Structural bases for the catalytic mechanism of Ni-containing carbon monoxide dehydrogenases†

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Significant progress has been made recently in our understanding of the structure/function relationships of the catalytic C-cluster of carbon monoxide dehydrogenases. Several structures of this enzyme have been reported, some of them at very high resolution. One recurrent problem, however, is the high degree of heterogeneity within each structure, as well as between the different X-ray models. Here, we have tried to relate the structural data with the wealth of spectroscopic and biochemical information gathered over many years. As a result, we propose a catalytic cycle that is consistent with both observations and stereochemistry. We also give alternatives to one of the most difficult aspects of the cycle, namely, the location of the two electrons in the most reduced state of the C-cluster.

1 Introduction

In anaerobic microorganisms the reduction of CO₂ and the oxidation of CO is mediated by the Ni-containing C-cluster of the enzyme CO dehydrogenase (CODH) according to:

\[
\text{CO} + \text{OH}^- \leftrightarrow \text{CO}_2 + 2e^- + \text{H}^+ \quad (1)
\]

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Ni-containing CODH’s can be divided into four classes.¹ Class I and II enzymes are found in methanogenic archaea such as Methanosarcina thermophila, class III enzymes are normally found in acetogens such as Moorella thermoacetica, whereas class IV enzymes are found in anaerobic bacteria such as Rhodospirillum rubrum and Carboxydothermus hydrogenoformans. Class I–III enzymes are bifunctional as they also catalyze either the synthesis or the decarbonylation of acetyl-CoA, a universal source of carbon and energy to the cell, according to:

\[
\text{CH}_3\text{CO}^{\text{III}}\text{FeSP} + \text{CO} + \text{CoA-SH} 
\leftrightarrow \text{CH}_3\text{CO}\text{SCoA} + \text{CoFeSP} + \text{H}^+ \quad (2)
\]

where CoFeSP is a heterodimeric corrinoid–cobalt–iron–sulfur cluster protein.

In methanogens, the different enzymes form a multimeric αβδε acetyl-CoA decarboxylase/synthase (ACDS) complex where α has CODH activity, β is the acetyl-CoA synthase (ACS) and γδε corresponds to CoFeSP.² In acetogens, ACS activity is located in the β subunit (homologous to β in ACDS) whereas the CODH activity is found in the β subunit (homologous to α in ACDS), both subunits forming an αβ tetramer.³ These microorganisms use the Wood–Ljungdahl pathway to synthesize an acetyl group from two CO₂ molecules.⁴ One of these molecules is fully reduced to a corrinoid-bound methyl group that is subsequently transferred to ACS whereas the

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Juan C. Fontecilla-Camps received his undergraduate training at the University of Concepcion, Chile, and his Ph. D. degree in protein crystallography at the University of Alabama in Birmingham under the supervision of Charles E. Bugg in 1980. Subsequently, he moved to Marseilles, France, where he became group leader and then head of a protein crystallography laboratory established by the CNRS and the University of Aix-Marseille. In 1991, he took a job with the French Commissariat l’Energie Atomique (CEA) in Grenoble. After having worked on the crystal structure of animal toxins, in 1993 he turned his attention to the structural biology of hydrogenases and other metalloenzymes. He and his group have solved the structures of NiFe and Fe-only hydrogenases, pyrivate–ferrodoxin oxidoreductase and, very recently, carbon monoxide dehydrogenase/acytetyl-Coenzyme A synthase. In 2000, he was awarded the medal of the European section of the International Society of Biological Inorganic Chemistry for his work on hydrogenases. He is also “Chevalier dans l’Ordre des Palmes Académiques”.  

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second CO$_2$ is only partially reduced to CO by CODH according to eqn. (1). Metal-based insertion/migration of the methyl and CO groups at the ACS A-cluster produces an acetyl group that binds CoA according to reaction 2. Class IV CODHs are monofunctional and form a$_2$ dimers. In these organisms, external CO can be used as a source of carbon and reducing power. Aerobic bacteria such as *Oligotropha carboxidovorans* utilize molybdopterin- and copper-containing CODHs, which are unrelated to the nickel- and iron-containing CODHs found in anaerobic bacteria and archaea.

