Interaction of Potassium Cyanide with the [Ni-4Fe-5S] Active Site Cluster of CO Dehydrogenase from Carboxydothermus hydrogenoformans

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The Ni-Fe carbon monoxide (CO) dehydrogenase II (CODHII\textsubscript{Ch}) from the anaerobic CO-utilizing hydrogenogenic bacterium Carboxydothermus hydrogenoformans catalyzes the oxidation of CO, presumably at the Ni-(\(\mu_2\)S)-Fe1 subsite of the [Ni-4S-5S] cluster in the active site. The CO oxidation mechanism proposed on the basis of several CODHII\textsubscript{Ch} crystal structures involved the apical binding of CO at the nickel ion and the activation of water at the Fe1 ion of the cluster. To understand how CO interacts with the active site, we have studied the reactivity of the cluster with potassium cyanide and analyzed the resulting type of nickel coordination by x-ray absorption spectroscopy. Cyanide acts as a competitive inhibitor of reduced CODHII\textsubscript{Ch} with respect to the substrate CO and is therefore expected to mimic the substrate. It inhibits the enzyme reversibly, forming a nickel cyanide. In this reaction, one of the four square-planar sulfur ligands of nickel is replaced by the carbon atom of cyanide, suggesting removal of the \(\mu_2\)S from the Ni-(\(\mu_2\)S)-Fe1 subsite. Upon reactivation of the inhibited enzyme, cyanide is released, and the square-planar coordination of nickel by 4S ligands is recovered, which includes the reformation of the Ni-(\(\mu_2\)S)-Fe1 bridge. The results are summarized in a model of the CO oxidation mechanism at the [Ni-4Fe-5S] active site cluster of CODHII\textsubscript{Ch} from \textit{C. hydrogenoformans}.

The hydrogenogenic thermophilic bacterium \textit{Carboxydothermus hydrogenoformans} utilizes CO\textsuperscript{3} as a sole source of energy and carbon under anaerobic chemolithoautotrophic conditions (1, 2). The oxidation of CO is catalyzed by two Ni-Fe CO dehydrogenases, designated CODH\textsubscript{Ch} and CODHII\textsubscript{Ch}, according to the equation CO + H\textsubscript{2}O → CO\textsubscript{2} + 2 H\textsuperscript{+} + 2 e\textsuperscript{−}. Crystal structures of CODHII\textsubscript{Ch} in different functional states have been solved to 1.1 Å resolution (3, 4). The homodimeric enzyme contains five metal clusters, of which clusters B, B\textquoteleft, and a subunit-bridging cluster D are conventional cubane-type [4Fe-4S] clusters (3). The active site clusters C and C\textprime; in dithionite-reduced active CODHII\textsubscript{Ch} (CO oxidation activity ~14,000 \(\mu\)mol of CO oxidized min\textsuperscript{−1} mg\textsuperscript{−1} at 70 °C) have been modeled as [Ni-4Fe-5S] centers containing a Ni-(\(\mu_2\)S)-Fe1 subsite. Their integral nickel ion is coordinated by 4S ligands with square-planar geometry (3, 4). The nickel coordination by 4S ligands in CODHII\textsubscript{Ch} was also apparent from x-ray absorption spectroscopy (XAS) (5). A defined non-functional [Ni-4Fe-4S] form of cluster C can be produced by treatment of CODHII\textsubscript{Ch} with CO in the absence of low potential reductants, resulting in inactivation and the loss of the bridging \(\mu_2\)S (4).

It has been assumed that CODHII\textsubscript{Ch} catalyzes the oxidation of CO at the Ni-(\(\mu_2\)S)-Fe1 subsite of cluster C (3). The prime candidate for CO binding is the nickel ion because of its facile accessibility through the substrate channel and its empty apical coordination site (3). Fe1 is the presumed OH\textsuperscript{−} donor ligand in CO\textsubscript{2} formation (3, 6). The CODH\textsubscript{Mto} from the aerobic bacterium \textit{Oligotropha carboxidovorans} oxidizes CO at the Mo-(\(\mu_2\)S)-Cu subsite of the [Cu-S-MoO\textsubscript{2}] active site, in which copper and molybdenum are bridged by a cyanolyzable sulfane \(\mu_2\)S (7, 8). The enzyme is inactivated when \(\mu_2\)S is removed and reactivated when \(\mu_2\)S is reinserted (9). The Mo-(\(\mu_2\)S)-Cu subsite resembles the Ni-(\(\mu_2\)S)-Fe1 bridge in cluster C of CODHII\textsubscript{Ch}. The mechanism of CO oxidation based on the x-ray structure of [Cu-S-MoO\textsubscript{2}] CODH\textsubscript{Mto} with bound inhibitor \(n\)-butyl isocyanide involves a thiocarbonate-like intermediate state and proposes the binding of CO between the \(\mu_2\)S and copper (equivalent to nickel in CODHII\textsubscript{Ch}) and the binding of an OH\textsuperscript{−} group at molybdenum (equivalent to Fe1 in CODHII\textsubscript{Ch}) (7).

