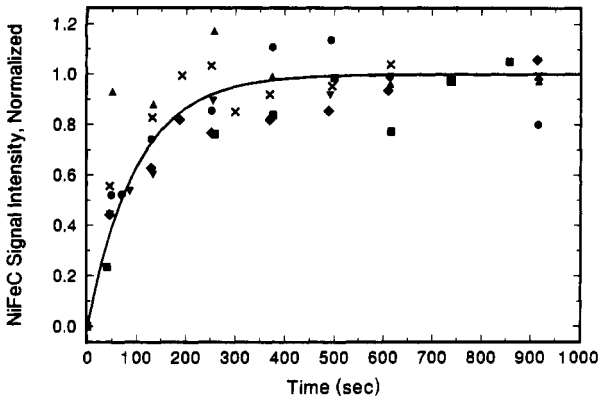



FIGURE 8: Kinetics of NiFeC signal development after adding CO to $CODH_{Ct}$. Aliquots of $CODH_{Ct}$ (25 $\mu$M $CODH_{Ct}$ $\alpha\beta$, batch no. 3 in 50 mM Tris, pH 8.0) were used to completely fill short EPR cuvettes with quartz chips, similar to those described (Shin & Lindahl, 1992a). Dithionite was added to each sample, mixed for 30 s, and incubated 5 min at ca. 27 °C. Samples were cooled to 1 °C. After 10 min, CO saturated buffer was added, and the solution was mixed for 30 s and frozen after various incubation times. (X's) 100 $\mu$L of 1 atm CO gas and 5 $\mu$L of 100 mM dithionite were added (900 $\mu$M CO, 1 mM $S_2O_4^{2-}$). NiFeC signal intensities were normalized by dividing by the average of the last eight values, 0.24 spin/$\alpha\beta$. (Circles) 15 $\mu$L of CO-saturated buffer and 5 $\mu$L of 100 mM dithionite were added (27 $\mu$M CO, 1 mM $S_2O_4^{2-}$). NiFeC signal intensities were normalized by dividing by the average of the last six values, 0.24 spin/$\alpha\beta$. (Down-Triangles) 30 $\mu$L of CO saturated buffer and 5 $\mu$L of 100 mM dithionite were added (54 $\mu$M CO, 1 mM $S_2O_4^{2-}$). NiFeC signal intensities were normalized by dividing by the average of the last five values, 0.21 spin/$\alpha\beta$. (Diamonds) 30 $\mu$L of CO saturated buffer and 5 $\mu$L of 10 mM dithionite were added (54 $\mu$M CO, 100 $\mu$M $S_2O_4^{2-}$). NiFeC signal intensities were normalized by dividing by the average of the last four values, 0.28 spin/$\alpha\beta$. (Squares) 30 $\mu$L of CO saturated buffer and 5 $\mu$L of 10 mM dithionite were added (54 $\mu$M CO, 100 $\mu$M $S_2O_4^{2-}$). NiFeC signal intensities were normalized by dividing by the average of the last five values, 0.13 spin/$\alpha\beta$. (Up-Triangles) 30 $\mu$L of CO saturated buffer and 5 $\mu$L of 4.6 mM dithionite were added (54 $\mu$M CO, 50 $\mu$M $S_2O_4^{2-}$). NiFeC signal intensities were normalized by dividing by the average of the last five values, 0.24 spin/$\alpha\beta$. The solid line is a simulation to the equation $[NiFeC]_t/[NiFeC]_{final} = 1 - \exp(-kt)$, where $[NiFeC]_t/[NiFeC]_{final}$ is the proportion of NiFeC signal developed at any time $t$, and $k = 0.01\ s^{-1}$.

$CODH_{Ct}$, the $C^{n+}/C^{(n-2)+}$ couple would be expected to have a redox potential near that of $CO/CO_2$. In addition, since the oxidation state of $C^{(n-2)+}$ and $C^{n+}$ differ by two electrons, the spin states of these forms of the C-cluster should both be either even or odd.

We propose that (i) $C_{1.82}$ and $C_{1.86}$ correspond to $C^{n+}$ and $C^{(n-2)+}$, respectively, (ii) the $C_{1.82}/C_{1.86}$ conversion arises from a two-electron reduction of the C-cluster, and (iii) $E^{o\prime}$ for the $C^{n+}/C^{(n-2)+}$ couple is the potential associated with the $C_{1.82}/C_{1.86}$ conversion, ca. −530 ± 35 mV. This proposal is summarized by reaction 2.

$$[C_{1.82}]^{n+} + CO + OH^- \rightleftharpoons [C_{1.86}]^{(n-2)+} + CO_2 + H^+ \quad (2)$$