2 The structure of the C-cluster

Crystal structures of nickel and iron-containing CODHs have been reported for *C. hydrogenoformans* (Ch),* R. rubrum* (Rr)* and the bifunctional *M. thermoacetica* CODH/ACS$_{Mt}$.*1–11* These homodimeric CODHs contain a total of five FeS clusters (Fig. 1a). The most exposed D-cluster that was first detected by X-ray crystallography, is coordinated by two cysteine residues (Fig. 1a). The most exposed D-cluster that was first detected by X-ray crystallography, is coordinated by two cysteine residues. In anaerobic bacteria and archaea.

Before going any further into the structural analysis it is necessary to introduce some nomenclature on the various redox states of the C-cluster. A diamagnetic state C$^{red_1}$ is obtained at potentials below $-200$ mV. One-electron reduction of C$_{C}$ produces the C$^{ox_1}$ state that converts to C$^{ox_2}$ upon addition of CO or other reductants. At pH 7 the midpoint potential of the C$^{ox_2}$ state is $-520$ mV, close to the E° of $-512$ mV of the CO/CO$_2$ redox couple of reaction 1. Both C$^{ox_1}$ states exhibit EPR spectra typical for an S = 1/2 spin state. Another undetected diamagnetic state called C$^{red_2}$ is postulated to arise from the one-electron reduction of C$^{ox_2}$ or from the one-electron oxidation of C$^{red_2}$.

All the CODH atomic models reported so far have been obtained from crystals grown under strictly anaerobic conditions and, in most cases, reducing agents have been added to the crystallization solutions (Table 1). High temperature factors and/or partial occupation for the C-cluster atoms in the refined models indicate the presence of structural heterogeneity in all the structures deposited with the Protein Data Bank. By far, the best resolutions and refinement statistics have been reported for the mono-functional CODH$_{Ch}$ enzyme.*1–4* In four of the five CODH$_{Ch}$ structures an inorganic sulfur atom (labeled S2) has been modeled at the E2 site (Fig. 1b). This labile sulfur atom is absent from the structures of the Rr and Mt enzymes. Most CO-treated crystals show the presence of a ligand at the apical E1 coordination site of the Ni, located at the end of the hydrophobic tunnel. This ligand is tentatively assigned as partially occupied CO in CODH$_{Mt}$ and as an unknown species in CODH$_{Ch}$.

Recently, Dobbek *et al.* have reported that incubation of CODH$_{Ch}$ with CO leads to rapid inactivation, especially when...
no other reducing agents are present. These authors observed a positive correlation between the presence of the inorganic Ni–Fe bridging S2 atom and the enzymatic activity of the corresponding crystalline enzyme; they reported that both the S2 ligand and the activity were lost after prolonged CO treatment. From these observations Dobbek et al. concluded that C-clusters that lack S2 represent non-functional states. However, Feng and Lindahl have shown that sulfide reversibly inactivates both CODHRr and CODHMt and that addition of Na2S to the Cred1 state leads to changes in its EPR spectrum. The inhibitory effect of sulfide can be reversed by reduction with CO and dithionite because, as with many other anions, sulfide will not bind to the Cred2 state. For instance, CN− that is known to interact with FCII, as indicated by Mössbauer spectroscopy and ENDOR results, can be dissociated from the C-cluster under the same reducing conditions. Moreover, CODH is known to reduce the CO2 analog COS to CO and SH−, in a reaction equivalent to (1). Since the bridging E2 site is likely to bind OH− during catalysis it should also bind the analog SH−. Taken together, these observations suggest that the bridging S2 in CODHCh is a signature of the sulfide-inhibited state reported by Feng and Lindahl and that that center mimics the OH− bound C-cluster.