Structures of cluster C of Ni-Fe CODHs from \textit{Rhodospirillum rubrum} (CODH\textsubscript{Rr}) (10) and \textit{Moorella thermoacetaica} (CODH\textsubscript{Mto}) (11, 12) also showed the positions of the five metal ions in cluster C of CODHII\textsubscript{Ch} but did not reveal the bridging \(\mu_2\)S. Since sulfur sulfide was found to inhibit CODH\textsubscript{Rr} and CODH\textsubscript{Mto}, it has been concluded that cluster C with the bridging \(\mu_2\)S, as has been observed in CODHII\textsubscript{Ch} from \textit{C. hydrogenoformans} (3, 4), might represent an inhibited form (13). On the other hand, it has been shown that the [Ni-4Fe-4S] cluster mis-
ing the bridging $\mu_2S$ is an inactivated decomposition product originating from the [Ni-4Fe-5S] cluster (4). A catalytic mechanism suggesting the apical binding of CO at nickel and the coordination of OH\(^{-}\) in the bridging position between the nickel and Fe1 ions was proposed for CODH\(_{Fr}\) and CODH\(_{Mt}\) (13–15).

To clarify some of these aspects and get further insights into the mechanism of CO oxidation, we were interested to study how CO interacts with cluster C of CODH\(_{IICh}\) and how the bridging $\mu_2S$ might be involved in catalysis. Although some crystal structures have modeled apical CO at the nickel ion, the occupancies of CO were very low in CODH\(_{Mt}\) (12), or the potential CO was modeled apical CO at the nickel ion, the occupancies of CODH\(_{IICh}\) (3), and inhibits the oxidation of CO by Ni-Fe CODHs has been attributed to the binding of cyanide plus 2 mM DTT, 4 mM Ti(III) citrate, 2 mM DTT or without reductants under an atmosphere of CO or N\(_2\). Inhibition was initiated by the addition of KCN. Aliquots were removed with time and analyzed for CO oxidation activity.

**Reactivation of Cyanide-inhibited Reduced CODH\(_{IICh}\)**

Cyanide-inhibited reduced CODH\(_{IICh}\) was prepared by treatment of the enzyme with KCN under non-turnover conditions. CODH\(_{IICh}\) (109 $\mu$g) was incubated for 20 min at 23 °C under N\(_2\) in 1 ml of buffer A containing 75 $\mu$M KCN, 4 mM dithionite, and 4 mM DTT. Such treatment resulted in complete loss of CO oxidation activity. To lower the concentration of KCN in reactivation assays to non-inhibitory 0.07 $\mu$M, samples of inhibited CODH\(_{IICh}\) were diluted 11 times in buffer A containing 2 mM DTT under N\(_2\). For reactivation, 10 $\mu$l of diluted samples were added to 1 ml of buffer A without reductants or with 4 mM dithionite plus 2 mM DTT, 4 mM Ti(III) citrate, or 2 mM DTT under CO or N\(_2\). Reactivations were performed at 23, 50, and 70 °C.

**Effect of Sodium Sulfide on CODH\(_{IICh}\)**

Sodium sulfide (Na\(_2\)S) was added to CODH\(_{IICh}\) activity assays under turnover conditions, to the reactivation assays of cyanide-inhibited reduced CODH\(_{IICh}\) and to the as isolated CODH\(_{IICh}\) under non-turnover conditions.

**Preparation of Samples for XAS—Dithionite-reduced CODH\(_{IICh}\)**

(CODH-DT, 15,400 units mg\(^{-1}\)) was produced by treatment of as isolated enzyme under N\(_2\) with 4 mM dithionite for 5 min at 23 °C followed by concentration under N\(_2\) to 99 mg ml\(^{-1}\). CO-treated dithionite-reduced CODH\(_{IICh}\), (CODH-CO, 14,600 units mg\(^{-1}\)) was produced by incubation of as isolated enzyme under CO in the presence of 4 mM dithionite for 1 h at 50 °C followed by concentration under CO to 85 mg ml\(^{-1}\). For dithionite-reduced CODH\(_{IICh}\) reversely inhibited by cyanide (CODH-CN\(^{a}\) and CODH-CN\(^{b}\)), the as isolated enzyme was diluted under N\(_2\) with buffer A containing 4 mM dithionite and 2 mM DTT to 0.25 (CODH-CN\(^{a}\)) or 0.46 mg ml\(^{-1}\) (CODH-CN\(^{b}\)). KCN was added to the final concentrations of 1 mM.
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(CODH-CN\textsuperscript{a}) and 200 \mu M (CODH-CN\textsuperscript{b}). After a 40-min incubation at 23 °C with gentle stirring, the activity was completely inhibited. The samples were concentrated under N\textsubscript{2} to 64 (CODH-CN\textsuperscript{a}) and 132 mg ml\textsuperscript{-1} (CODH-CN\textsuperscript{b}) and displayed activities of 700 and 44 units mg\textsuperscript{-1}, respectively. CODH-CN\textsuperscript{a} and CODH-CN\textsuperscript{b} were reversibly inhibited since they could be reactivated to 5,400 and 13,100 units mg\textsuperscript{-1}, respectively, upon incubation at 70 °C under CO or N\textsubscript{2} in buffer A containing 4 mM Ti(III) citrate or 4 mM dithionite plus 2 mM DTT.