Dithionite and other low-potential reductants can also reduce $C_{1.82}$ to $C_{1.86}$, as well as $C_{S=0}$ to $C_{1.82}$. Thus, the overall redox properties of the C-cluster are proposed to be

$$[C_{S=0}]^{(n+1)+} + e^- \rightleftharpoons [C_{1.82}]^{n+} \quad (3)$$

$$[C_{1.82}]^{n+} + 2e^- \rightleftharpoons [C_{1.86}]^{(n-2)+} \quad (4)$$

*The Effect of CN⁻ on the C-Cluster.* The $g_{av} = 1.72$ signal is obtained when $CN^-$ binds to the $C_{1.82}$ form of the C-cluster (Figure 1). No analogous shift in the $g_{av} = 1.86$ signal occurs immediately after $CN^-$ is added to enzyme in the $C_{1.86}$ form (Figure 3B). This suggests that $CN^-$ does not bind to the $C_{1.86}$ form of the C-cluster. Accordingly, dithionite probably temporarily protects the enzyme from the effects of $CN^-$ by reducing the C-cluster from the form that binds $CN^-$ ($C_{1.82}$) to the form that does not ($C_{1.86}$). Protection is temporary because $C_{1.82}$ and $C_{1.86}$ are in redox equilibrium and $CN^-$ binds to the small fraction of $C_{1.82}$ in dithionite-reduced samples. This binding slowly "pulls" the remaining $C_{1.86}$ molecules to the $C_{1.72}$ form (Figure 3).

*The Competitive Binding Proposal.* How might the ability of CO to reactivate $CN^-$-inhibited enzyme, and the inability of dithionite to do so (Figure 2), be explained? $CODH_{Rr}$ and the CODHs from other organisms are also inhibited by $CN^-$ and reactivated by CO. This phenomenon led Grahame and Stadtman (1987) to the reasonable conclusion that CO and $CN^-$ compete for the same binding site and that CO reactivates the enzyme by outcompeting $CN^-$ for that site. We will call this the competitive binding proposal (CBP), defined by reactions 5 and 6.

$$CODH\text{–}CN^- \rightleftharpoons CODH + CN^- \quad (5)$$

$$CODH + CO \rightleftharpoons CODH\text{–}CO \quad (6)$$

The dissociation constant for $CN^-$ is $K_{CN} = k_{off}/k_{on}$. We suggest that the forms of the enzyme in reactions 5 and 6 have the C-cluster forms given in reactions 7 and 8.

$$[C_{1.72}]^{n+}\text{–}CN^- \rightleftharpoons [C_{1.82}]^{n+} + CN^- \quad (7)$$

$$[C_{1.82}]^{n+} + 2CO + OH^- \rightleftharpoons [C_{1.86}]^{(n-2)+}\text{–}CO + H^+ + CO_2 \quad (8)$$

According to the CBP, CO reactivates $CN^-$-inhibited $CODH_{Ct}$ by binding $C_{1.86}$. Dithionite does not reactivate the enzyme because it does not bind $C_{1.86}$.

The only problem with this scenario is that CO does not appear to bind $C_{1.86}$. The $g_{av} = 1.86$ signal can be obtained by reducing $CODH_{Ct}$ with either CO, dithionite, or low-potential redox mediators (Lindahl et al., 1990a). CO is a strong-field ligand, known to dramatically affect the EPR signals of complexes to which it binds. If CO was bound to $C_{1.86}$, the EPR signal obtained by reducing the enzyme with dithionite or low-potential redox mediators should be significantly different. Yet the $g_{av} = 1.86$ signals obtained with each of these reagents are indistinguishable [compare Figures 3A and 4J and Figure 2 of Lindahl et al. (1990a)]. This insight was the first indication that the CBP was incorrect.

*Unrealized Implications of the Competitive Binding Proposal.* If the CBP was correct, and reactions 7 and 8 actually occurred, then the rate of reactivation would equal (ignore the charges on the various states)

$$-d[C_{1.72}\text{–}CN]/dt = k_{off}[C_{1.72}\text{–}CN] - k_{on}[C_{1.82}][CN] \quad (9)$$

The maximum rate of reactivation would occur when the second term of reaction 9 was eliminated. The ability of CO to reverse $CN^-$ inhibition arises (according to the CBP) because CO binds $C_{1.82}$, forming $C_{1.86}$–CO, and thus effectively eliminates the second term of reaction 9. In the limit, the $C_{1.82}$ concentration would equal 0 during reactivation, and the reactivation rate would be given by the first term of reaction 9 ($k_{off}[C_{1.72}\text{–}CN]$).

The second term of reaction 9 could also be eliminated or rendered insignificant by removing free $CN^-$ from solution and diluting the system significantly. In this case, the initial rate of reactivation should also be dominated by the first term

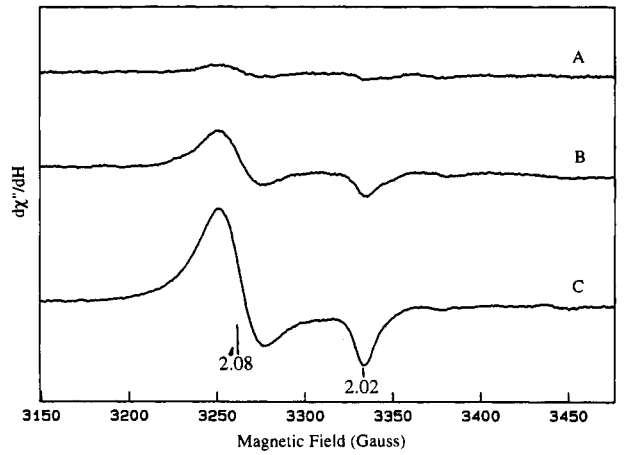
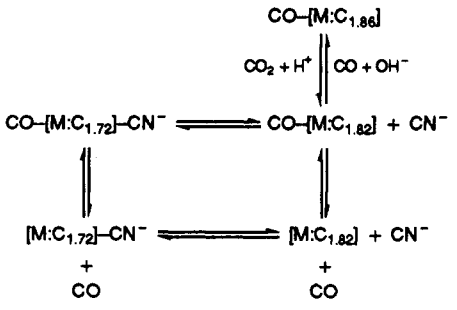