A comparison of the various refined C-cluster structures shows that the [3Fe–4S] sub-site is relatively well ordered whereas S1, Fe1 (the putative Feu or FCII) and especially the Ni are either significantly agitated or only partially occupied (Table 1, Fig. 2). In the crystal structure of CO-treated CODHr the C-cluster has been modeled as a distorted [Ni–3Fe–4S] cubane. The Ni coordination environment includes a bridging cysteine ligand to Fe1 and it may be described as distorted trigonal bipyramidal, with an exogenous ligand that could be CO, bound opposite to S1. Fe1 has a distorted tetrahedral coordination. In all the CODHCh structures the C-cluster adopts a more open conformation with Ni–S1 distances that are 1.1–1.5 Å longer than in CODHr. The Ni has either distorted square planar or square pyramidal coordination whereas Fe1 retains the distorted tetrahedral coordination. In three of the deposited structures, PDB codes ISU6, ISU7 and ISUF, an apical Ni ligand has been modeled as water (although in the latter structure it could be CO, given the experimental conditions). Alternative positions for some Fe ions and cysteine side chains were observed in the CODHCh structures refined at very high resolution. An alternative position for Fe1 in the ISUF model (not shown) would convert the [3Fe–4S] sub-site into an approximately regular [4Fe–4S] cubane when Ni is absent. It is known that Ni-depleted C-clusters are not catalytically competent and, until recently, they have been considered to have spectroscopic characteristics similar to those of a $S = 3/2$ [4Fe–4S] cluster. However, as further discussed below, very recent MCD and Raman data have challenged this idea.

In the first reported CODH/ACSMt structure (PDB code 1MJG) both the C-cluster Fe1 and Ni ions are coordinated by only three ligands, a rather uncommon situation for these metal ions. This C-cluster structure has not been described in the literature but the Ni–Fe distance of 2.7 Å is compatible with the presence of a bridging hydride, which would not be detectable at 2.2 Å resolution. A bridging hydride would bring the number of Fe and Ni ligands to four, a much more plausible proposition. The largest displacement of the Ni with respect to S1 and Fe1 is the one found by us in the structure of another CODHCh.

Fig. 2 Crystallographic C-cluster models of CODH. (a) Rr, (b–f) Ch, (g–h) Mt, with PDB codes 1JQK, 1JJY, ISU6, ISU7, ISU8, ISUF, 1MJG and 1OAO, respectively, (h) Mt, another crystal of the same form as (h), refined at 2.5 Å resolution. See also Table 1.
CoA synthesis by CODH/ACSMt in the presence of reductants and nas hydrogenase.

To the assay and showed that there was no acetyl-CoA synthesis. Out the possibility that Hb would reversibly bind CO under their containing protein did not affect acetyl-CoA synthesis. To rule (Hb) as a CO scavenger, they also showed that this heme-group of acetyl-CoA according to reaction 1 and the reaction such as Ti(III) citrate or methyl viologen, was 40 times faster under CO/CO2 than under CO/Ar.

The first hint pointing out the existence of tunnels connecting the C- and A-cluster came from the work by Grahame and DeMoll who, in 1995, concluded that the physiological substrate of ACDS from M. barkeri was CO2 and not CO. This conclusion was based on the fact that CO-dependent reactions had a rate of acetyl-CoA synthesis that was about one-half of the way obtained using CO2 plus reduced ferredoxin. These authors postulated that this was due to the artificial nature of CO as a substrate. However, as we shall see below, CO is the natural substrate of the A-cluster when supplied endogenously. Synthesis or cleavage of acetyl-CoA depended on the amount of H2 present and on a specific hydrogenase.

In 1999, Maynard and Lindahl showed that the rate of acetyl-CoA synthesis by CODH/ACSMt in the presence of reductants such as Ti(III) citrate or methyl viologen, was 40 times faster under CO/CO2 than under CO/Ar. Furthermore, the rate did not depend on the presence of CO. Using hemoglobin (Hb) as a CO scavenger, they also showed that this heme-containing protein did not affect acetyl-CoA synthesis. To rule out the possibility that Hb would reversibly bind CO under their experimental conditions Maynard and Lindahl supplied Hb-CO to the assay and showed that there was no acetyl-CoA synthesis. Their conclusion was that, physiologically, it is CO from the reduction of CO2 at the C-cluster that combines with CH2 at the A-cluster to generate acetyl-CoA and that a tunnel connects both active centers.