CODH\textsubscript{IICh} reactivated after the inhibition by cyanide (CODH-react., 13,100 units mg\textsuperscript{-1}) was prepared from the concentrated CODH-CN\textsuperscript{b}. To remove the unbound cyanide, CODH-CN\textsuperscript{b} (70 \mu l) was dissolved in 0.5 ml of buffer A under N\textsubscript{2} containing 4 mM dithionite and 2 mM DTT and concentrated to 50 \mu l by ultrafiltration under N\textsubscript{2}. The procedure was repeated two times. For the reactivation, the concentrated enzyme (50 \mu l) was dissolved in 30 ml of buffer A with 4 mM dithionite and 2 mM DTT to a protein concentration of 0.3 mg ml\textsuperscript{-1} and incubated at 70 °C under N\textsubscript{2} for 40 min. Reactivated CODH\textsubscript{IICh} was concentrated under N\textsubscript{2} to 157 mg ml\textsuperscript{-1}, yielding sample CODH-react.

Ni-K Edge XAS Measurements—For XAS measurements, 25 \mu l of each sample were filled into plastic cells covered with Kapton foil. Cells were sealed and kept in liquid N\textsubscript{2}. The Ni-K edge XAS data, 8.2–9.2 keV, were collected in fluorescence mode with a silicon (111) monochromator at the EMBL beamline D2 (DESY, Hamburg, Germany). Harmonic rejection was achieved by a focusing mirror (cut-off energy 20.5 KeV) and a monochromator detuning to 70% of its peak intensity. The sample cells were kept at ~20 K in a two-stage Diplex cryostat. Automated data reduction, such as normalization and extraction of the fine structure, was performed with KEMP (24) assuming an energy threshold \( E_{d,Ni} = 8,333 \text{ eV} \). Sample integrity during exposure to synchrotron radiation was checked by monitoring the position and shape of the absorption edge on sequential scans. No change in redox state or metal environment was detectable.

X-ray Absorption near Edge Structure (XANES) Analysis—The Ni-K edge position was defined at the energy corresponding to a normalized absorbance of 0.5 (25). The extracted 1s \rightarrow 3d pre-edge feature, isolated by subtracting an arctangent function and a first order polynomial to the rising edge (25), was fitted to a single Gaussian function centered at ~8,332 eV. Its intensity corresponds to the integrated area of the Gaussian function.

Extended X-ray Absorption Fine Structure (EXAFS) Analysis—The extracted Ni-K edge (20~700 eV) EXAFS were converted to photoelectron wave vector k-space and weighted by \( k^3 \). The spectra were analyzed with EXCURV98 (26), refining the theoretical EXAFS for defined structural models based on the curved-wave theory. In addition to single scattering contributions, multiple scattering units were defined for linear Ni–C–N and square-planar Ni–4S. Parameters of each structural model, namely the atomic distances (\( R \)), the Debye-Waller factors (2\( \sigma^2 \)), and a residual shift of the energy origin (EF), were optimized, minimizing the fit index (\( \Phi \)) while keeping the number of free parameters below those of the independent points (26, 27). Throughout the data analysis, the amplitude reduction factor was kept at 1.0. The reduced \( \chi^2 \) test verified the significance of an additional ligand in the models (26).

RESULTS AND DISCUSSION

Inhibition of CODH\textsubscript{IICh} by Potassium Cyanide—Potassium cyanide inhibits CO oxidation activity of CODH\textsubscript{IICh}, under catalytic (turnover) conditions in the presence of CO and electron acceptor methyl viologen (Figs. 1, A and B, and 2A) as well as under non-turnover conditions in the absence of CO and acceptor (Fig. 1, C and D). The rate of inhibition depends on time (Fig. 1, A–D), the cyanide concentration (Fig. 1, A–C), and the incubation temperature (Fig. 1, A and B). Since CODH\textsubscript{IICh} displays a high temperature optimum for activity (2), the temperature dependence of inhibition indicates a similar mode of interaction of cyanide and CO with the active site. This is supported by the double reciprocal plot of initial activity versus CO concentration as a function of cyanide concentration, revealing a pattern characteristic of competitive inhibition and a \( K_I \) of 21.7 \mu M cyanide (Fig. 2A).