FIGURE 9: EPR spectra of $CN^-$-inhibited, dithionite-reduced $CODH_{Ct}$ frozen at various times after exposure to 1 atm CO. A glass tube (14 mm diameter × 15 cm long) containing a stir bar was sealed at one end and attached to a stopcock at the other. The open end of the stopcock was sealed with a rubber septum. To a sample of $CODH_{Ct}$ (6 mL of 25 $\mu$M, batch no. 3 in 50 mM Tris, pH 8.0) in the tube was added 25 $\mu$L of 0.25 M $S_2O_4^{2-}$ followed by 5 equiv/$\alpha\beta$ of KCN (73 $\mu$L of 10 mM). After 1 h, the solution exhibited no CO oxidation activity. The stirred solution was cooled to 1 °C, and then the gas phase of the apparatus was replace with 1 atm of CO. Aliquots were removed at various times after adding CO and were either assayed for CO oxidation activity or transferred to a cooled EPR tube and frozen immediately. (A) Aliquot frozen 5.3 min after adding CO. The NiFeC signal intensity was 0.013 spin/$\alpha\beta$. An aliquot removed soon afterward (at 8.3 min) had no activity. (B) Aliquot frozen 22 min after adding CO. The NiFeC signal had an intensity of 0.038 spin/$\alpha\beta$. An aliquot removed 2 min later had an activity of 14 units/mg. (C) Aliquot frozen 208 min after adding CO. The NiFeC signal had an intensity of 0.11 spin/$\alpha\beta$. An aliquot removed 2 min later had an activity of 140 units/mg. The final specific activity after reactivation was 400 units/mg, and the NiFeC intensity corresponded to 0.13 spin/$\alpha\beta$. The data from this experiment are plotted as the diamonds in Figure 10. EPR conditions: microwave frequency, 9.46 GHz; microwave power, 80 mW; temperature, 130 K; gain, $1 \times 10^5$; time constant, 0.327 s.

of reaction 9 (i.e., reactivation should occur at a rate similar to that obtained by adding CO). In contrast to this expectation, $CN^-$-inhibited $CODH_{Ct}$ did not reactivate when free $CN^-$ was removed and the enzyme was diluted 100-fold (Figure 5). This indicates that $k_{off}$ is actually very small (i.e., $CN^-$ binds very tightly) and that the rate of reactivation obtained by adding CO is not dictated by $k_{off}$. This experiment provided the second indication that the CBP was incorrect.

*The Modulative Binding Proposal.* CO also accelerates the dissociation of $CN^-$ from the CO oxidation site of $CODH_{Rr}$ (Ensign et al., 1989b; Hyman et al., 1989). Hyman et al. proposed that CO binds to two sites on $CODH_{Rr}$: the CO oxidation active site (where $CN^-$ binds) and a "modulator site" that serves, when bound with CO, to accelerate the dissociation of $CN^-$ from the active site. We define the modulative binding proposal for $CODH_{Ct}$ by the following scheme, where M is the modulator and C is the C-cluster.




(10)