Seravalli and Ragsdale provided additional evidence for the existence of such a channel.26 They studied the isotopic exchange reaction between labeled 13CO and the unlabeled carbonyl group of acetyl-CoA according to reaction 1 and the reaction below, catalyzed by the A-cluster:

\[(\text{CH}_3)_2\text{C}=\text{O} + \text{CO} \rightarrow (\text{CH}_3)_2\text{C}=\text{O} + \text{CO} \] (3)

They observed that 33% of the exchange reaction could still take place in the presence of a high concentration of Hb, when all the solution CO should have been complexed. From this they concluded that under their conditions exchange was mediated by CO2-derived CO traveling through a tunnel connecting the C- and A-clusters. Although high levels of external CO did not inhibit incorporation of labeled carbon from CO2 to acetyl-CoA, the exchange represented only 60% of the expected value, probably because some CO formed from unlabeled acetyl groups at the A-cluster, migrated back to the C-cluster and was oxidized to CO2. These authors concluded that CO generated at the C-cluster is prefered to CO from solution and that tunnels are advantageous for animal hosts because the potentially toxic CO remains confined to the enzyme interior.

The crystallographic studies of CODH/ACSMt have confirmed the existence of these extensive tunnels.10-11 However, this tunneling system poses a major problem, namely, how does CO gain access to the C-cluster. We postulated that CO2 could diffuse from the A-cluster because this center can be accessible to the medium when CoFeSP delivers the methyl group to the Ni ion.21 However, Tan et al. have very recently shown that (putatively) blocking the tunnel connecting the C- and A-clusters by three mutations, A222L, A265M and A110C, does not prevent significant CO oxidation activity (that involves diffusion of CO2 to the external medium).22 Additional mutations at other potential tunnels regions, transient and thus not detected by X-ray crystallography, will be required before this issue is settled. Determining which residues should be mutated may be helped by molecular dynamics studies. It should also be noted that the tunnels observed in Rr and Ch CODHs are different from those found in CODH/ACSMt but in every case the tunnel points to the same apical coordination site (E1) of the Ni ion of the C-cluster (Fig. 3). At any rate, CO2 transit through the protein matrix is unlikely as we have shown to be the case for H2 diffusion in NiFe hydrogenases.23

Lindahl and co-workers have defined two modes of operation for CODH/ACSMt: the “reductase” (i.e. CO2 reduction) and the “synthase” (i.e. acetyl-CoA synthesis) modes.24 The fact that Hb had no effect on the kinetics under “reductase” mode indicated that there is no equilibrium between external and internal CO. Under this mode, CO synthesis is less efficient. Indeed, it has been shown that Kcat/Keq ratios of CO2 reduction to CO differ by a factor of 100 depending on whether the bi-functional enzyme is producing acetyl-CoA or just reducing CO2. In the presence of an excess of CH2=CoFeSP (which would stabilize the open a-subunit and close the tunnel) the amount of external CO decreased, as measured by CO-Hb formation. This effect has been assigned to coupling between the A and C clusters.25 However, the crystal structure indicates that either electronic or conformational changes induced by small effectors such as CO are unlikely to connect the two active centers given the long distance that separates them.26-31 Instead, we believe that any correlated effects have to be attributed to the concentrations of substrate and product traveling through the tunnels and possibly having a crowding inhibitory effect depending on the conformational changes that the a subunit undergoes that affects opening and closing of the tunnel connecting the A and the C clusters.
The time dependence of the IR band decay the rates of (4) and (5) showed that the Ni ion in the C-cluster was not part of a NiFe$_4$S$_4$ cluster. A 2.7 Å distance from the nickel ion appeared (Fe1$^-$) and the Ni–S distances were more homogeneous because it can also be a ligand to Ni but as an electron donor because it can also be reduced. The strongest band occurred at 1996 cm$^{-1}$, and the Ni–S bands gradually disappeared with time, but with heterogeneity observed in the crystal structures of CO-treated CODHCh. Different CO complexes are formed, consistent with the C-cluster geometry of the Fe–S site. This also applies to the Zn-substituted C-cluster.