The inhibition by cyanide under non-turnover conditions greatly depends on the redox state of CODH\textsubscript{IICh} (Fig. 1, C and D). Reduced CODH\textsubscript{IICh} incubated with low potential reductants dithionite or Ti(III) citrate (redox potential \( \approx -500 \text{ mV} \)) is inhibited more strongly than the more oxidized enzyme incubated with the weak reductant DTT (\( \approx -330 \text{ mV} \)) or without reductants. This indicates an efficient interaction of cyanide with the highly reduced cluster C of CODH\textsubscript{IICh} at redox potentials of \( \approx -500 \text{ mV} \). Obviously, CO also interacts with cluster C of CODH\textsubscript{IICh} at very low potentials since the oxidation of CO in *C. hydrogenoformans* (\( -520 \text{ mV} \)) is coupled to the reduction of protons to H\textsubscript{2} (\( -410 \text{ mV} \)). Therefore, cyanide interacts with the reduced cluster C of CODH\textsubscript{IICh} in a similar fashion as the substrate CO.

CO protects reduced CODH\textsubscript{IICh} against inhibition by potassium cyanide since there is no decrease of activity under CO in contrast to complete inhibition under N\textsubscript{2} (Fig. 1D), whereas the oxidized enzyme is not protected by CO (Fig. 2B). The protection by CO suggests that cyanide and CO share a common binding site at the reduced cluster C. The effects of temperature (Fig. 1, A and B), redox dependence (Fig. 1, C and D), protection by CO (Fig. 1D), and competitive character of inhibition with respect to CO (Fig. 2A) suggest that the inhibition of reduced CODH\textsubscript{IICh} by cyanide is due to the occupation of the CO binding site.

Reactivation of Cyanide-inhibited Reduced CODH\textsubscript{IICh}—Inhibition of dithionite- or Ti(III) citrate-reduced CODH\textsubscript{IICh} by potassium cyanide is fully reversible since the enzyme can be completely reactivated (Fig. 1, E and F). CO, high temperature (70 °C), and the presence of dithionite or Ti(III) citrate accelerate the reactivation and increase the maximum level of regained activity as compared with reactivation under N\textsubscript{2} at lower incubation temperatures (23 or 50 °C) and in the absence of low potential reductants (Fig. 1, E and F).

The significant acceleration of reactivation under CO (Fig. 1, E and F), which apparently displaces cyanide at the CO binding site, indicates again that the interaction of reduced CODH\textsubscript{IICh} with cyanide mimics its interaction with CO. The effect of CO is evident at 23 and 50 °C (Fig. 1E). At 70 °C, the effect of CO is...
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FIGURE 1. A and B, inhibition of CODHIICh by potassium cyanide under turnover conditions. Assays in A contained 0.87 ng ml⁻¹ CODHIICh, and 0 (○), 50 (●), 100 (▲), 250 (■), or 1,000 (▲) μM KCN at 70 °C. 100% activity in A and C–H corresponds to 14,800 units mg⁻¹. Assays in B contained 5.35 ng ml⁻¹ CODHIICh, and 0 (○), 1 (●), 5 (▲), 10 (■), or 20 (▲) mM KCN at 23 °C. 100% corresponds to 1,400 units mg⁻¹. C and D, inhibition of CODHIICh, by potassium cyanide under non-turnover conditions. Assays in C contained 1.4 μg ml⁻¹ CODHIICh, under N₂, 4 mM dithionite (filled symbols), or no reductants (open symbols) and 0 (○), 10 (▲), or 100 (■) mM KCN at 23 °C. Assays in D contained 0.19 μg ml⁻¹ CODHIICh, under N₂ (filled symbols) or CO (open symbols), 15 μM KCN, and 4 mM dithionite plus 2 mM DTT (●), or no reductants (▲) at 23 °C. E and F, reactivation of cyanide-inhibited CODHIICh. Assays contained 0.1 μg ml⁻¹ of inhibited CODHIICh under CO (filled symbols) or N₂ (open symbols). Reactivation in E was performed in the presence of 4 mM dithionite plus 2 mM DTT at 23 °C, E and F, 50 (▲), or 70 °C (■). Reactivation in F was performed in the presence of 4 mM dithionite plus 2 mM DTT (●), 4 mM Ti(III) citrate (▲), 2 mM DTT (■), or without reductants (▲, △) at 70 °C. G and H, effect of sodium sulfide on CODHIICh. Reactivation assays in G contained 0.12 μg ml⁻¹ of cyanide-inhibited CODHIICh. Assays in H were under non-turnover conditions and contained 0.12 μg ml⁻¹ of as isolated CODHIICh. Assays were performed in the presence of 4 mM dithionite and 2 mM DTT (●), 4 mM dithionite, 2 mM DTT, and 0.2 mM Na₂S (▲), 4 mM dithionite, 2 mM DTT, and 1.0 mM Na₂S (■), or 1 mM Na₂S (▲) at 70 °C.