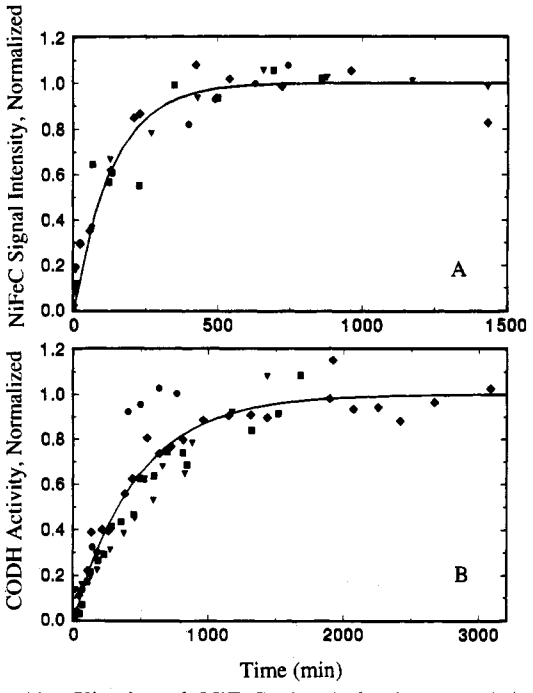



FIGURE 10: Kinetics of NiFeC signal development (A) and reactivation (B) after adding CO to $CN^-$-inhibited, dithionite-reduced $CODH_{Ct}$ at 1 °C. Experiments were performed as described in the legend to Figure 9. (Circles) 28 $\mu$M $CODH_{Ct}$ $\alpha\beta$ from batch no. 3, 1 mM $S_2O_4^{2-}$, 130 $\mu$M KCN, and 0.9 mM CO. Activities have been normalized, by dividing by the average of the last four activity points, 460 units/mg. Plotted NiFeC intensity data were normalized, by dividing by the average of the last four points, 0.095 spin/$\alpha\beta$. (Triangles) 28 $\mu$M $CODH_{Ct}$ $\alpha\beta$, 1 mM $S_2O_4^{2-}$, 130 $\mu$M KCN, and 0.9 mM CO. Plotted activities have been normalized, by dividing by the average of the last three activity points, 410 units/mg. Plotted NiFeC intensity data were normalized, by dividing by the average of the last three points, 0.21 spin/$\alpha\beta$. (Squares) Sample contained 24 $\mu$M $CODH_{Ct}$ $\alpha\beta$, 1 mM $S_2O_4^{2-}$, 73 $\mu$M KCN, and 0.9 mM CO. Activities have been normalized, by dividing by the average of the last four activity points, 370 units/mg. Plotted NiFeC intensity data were normalized, by dividing by the average of the last four points, 0.17 spin/$\alpha\beta$. (Diamonds) 24 $\mu$M $CODH_{Ct}$ $\alpha\beta$, 1 mM $S_2O_4^{2-}$, 120 $\mu$M KCN, and 0.9 mM CO. Activities have been normalized, by dividing by the average of the last eight activity points, 340 units/mg. Plotted NiFeC intensity data were normalized, by dividing by the average of the last five points, 0.13 spin/$\alpha\beta$. The solid line in A is a simulation to the equation $[\text{activity}]_t/[\text{activity}]_{final} = 1 - \exp(-kt)$, where $[\text{activity}]_t/[\text{activity}]_{final}$ is the proportion of reactivated enzyme at any time $t$, and $k = 2.5 \times 10^{-5}$ s$^{-1}$. The solid line in B is a simulation to the same equation (except for substituting NiFeC intensity for activity), where $k = 1.2 \times 10^{-4}$ s$^{-1}$. The difference between the NiFeC development and reactivation ($k = 9.5 \times 10^{-5}$ s$^{-1}$) reflects the apparent first-order rate constant for the reduction of $NiFe_{ox}$ by a mechanism independent of the C-cluster.

According to this proposal, $CN^-$ binds very tightly to C unless CO is bound at M, in which case $CN^-$ readily dissociates.

Hyman et al. (1989) found that $CO_2$, COS, $CS_2$, and $SO_2$ accelerate $CN^-$ dissociation from $CODH_{Rr}$, and suggested that these molecules also bind at a modulator site. We found (Figure 6) that $CODH_{Ct}$ behaved analogously with $CO_2$ and $CS_2$ (we have not examined the effects of COS and $SO_2$). Since both $CO_2$ and $CS_2$ required dithionite to be effective, the modulator in $CODH_{Ct}$ appears to be a redox-active species which can be reduced by dithionite and bound by $CO_2$ or $CS_2$ only in its reduced form.

The ability of $CS_2$ to modulate $CN^-$ binding at the C-cluster is especially important, because it provides a third argument against the CBP. The CBP requires that $CN^-$ and $CS_2$ bind at the same site of the C-cluster (the active site). Since $CN^-$ binds tightly, $CS_2$ would be required to bind even more tightly

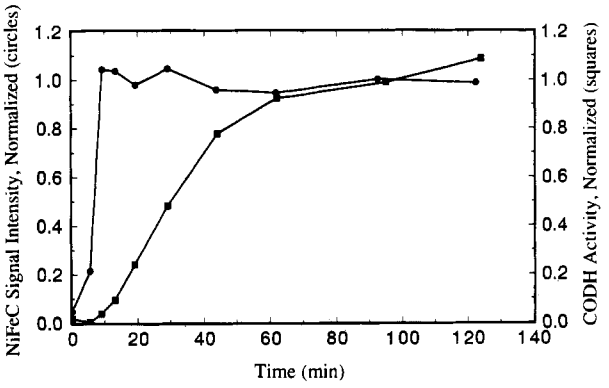



FIGURE 11: Kinetics of NiFeC signal development and reactivation after adding CO to $CN^-$-inhibited, dithionite-reduced $CODH_{Ct}$ at 27 °C. The experiment is described in the legend to Figure 4. Activities (squares) were normalized by dividing by the average activities for the last three points, 310 units/mg. NiFeC signal intensities (circles) were normalized by dividing by the average intensities for the last eight points, 0.14 spin/$\alpha\beta$.

(since it would displace $CN^-$ as it reactivated the enzyme). But this would render $CS_2$ a potent competitive inhibitor of CO oxidation, which it does not appear to be.

Which species in $CODH_{Ct}$ is the modulator? CO binds to the NiFe complex, so the NiFe complex could have been the modulator. However, our data provide evidence against this, because phenanthroline-treated enzyme, containing a non-functional NiFe complex devoid of Ni, reactivated in essentially the same manner as native enzyme (Figure 6).

Might the C-cluster itself be the modulator? Although our data indicate that $CN^-$ and the CO used in reactivation do not bind at the *same site* of the C-cluster, they do not exclude them binding different metal ions of the C-cluster or even different coordination sites of the same metal ion. CO could replace $CN^-$ by an associative mechanism and accelerate the dissociation of $CN^-$ indirectly by weakening the adjacent M–$CN^-$ bond. This proposal is simple and therefore appealing. Unfortunately, we are unable to explain how $CO_2$ and $CS_2$ reactivate the enzyme using this proposal. The problem is that $CO_2$ and $CS_2$ can only reactivate $CN^-$-inhibited enzyme reduced with dithionite (in the $C_{1.72}$ form), yet dithionite cannot reduce the $C_{1.72}$ form of the C-cluster (Figure 1C). Therefore how could $CO_2$ and $CS_2$ reactivate by binding $C_{1.72}$?