FIGURE 2. A, competitive inhibition of CO oxidation by CODHIICh under turnover conditions in the presence of CO, methyl viologen, and cyanide. KCN was added to serum-stoppered cuvettes for the assay of CO oxidation activity prior to the addition of CODHIICh. Reactions were initiated by the addition of 10 μl of stock enzyme solution (0.144 μg ml⁻¹). The different CO concentrations were established by adding the appropriate amounts of CO-saturated reaction mixture to assays containing the same reaction mixture saturated with N₂. Vₐ indicates the initial activity in units mg⁻¹. KCN concentrations in the cuvettes were 0 (○), 24.6 (▲), and 49.0 (■) μM. B, effect of CO on the inhibition of oxidized CODHIICh by cyanide under non-turnover conditions. CODHIICh (0.19 μg ml⁻¹) was incubated with 2 μM KCN in the absence of reductants under an atmosphere of CO (●) or N₂ (○) at 23 °C. C, effect of sodium sulfide on the activity of CODHIICh under turnover conditions in the presence of CO and methyl viologen. Na₂S was added to serum-stoppered cuvettes for the assay of CO oxidation activity containing 1.05 ng ml⁻¹ CODHIICh; 100% activity corresponds to 14,800 units mg⁻¹.

Low potential reductants are required for fast and complete reactivation (Fig. 1F). Inhibited CODHIICh regains initial activity after a 15–25-min incubation at 70 °C with dithionite or Ti(III) citrate under CO or N₂. In contrast, slower and partial reactivation to 30–50% of the initial activity occurs with DTT or without reductants (Fig. 1F).
The reactivation patterns discussed above further substantiate that the inhibition of reduced \( \text{CODH}_{\text{IICh}} \), originates from the occupation of the CO binding site by cyanide. This inhibition is not due to any decomposition of cluster C since the activity can be completely recovered (Fig. 1, E and F).

**Effect of Sodium Sulfide on \( \text{CODH}_{\text{IICh}} \)**—Sodium sulfide has no effect on the reactivation of cyanide-inhibited \( \text{CODH}_{\text{IICh}} \) in the presence of dithionite (Fig. 1G). Partial reactivation with sulfide alone (Fig. 1G) is brought about by its function as a strong reductant and not as a sulfur source since the enzyme can be completely reactivated in the presence of \( \text{Ti(III)} \) citrate alone (Fig. 1F). Sulfide does not inhibit \( \text{CODH}_{\text{IICh}} \) under non-turnover conditions in the presence or absence of dithionite (Fig. 1H) as well as under turnover conditions (Fig. 2C). Apparently, sulfide does not affect CO oxidation by \( \text{CODH}_{\text{IICh}} \), which is in contrast to the reported inhibition of \( \text{CODH}_{\text{Rr}} \) and \( \text{CODH}_{\text{Mr}} \) by sulfide and to the suggested inhibitory role of the bridging \( \mu_2\text{S} \) (13).

**XAS of Dithionite-reduced \( \text{CODH}_{\text{IICh}} \)**—Ni-K edge XAS on highly active dithionite-reduced \( \text{CODH}_{\text{IICh}} \) (\( \text{CODH-DT}, 15,400 \text{ units mg}^{-1} \)) reveals the nickel coordination in functional \( \text{CODH}_{\text{IICh}} \) in solution. The XANES spectrum (Fig. 3A) resembles that of the four-coordinate square-planar complexes of nickel (25). It shows a small shoulder near 8,337 eV, which has been observed in tetragonal geometries lacking one or more axial ligands and has been assigned to a 1s \( \rightarrow 4p_z \) transition (with shakedown contributions). The spectrum exhibits a very weak 1s \( \rightarrow 3d \) pre-edge peak centered at \(-8,332 \text{ eV}\). The normalized integrated area of this peak is 0.030 eV. The 1s \( \rightarrow 3d \) transition is dipole-forbidden; however, it can gain intensity due to p-d mixing in non-centrosymmetric geometries. Thus, planar complexes will feature weak transitions with areas of \(-0.0–0.029 \text{ eV}\), whereas the tetrahedral ones will display stronger transitions with areas of \(-0.08–0.114 \text{ eV}\) (25). In \( \text{CODH-DT} \), the combination of weak 1s \( \rightarrow 3d \) transition and a shoulder on the rising edge indicates that the nickel ion is four-coordinate with a square-planar geometry. The edge energy of 8,338.5 eV is consistent with a \( \text{Ni}^{2+} \) oxidation state of the nickel ion (25).

The EXAFS spectrum provides further insight into the metal coordination (Fig. 3B, trace a). The amplitude envelope of the oscillations, e.g. its maximum at \(-6.5 \text{ Å}^{-1} \), is indicative of the presence of elements heavier than oxygen and nitrogen in the vicinity of the absorber atom. The lack of the beat node-like change in the EXAFS amplitude marks a homogenous ligand sphere. This is further substantiated by the Fourier transform of the EXAFS data showing one dominant peak at \(-2.2 \text{ Å} \) and small contributions at \(-2.8 \text{ and } -4.4 \text{ Å}\) (Fig. 3C, trace a). Both 2.2 and 4.4 Å peaks could be best fitted with four Ni–S interactions at 2.23 Å in the square-planar geometry (Table 1). No further interactions with light atoms, e.g. Ni–O, at shorter bond lengths were required for a good fit.