The only hypothesis we can presently devise, that is compatible with all relevant data, is that the modulator is (i) an autonomous redox-active species, (ii) magnetically separated from the C-cluster and the NiFe complex, (iii) able to bind CO in its oxidized form and $CO_2$ and $CS_2$ in its reduced form, and (iv) able, once bound, to accelerate the dissociation of $CN^-$ from the C-cluster, apparently by inducing a protein conformational change.

*Relation of the C-Cluster to the $A_{420}$-Sensitive Fe-S Clusters.* The ability of dithionite to reduce $A_{420}$-sensitive Fe-S clusters when $CN^-$ is bound at the C-cluster, and the inability of CO to do so (Figure 7), suggests that (i) CO reduces the $A_{420}$-sensitive Fe-S clusters via the C-cluster, (ii) $CN^-$-binds at the active site of the C-cluster and prevents CO from binding, (iii) dithionite can reduce the $A_{420}$-sensitive clusters either directly or via another redox site, and (iv) during the catalytic oxidation of CO, electrons are transferred from the C-cluster to the $A_{420}$-sensitive Fe-S clusters and eventually out of the enzyme to external redox agents. Thus, there appears to be an intraenzymic electron-transfer pathway from the C-cluster to the $A_{420}$-sensitive Fe-S clusters as shown in reaction 11.[3]

$$[C_{1.86}]^{(n-2)+} + 2[Fe_4S_4]^{2+} \rightleftharpoons [C_{1.82}]^{n+} + 2[Fe_4S_4]^{1+} \quad (11)$$

We propose that the $A_{420}$-sensitive Fe-S clusters are used to transfer electrons between other redox sites in the enzyme and external redox agents, as shown using $MV^{2+/1+}$ in reaction 12, and that dithionite reduces the $[Fe_4S_4]^{2+}$ clusters directly.

$$[Fe_4S_4]^{1+} + MV^{2+} \rightleftharpoons [Fe_4S_4]^{2+} + MV^{1+} \quad (12)$$

*Relation of the C-Cluster to the NiFe Complex.* To generate the NiFeC state, $NiFe_{ox}$ must be reduced by an electron and bound by CO. The electron used for reduction *can* originate from the oxidation of CO (i.e., from the C-cluster) (Shin & Lindahl, 1992a). We addressed the following questions: *Must* the electron originate from the C-cluster? Does reduction or CO-binding control the rate of NiFeC development? Is the reduction of the NiFe complex part of the CO oxidation catalytic cycle?

The ability of $CN^-$ to inhibit the rate at which the NiFeC signal developed in the presence of 1 atm CO (compare Figures 8 and 10, top panel) suggests that reduction, not CO-binding, controls the rate of NiFeC development and that the electrons used for reduction originate *predominantly* from the C-cluster. Thus, $CODH_{Ct}$ appears to contain an intramolecular electron transfer pathway from the C-cluster to the NiFe complex, as shown in reaction 13.[3]

$$[C_{1.86}]^{(n-2)+} + [NiFe_{ox}]^{m+} + [X]^{z+} \rightleftharpoons [C_{1.82}]^{n+} + [NiFe_{red}]^{(m-1)+} + [X]^{(z-1)+} \quad (13)$$

Given that the rate at which the NiFeC signal develops reflects the rate of reducing the complex, we compared the rates of NiFeC development (Figure 8) and CO oxidation to determine whether reducing $NiFe_{ox}$ was part of the CO oxidation mechanism. The fact that the NiFeC signal developed ca. 2000 times slower than CO oxidized indicates that reducing the NiFe complex is *not* a step in the catalytic mechanism used to oxidized CO, and it suggests that the intraenzymic electron pathway connecting the C-cluster to the $A_{420}$-sensitive Fe-S clusters does not include the NiFe complex.

*The NiFe Complex Can Be Reduced Independently of the C-Cluster.* The experiment where $CN^-$ inhibited the development of the NiFeC signal (Figure 10) was complicated by the ability of CO to accelerate the loss of $CN^-$ from the C-cluster and reactivate the enzyme. If the electrons used to reduce the NiFe complex originated *exclusively* from the C-cluster, the rate of NiFeC development would have equaled the rate of reactivation. That the rate of NiFeC development was nearly 5 times *faster* than reactivation (Figures 10 and 11) indicates that the NiFe complex of $CN^-$-inhibited molecules *can be reduced slowly* by some reductant other than CO. This reductant was probably dithionite.[4] Dithionite may have reduced $NiFe_{ox}$ directly, or indirectly through another intraenzymic electron pathway (e.g., via $A_{420}$-sensitive

[3] Since each $[Fe_4S_4]^{2+}$ undergoes $n = 1$ redox, and the C-cluster appears to undergo $n = 2$ redox, there must be some means by which an $n = 2$ process becomes two $n = 1$ processes. Because we do not know how this is done, reaction 11 is to be considered a stoichiometric reaction, not an elementary mechanistic step, and we leave open the question of whether the $2[Fe_4S_4]^{2+}$ are two different clusters or one cluster. A similar problem arises with reaction 13, since the NiFe complex undergoes $n = 1$ redox. The stoichiometry indicates that another species $[X]^{z+}$ must be reduced as well. We leave open the question of whether X is another $NiFe_{ox}$, an $[Fe_4S_4]^{2+}$ cluster, or some other species.