The Ni–S bond lengths depend on the nickel geometry. In four-coordinate \( \text{Ni}^{2+} \) complexes containing thiolate ligands, the Ni–S bond lengths range from 2.14 to 2.24 Å for approximately square-planar geometries and from 2.26 to 2.33 Å for tetrahedral geometries (28). The Ni–S distances in \( \text{CODH}_{\text{IICh}} \) (Table 1) are in the range for square-planar complexes. Independent evidence for this coordination arises from the multiple scattering via the central nickel atom within the Ni-4S system, visible at about 4.4 Å in the Fourier transforms. Such features only occur when the scattering vector is close to 180°.

To identify the potential contributors to the \(-2.8 \text{ Å}\) peak in the Fourier transform spectrum, two different scenarios have been considered. Based on the crystal structure of functional \( \text{CODH}_{\text{IICh}} \), the most probable ligands to nickel at this distance are 2Fe ions at \(-2.8 \text{ and } -2.9 \text{ Å}\) (3). To test their presence, a single 2Fe shell was modeled first, but its refinement resulted in a relatively high Debye-Waller factor, and thus, had to be discarded. Followed by that, a single 1Fe shell at \(-2.7 \text{ Å}\) was fitted (supplemental Table 1). The inclusion of a second iron contri-
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**TABLE 1**

EXAFS refinement parameters for different CODHIICh samples

| Ligand Atom | Nickel Site | 1s Transition | 2pz Transition |
|-------------|-------------|---------------|---------------|
| 1 Ni—4S     | 2.23 (2)    | 0.0095 (3)    | 9.9 (4)       |
| 1 Ni—Fe     | 2.71 (2)    | 0.020 (4)     |               |
| 1 Ni—Fe     | 2.99 (2)    | 0.020 (5)     |               |

*Note: Values in parentheses represent statistical errors (± standard deviation) of the least square refinement.*

bution at ~2.9 Å significantly improved the fit, as shown by the 5% drop of the fit index (Table 1). The data published previously on the as isolated CODHIICh, did not show any evidence for Ni-Fe contribution(s), whereas for the Ni treated and Ti(III) citrate-reduced samples, only one Ni-Fe interaction at ~2.7 Å was detected (5). The lack of the Ni-Fe contributions or their weak signal was then attributed to the destructive interference between 2.7 and 2.9 Å Ni-Fe components within the ~5–10 Å⁻¹ range (5). In the present studies, a partial cancellation of both Ni-Fe signals takes place as well (especially between 5 and 8 Å⁻¹). However, a longer photoelectron wave number (k) range as compared with the previous XAS data on CODHIICh (5) ensures that both of the components can be detected. The lack of any substantial numerical correlation during the EXAFS data refinement between the structural parameters of both iron shells further supports this statement.

The model comprising 4S atoms at 2.23 Å in a square-planar geometry and 2Fe atoms at 2.71 and 2.59 Å (Table 1) correlates well with the crystal structure of functional CODHIICh (3, 4). However, the 2.71 Å Ni–Fe distance is shorter than the shortest Ni–Fe bond (2.82 Å) found by x-ray crystallography (3, 4). It may reflect a redox-dependent conformational change in cluster C due to the slightly different redox state of the protein in samples studied by XAS and crystallography as it was previously suggested (5).

**XAS of CO-treated Dithionite-reduced CODHIICh**—XAS on highly active CO-treated dithionite-reduced CODHIICh (CODH-CO, 14,600 units mg⁻¹) was performed to determine the effect of CO on the nickel coordination and to identify the binding position of CO. XAS revealed no change in nickel geometry and ligand sphere composition upon CO treatment. The Ni-K edge spectrum is almost identical to that of the CODH-DT with a small shoulder at ~8,337 eV and a pre-edge peak at ~8,332 eV (Fig. 3A). The edge energy has not changed and is consistent with the Ni²⁺ state. EXAFS demonstrated that the nickel coordination has not been altered by CO treatment (Fig. 3, B and C, traces b). The final structural model is consistent with the model for CODH-DT and comprises 4S atoms at 2.23 Å in a square-planar geometry and 2Fe atoms at 2.71 and 2.96 Å (Table 1). A single 1Fe shell at ~2.7 Å was tested as well, but the fit was significantly worse, as demonstrated by the 8% increase of the fit index value, as compared with 2Fe model (supplemental Table 1). The obtained results are similar to the data on CO-treated CODHIICh published previously (5). However, the average Ni–S bond length found in this study is slightly shorter (2.23 (2) Å versus 2.252 (3) Å) and has a lower Debye-Waller factor (0.0080 (3) versus 0.0155 Å²), which indicates the lower structural disorder of the 4S shell in the present CO-treated CODHIICh sample. As the crystal structure of functional CODHIICh, briefly treated with CO (4), the model for CODH-CO indicates the presence of μ₂-S and the absence of bound CO. Therefore, after turnover of CO, cluster C remains in the functional state with 4S ligands at nickel. Since a carbon atom was not apparent in the vicinity of the nickel ion, the reaction product CO₂ obviously leaves the active site very quickly without the formation of a stable carboxyl intermediate.