$Fe_4S_4$ clusters). Dithionite could not have reduced $NiFe_{ox}$ via the C-cluster pathway, because $CN^-$ was bound at the C-cluster while $NiFe_{ox}$ was being reduced, and dithionite does not appear able to reduce the C-cluster with $CN^-$ bound (Figure 1C).

Other studies also indicate that electrons from low-potential reductants like dithionite or viologens can be used to reduce the NiFe complex. $NiFe_{ox}$ was reduced by low-potential, electrochemically reduced redox mediators in the presence of $CO_2$ (Lindahl et al., 1990a) and acetyl-CoA (Gorst & Ragsdale, 1991). $NiFe_{ox}$ appears to be reduced to an EPR-active state by dithionite in the presence of $CS_2$ (Stephen W. Ragsdale, personal communication).

These molecules (CO, $CO_2$, $CS_2$, and acetyl-CoA) are either changing the thermodynamics or kinetics of the reduction process. Shin and Lindahl (1992a) argued that CO had to bind $NiFe_{ox}$ before the NiFe complex could be reduced by dithionite (they thought $E^{o\prime}$ for the $NiFe_{ox}/NiFe_{red}$ couple might be more negative than $E^{o\prime}$ for the dithionite/bisulfite couple and that the binding of CO to $NiFe_{ox}$ might increase $E^{o\prime}$ enough to render dithionite an effective reductant). Accordingly $CS_2$ and acetyl-CoA may bind $NiFe_{ox}$ as well (the $CO_2$ in the experiment cited above may have been reduced to CO and the CO may have bound). Alternatively, these molecules might be lowering a kinetic barrier to reduction. Given our evidence that CO, $CO_2$, and $CS_2$ bind at the modulator, we raise the possibility that the modulator may need to be bound before electrons from dithionite can reduce $NiFe_{ox}$ (i.e., the modulator might control intraenzymic electron pathways to the NiFe complex). Further studies are needed to evaluate these possibilities.

*Mechanism of CO → $CO_2$ Oxidation.* A model describing the mechanism of CO oxidation by $CODH_{Ct}$ is illustrated in Figure 12 and is defined by the following statements: The C-cluster is the active site for CO oxidation and $CO_2$ reduction. It can be stabilized in three oxidation states: an oxidized state ($C_{S=0}$), a one-electron-reduced state ($C_{1.82}$), and a three-electron-reduced state ($C_{1.86}$). CO and dithionite can each reduce $C_{S=0}$ stoichiometrically, resulting in $C_{1.82}$. This reduction is not part of the CO oxidation catalytic cycle. With the C-cluster in the $C_{1.82}$ form, the enzyme can oxidize CO catalytically. CO binds $C_{1.82}$, reacts with $OH^-$, becomes $CO_2$ as it transfers two electrons to $C_{1.82}$, and dissociates from $C_{1.86}$. The electrons are transferred from $C_{1.86}$ to the $A_{420}$-sensitive Fe-S clusters (reforming $C_{1.82}$), and then to external electron acceptors. The enzyme can oxidize CO in the $C_{1.82}$ form only; CO can neither bind to, nor can it be oxidized by, $C_{1.86}$.

The catalytic reaction operates in reverse, reducing $CO_2$ to CO. External reductants such as low-potential viologens or dithionite reduce the Fe-S clusters, which in-turn reduce $C_{1.82}$ to $C_{1.86}$. Two additional electrons from external reductants rereduce the Fe-S clusters. In this fully reduced enzyme state, $CO_2$ binds and removes two electrons from $C_{1.86}$, protonates, loses $OH^-$, and then dissociates from $C_{1.82}$ as CO. The Fe-S clusters reduce $C_{1.82}$ to $C_{1.86}$; then two additional electrons from external electron donors rereduce the Fe-S clusters, and $CO_2$ reduction can begin again.

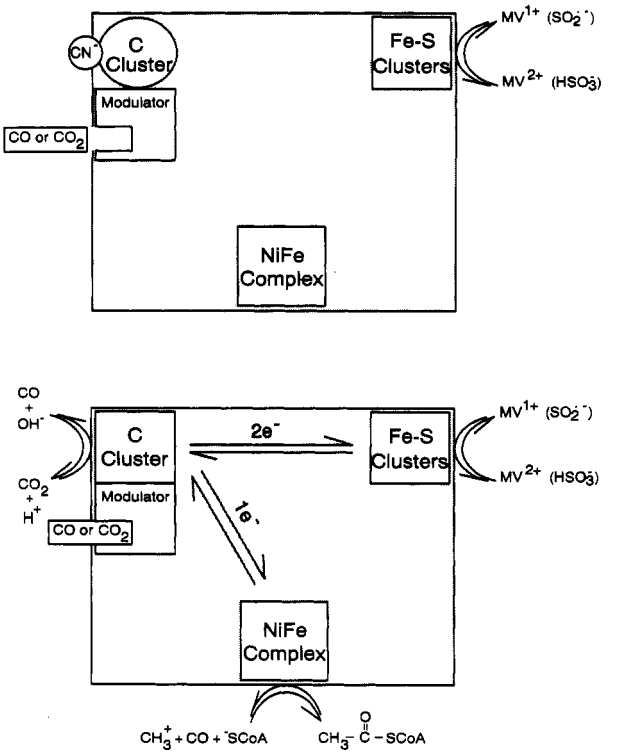



FIGURE 12: Model of CO oxidation, cyanide inhibition, and the reduction of the NiFe complex in $CODH_{Ct}$. (Upper Drawing) With $CN^-$ bound at the C-cluster and nothing bound at the modulator, the Fe-S clusters can be reduced by external reductants such as dithionite, but neither CO oxidation nor the reduction of the NiFe complex can occur. (Lower Drawing) With CO or $CO_2$ bound at the modulator, $CN^-$ dissociates from the C-cluster, allowing CO oxidation and reduction of the NiFe complex to occur. Electrons from the oxidation of CO at the C-cluster pass through the Fe-S clusters and then to external electron acceptors such as methyl viologen. The reactions occur reversibly. The NiFe complex can be reduced by a pathway originating from the C-cluster. Once reduced, the NiFe complex is able to catalyze the synthesis of acetyl-CoA.

The thermodynamics implied by this part of the model should be considered. The redox potential for the $CO/CO_2$ couple at pH 7 is −520 mV, while that for $C_{1.82}/C_{1.86}$ is −530 ± 35 mV. Thus, reducing $C_{1.82}$ to $C_{1.86}$ with CO occurs with little change in free energy. $E^{o\prime}$ for the $[Fe_4S_4]^{2+/1+}$ clusters is ca. −440 ± 35 mV, indicating that these clusters are spontaneously reduced by $C_{1.86}$. Reducing external reductants such as methyl viologen ($E^o$ = −440 mV) by the Fe-S clusters occurs with little change in free energy. In summary, the model proposed for CO oxidation is congruent with the redox potentials of the relevant clusters. The thermodynamics of the reverse reaction, $CO_2$ reduction, are less favorable under standard conditions, and this may result in a slower rate of $CO_2$ reduction *vis-à-vis* CO oxidation.

*Internal Electron Pathway Used To Reduce the NiFe Complex.* Our model includes the following statements as well: The NiFe complex is the active site for the synthesis of acetyl-CoA and various exchange reactions. It is stable in two redox states: an oxidized state that is diamagnetic ($NiFe_{ox}$) and a one-electron-reduced, CO-bound state that yields the NiFeC EPR signal. $NiFe_{ox}$ is reduced predominantly by electrons originating from the C-cluster. This reduction cannot be part of the CO oxidation catalytic cycle, as it is 2000 times slower than CO oxidation. $NiFe_{ox}$ can be reduced with $CN^-$ bound at the C-cluster, but the rate is 100 times slower. The

[4] We considered that the reductant was those few $CODH_{Ct}$ molecules that reactivated early in the experiment. No more than a very small fraction of the total $CODH_{Ct}$ would be needed to reduce all remaining $CN^-$-inhibited $CODH_{Ct}$ since the rate at which they would oxidize CO, and thus the rate at which they could reduce the NiFe complexes of those $CN^-$-inhibited molecules, would be much faster than the $CN^-$-inhibited molecules could reactivate. We discount this possibility, however, because the $A_{420}$-sensitive Fe-S clusters would have likely been reduced rapidly during reactivation *if* reactivated $CODH_{Ct}$ molecules could reduce $CN^-$-inhibited ones. As shown in Figure 7, the $A_{420}$-sensitive clusters were reduced only at a rate comparable to the rate of reactivation.

reductant for this process is probably dithionite, reducing $NiFe_{ox}$ either directly or indirectly through species other than the C-cluster (possibly through the $A_{420}$-sensitive Fe-S clusters).

The complex remains reduced and catalytically active as long as the C-cluster and Fe-S clusters also remain reduced. The C-cluster and Fe-S clusters, involved in the CO oxidation catalytic cycle, constantly undergo redox changes as electrons are transferred from CO to methyl viologen. It might be argued that the NiFe complex would oxidize and reduce synchronously with fluctuating redox levels of the C-cluster. However, we suspect that it does not, because the rate of NiFe complex reduction is far too slow to respond fast enough to such changes. Rather, the proportion of NiFe complexes in the reduced state during CO oxidation catalysis is probably determined by the time-averaged redox level of the C-cluster.

*Comparing This Model to That Proposed by Kumar et al. (1993).* After this paper had been submitted for publication, Kumar et al. (1993) submitted and recently published a pre-steady-state kinetic study of the reaction of $CODH_{Ct}$ (in the partially reduced $C_{1.82}$ state)[5] with CO. They found that $C_{1.82}$ converted to $C_{1.86}$ faster ($k_{obs} \sim 400\ s^{-1}$ at 5 °C) than the $g_{av} = 1.94$ signals developed ($k_{obs} \sim 60\ s^{-1}$ at 5 °C) and much faster than the NiFeC signal developed ($k_{obs} = 0.16\ s^{-1}$ at 25 °C). The NiFe complex was reduced ca. 1800 times slower than $k_{cat}$ for CO oxidation. These results are compatible with our results and they support our model. They indicate that CO reduces $C_{1.82}$ faster than $C_{1.86}$ reduces the $A_{420}$-sensitive Fe-S clusters.