**XAS of Dithionite-reduced CODHIICh Reversibly Inhibited by Cyanide**—XAS on dithionite-reduced CODHIICh reversibly inhibited by cyanide (CODH-CN⁰ with 700 units mg⁻¹ and CODH-CN⁰ with 44 units mg⁻¹) elucidates cyanide binding to cluster C. The XANES patterns of both samples almost line up with each other (Fig. 3A) but differ significantly from those of CODH-DT and CODH-CO, indicating significant changes of structure and/or ligand composition of the nickel site. Both XANES spectra also exhibit features of CODH-DT and CODH-CO, i.e. weak 1s → 3d pre-edge peak and a small shoulder due to the 1s → 4p transition. However, the position of the shoulder is shifted slightly toward lower energies, and the edge energy...
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increases by $\sim 1.2$ eV. This edge shift could indicate an increase in the nickel oxidation state. However, the preserved low intensities of the $1s \rightarrow 3d$ transition ($0.031$ and $0.036$ eV for CODH-CN$^a$ and CODH-CN$^b$, respectively) exclude such a possibility and are consistent with the Ni$^{3+}$ oxidation state of the nickel ion (25). Instead, the change in the hardness of some of the donor atoms is more likely. A general shift to lower edge energies has been observed for complexes with increasing numbers of sulfur-donor ligands (25). Thus, the observed changes indicate that cyanide substitutes for one of the sulfur ligands without affecting the square-planar geometry of the nickel site.

In the EXAFS spectra of CODH-CN$^a$ and CODH-CN$^b$, the sharp oscillations at $\sim 6.5$ Å$^{-1}$, present in CODH-DT and CODH-CO, are diminished (Fig. 3B, traces c and d), and a beat node-like change in the regular sinusoidal pattern emerges. This indicates heterogeneous ligand sphere, most probably caused by cyanide binding, and is visualized in both Fourier transform spectra (Fig. 3C, traces c and d). As compared with CODH-DT and CODH-CO, a small peak at $\sim 1.8$ Å emerges, whereas the $\sim 2.8$ Å contribution is replaced by a broad peak centered at $\sim 3.0$ Å. The 4.4 Å peak marking a 4S square-planar geometry in CODH-DT and CODH-CO decreases to the noise level. The 2.2 Å peak intensity decreases by $\sim 30\%$. These features are likely caused by a cyanide ligand replacing one of the sulfur ligands. Based on the reduced $\chi^2$ test (26), the best fit among all considered models was obtained for a nickel ion coordinated by three sulfur atoms at $\sim 2.20$ or $\sim 2.23$ Å and one CN group with a Ni–C distance of 1.81 or 1.84 Å for CODH-CN$^a$ and CODH-CN$^b$, respectively (Table 1). The refined Ni–C and C–N bond lengths are consistent with Ni$^{2+}$ complexes with cyanide ligands (29). Assuming Ni–C distances of 1.81 or 1.84 Å, Ni–N distances of 3.00 Å, and C–N distance in cyanide of 1.15–1.18 Å (29, 30), cyanide binds to the nickel ion of cluster C by its carbon atom in a linear fashion.

Therefore, in CODH$^{\text{III\_ch}}$, reversibly inhibited by cyanide, one of the Ni–S bonds is cleaved and one CN ligand is bound to nickel in square-planar geometry. This suggests that cyanide cleaves the labile bond between nickel and the bridging $\mu_2S$ (4) and binds to nickel at the coordination site previously occupied by the $\mu_2S$.

XAS of CODH$^{\text{III\_ch}}$, Reversibly Inhibited by Cyanide and Then Reactivated—XAS on CODH-react. (13,100 units mg$^{-1}$) determines the nickel coordination in highly active CODH$^{\text{III\_ch}}$ formed after reactivation of enzyme reversibly inhibited by cyanide. The Ni-K edge shape is almost identical to that of CODH-DT and CODH-CO, indicating the same square-planar geometry and oxidation state of nickel in CODH-react. (Fig. 3A). However, the normalized integrated area of the $1s \rightarrow 3d$ increases (0.040 eV), suggesting a slightly disordered geometry of the nickel site. EXAFS confirms this observation (Fig. 3B, trace e). As compared with CODH-DT, the intensity of the Ni–S backscattering contribution is lowered by $\sim 15\%$ (Fig. 3C, trace e), but multiple scattering contributions within the square-planar Ni-4S unit significantly improve the fit. Thus, a slightly disordered square-planar geometry of the nickel site is likely, especially because the average Ni–S bond length has not changed as compared with CODH-DT and CODH-CO (Table 1). This is consistent with the CODH$^{\text{III\_ch}}$ crystal structure where the partial occupance of the $\mu_2S$ has been observed (4) and refers to a minor component lacking the fourth sulfur ligand. Activities (15,400 units mg$^{-1}$) in CODH-DT versus 13,100 units mg$^{-1}$ in CODH-react.) indicate the presence of roughly 15% catalytically non-competent enzyme in CODH-react., which presumably contains a Ni-CN. Then, the reactivated component must be formed entirely as a NiS$_3$ site. Thus, the model for reactivated CODH$^{\text{III\_ch}}$ is similar to that of CODH-DT, comprising four square-planar sulfur at 2.23 Å and 2Fe at 2.69 and 2.97 Å (Table 1).