Kumar et al. explained their results with a model similar to ours, except that they assigned the $C_{1.82}/C_{1.86}$ conversion to the binding of CO, as in reaction 14, not to the reduction by CO as we propose in reaction 2.

$$[C_{1.82}]^{n+} + CO \rightleftharpoons [C_{1.86}]^{n+}\text{–}CO \qquad (14)$$

They also propose that, during catalysis, electrons are transferred from the C-cluster to the NiFe complex and the Fe-S clusters "as $CO_2$ is formed". Besides the freeze–quench results just mentioned, the evidence cited in support of their model included (i) that the $C_{1.82}/C_{1.86}$ conversion rate depended on the concentration of CO, (ii) that the UV–vis spectrum of the enzyme was unchanged during the $C_{1.82}/C_{1.86}$ conversion, and (iii) that $CO_2$ causes an apparent increase in the reduction potential of $C_{1.86}$ (Lindahl et al., 1990a).

The first two lines of evidence are compatible with our model as well. For if the $C_{1.82}/C_{1.86}$ conversion reflected the reduction of $C_{1.82}$, as we propose, the conversion rate might also depend on CO concentration. Whether the absence of spectral changes during the conversion indicates a lack of redox chemistry (or CO binding, for that matter) is not known, since redox changes (or CO binding) need not dramatically alter the spectral properties of the C-cluster. Reducing $C_{S=0}$ to $C_{1.82}$, for example, did not cause significant spectral changes at 420 nm (Shin et al., 1992).

How the experiment cited as their last piece of evidence relates to either model is unclear. Lindahl et al. (1990a) measured the apparent $E^{o\prime}$ for the $C_{S=0}/C_{1.82}$ couple and the $C_{1.82}/C_{1.86}$ conversion to be $-220 \pm 35$ mV and $-530 \pm 35$ mV, respectively (at pH 7.2, under argon). Under $CO_2$ (at pH 6.3) the $g_{av} = 1.82$ signal was not observed at any potential examined (0 to –650 mV), and the $g_{av} = 1.86$ signal developed in accordance with $E^{o\prime}$ of $-360 \pm 35$ mV. We are unsure how to interpret these results, though it would appear that $CO_2$ can bind the enzyme (to the modulator?) at potentials more positive than those required to reduce it to CO and that the redox properties of the C-cluster are affected when $CO_2$ is bound. Further experiments are needed to clarify the chemistry involved.

One irrefutable experimental result seems problematic for the model proposed by Kumar et al. (1993), namely, that adding dithionite or reduced viologens to the $C_{1.82}$ form of $CODH_{Ct}$ affords $C_{1.86}$. If the $C_{1.82}/C_{1.86}$ conversion reflects the binding of CO, why should the conversion occur in the absence of CO by adding reductants? In the unlikely event that dithionite or reduced viologens bound to the C-cluster, it seems almost inconceivable that they would bind in the same manner as CO.

Another consideration seems problematic for their model, namely, that an unlikely concerted reaction would be required if electrons are transferred from the C-cluster to the NiFe complex and the Fe-S clusters "as $CO_2$ is formed". As we understand it, $OH^-$ would react with $[C_{1.86}]^{n+}$–CO, forming $[C_{1.86}]^{n+}$–$COO^-$ after $H^+$ dissociated. Then, as $CO_2$ is formed, an electron pair would be delivered to $[C_{1.86}]^{n+}$, affording an unstable three-electron-reduced form we shall call $[C^{\bullet\bullet\bullet}]^{(n-2)+}$. As $[C^{\bullet\bullet\bullet}]^{(n-2)+}$ is formed, it rapidly reduces the Fe-S clusters and the NiFe complex, reforming $[C_{1.82}]^{n+}$. Since $CODH_{Ct}$ catalyzes the reduction of $CO_2$ as well as the oxidation of CO, the principle of microscopic reversibility requires that $CO_2$ must bind to and be reduced by $[C^{\bullet\bullet\bullet}]^{(n-2)+}$. However, the timing involved in reducing $[C_{1.82}]^{n+}$ to the unstable $[C^{\bullet\bullet\bullet}]^{(n-2)+}$ form (using electrons from the Fe-S clusters) and *simultaneously* binding $CO_2$ to $[C^{\bullet\bullet\bullet}]^{(n-2)+}$ seems to render this proposal highly unlikely. Since the three-electron-reduced C-cluster form in our model ($[C_{1.86}]^{(n-2)+}$) is stable, no such synchronization of events is required.

$CODH_{Ct}$ is among the most complicated metalloenzymes known, and both models undoubtedly oversimplify the properties of the C-cluster and the actual catalytic mechanisms used by the enzyme. Nevertheless, we must start somewhere. Fortunately, both models are highly testable, and we plan to test them in the future. We are equally excited about obtaining further support for either model or modifying them as new experimental results demand.

[5] Kumar et al. (1993) use a different nomenclature: The NiFe complex, $A_{420}$-sensitive Fe-S clusters, and C-cluster are called centers A, B, and C, respectively. $C_{1.82}$ is called C and $C_{1.86}$ is called C′.

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