CO Oxidation at the [Ni-4Fe-5S] Cluster of CODH$^{\text{III\_ch}}$—This study shows that cyanide is an inhibitor of CODH$^{\text{III\_ch}}$ because it competes with CO at the reduced [Ni-4Fe-5S] cluster. XAS indicates that the reversible inhibition of CODH$^{\text{III\_ch}}$ with 4S coordinated nickel (CODH-DT) results in a 3S and 1CN coordinated nickel (CODH-CN$^a$ and CODH-CN$^b$). The binding of cyanide to nickel cleaves the bond between the nickel ion and the bridging $\mu_2S$, which stays bound to Fe1 since after reactivation, the 4S coordination of nickel is reestablished (CODH-react.), and external sulfide is not required for reactivation (Fig. 1, F and G). The requirement of reduced conditions for reactivation (Fig. 1F) indicates that the Fe1-bound $\mu_2S$ should be in its S$^{2-}$ state to produce the bridge.

We feel that our data do not support a mechanism of CO oxidation at the [Ni-4Fe-5S] cluster of CODH$^{\text{III\_ch}}$ from C. hydrogenoformans involving binding of oxygen in a bridging position between nickel and iron (13–15) since this position will be occupied by sulfur in an isolated state or by CO after the binding of the substrate, and XAS did not identify an oxygen ligand to nickel in any of the examined states of the enzyme. On the other hand, since we have not captured any of the intermediates...
described in Fig. 4, the possibility of an oxygen atom, which transiently bridges the two metals during catalysis, cannot finally be ruled out. Our results also argue against an inhibitory role of the bridging \( \mu_2\)S in cluster C of CODHIII\text{Ch} (13). We did not observe an inhibition of CODHIII\text{Ch} by sulfide. As we cultivate \textit{C. hydrogenoformans} in the presence of 3.3 mM Na\textsubscript{2}S as a reductant of the growth medium, the compound is apparently not toxic to the bacteria.

In analogy to the dithionite-reduced, cyanide-inhibited, and reactivated states of CODHIII\text{Ch} analyzed by XAS, as well as in accordance with the structure-based mechanism of CODHIII\text{Ch} (3), we propose a mechanism of CO oxidation at the [Ni-4Fe-5S] cluster (Fig. 4). An incoming CO molecule reaches the cluster through the substrate channel ending at the nickel ion (Fig. 4A) where it binds, resulting in a square-pyramidal five-coordinate intermediate (Fig. 4B). A water molecule binds as OH\textsuperscript{−} to the histidine-coordinated Fe1 as proposed previously (6). The resulting CO to OH\textsuperscript{−} distance exceeds 4 Å, which does not allow interaction (3). The \( \mu_2\)S ligand dissociates from the nickel and remains bound to pentacoordinated Fe1 as \( \text{S}_2\text{O} \). A simultaneous rearrangement leads to a square-planar nickel intermediate with three sulfur and one CO ligand. This moves CO toward the OH\textsuperscript{−}, making a nucleophilic attack possible (Fig. 4C). The proposed rearrangement agrees with the models describing the conversion of CO by a nickel to sulfur rebound mechanism studied with model compounds containing four-coordinate nickel (31, 32). The nickel-bound CO undergoes a nucleophilic attack by the Fe1-bound OH\textsuperscript{−}, forming a nickel-bound carboxylic acid group. The carboxylic acid group is deprotonated by the Fe1-bound \( \text{S}_2\text{O} \), which is ideally situated to act as a catalytic base, resulting in a thiol group and a preformed CO\textsubscript{2} (Fig. 4D). The proton of the thiol group is subsequently transmitted via His-261 to His-93 and Lys-563 of the assumed proton transfer chain (33), whereas the sulfide stays bound to Fe1 (Fig. 4E). Dissociation of the nickel carboxyl generates two electrons that are delocalized on the [3Fe-4S] subcluster and transferred further through clusters B, B\textsubscript{2}, and D to the external electron acceptor. Simultaneously, the Ni-(\( \mu_2\))S–Fe1 bridge is being reformed (Fig. 4A), and a new reaction cycle can proceed. This mechanism resembles that of CODH\textsubscript{ox} from \textit{O. carboxydovorans}, containing a Cu-(\( \mu_3\))S–Mo bridge in the active site. In that enzyme, the insertion of CO between the copper, the sulfido-ligand, and the hydroxo-group at molybdenum, thereby forming a thio carbonate intermediate, has been proposed (7, 8).

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