Gu and co-workers reported no changes in the Ni absorption edge upon reduction of CODH$_{Ch}$ by either CO or T(III) treatment and, consequently, concluded that nickel does not get reduced under these conditions. However, both XANES and EXAFS provided evidence for a redox-dependent structural change in the NiFeS$_4$S$_4$-C-cluster. In the as-prepared CODH$_{Ch}$, the EXAFS could be simulated with 4 Ni–S bonds at 2.2 Å and no light atom was required. After CO treatment, a new feature at 2.7 Å from the nickel ion appeared (Fe1$^-$) and the Ni–S distances expanded to 2.25 Å. This effect does not depend on CO as a ligand to Ni but as an electron donor because it can also be observed when the enzyme is reduced with T(III) citrate. So, although nickel may not be the two-electron acceptor, reduction significantly modifies the structure of the C-cluster. It should be noted that similarly small changes in the absorption edge have been observed in NiFe hydrogenases although it is now widely accepted that nickel is found in at least two redox states: Ni(II) and Ni(III).

Charge delocalization over the thiolate and sulfide ligands may explain this discrepancy.

5 Infrared studies of CO binding to the C-cluster

A detailed infrared (IR) study of CO binding to CODH/ACSMt in the absence of external redox mediators was reported recently by Chen and co-workers. They detected at least seven IR bands between 1900 and 2100 cm$^{-1}$ suggesting that many different CO complexes are formed, consistent with the C-cluster heterogeneity observed in the crystal structures of CO-treated enzymes. The bands gradually disappeared with time, but with different decay rates. The strongest band occurred at 1996 cm$^{-1}$ and it was assigned to CO bound to the A-cluster (the NiFeC state), in agreement with previous results. This was also the last IR CO band to disappear. Using labeled $^{13}$CO, an additional band at 2278 cm$^{-1}$ appeared indicating the formation of $^{13}$CO that could be distinguished from the 2349 cm$^{-1}$ band arising from natural abundance $^{12}$CO present as a contaminant in the spectrometer. Consequently, all the added CO was gradually transformed to CO$_2$, according to the following two reactions:

$$\text{CODH}_{\text{Ch}} + \text{CO} + \text{H}_2\text{O} \leftrightarrow \text{CODH}_{\text{Ch}}^{\text{red}} + \text{CO}_2 + 2\text{H}^+$$  (4)

$$\text{CODH}_{\text{Ch}}^{\text{red}} + \text{H}_2 \leftrightarrow \text{CODH}_{\text{Ch}} + \text{H}_2$$  (5)

H$_2$ formation in (5) was postulated because protons were the only available electron acceptors in the experiment. This is also in agreement with the small but significant hydrogen evolution activity that has been reported for CODH/ACSMt. Based on the time dependence of the IR band decay the rates of (4) and (5) were estimated at 30 and 0.018 m$^{-1}$ min$^{-1}$, respectively, whereas the rate of CO$_2$ formation was about 30 min$^{-1}$. The expected enzymatic activity measured in the presence of redox mediators, is more than three orders of magnitude higher but this agrees with the relative rates of eqn. (5) and (4). Although the formation of H$_2$ suggests that there is at least a transient state of the C-cluster with a bound hydride, hydrogen evolution is clearly not part of the catalytic mechanism and the same may be true for the involvement of many of the CO-bound states. This will be further discussed below.

As no CO bands were detectable before CO treatment, there are no bound intrinsic CO molecules, in contradiction with an earlier proposal by Ludden and co-workers for CODH$_{Ch}$. The CO band at 2074 cm$^{-1}$ decayed the fastest probably because it corresponded to the initial binding of CO to oxidized enzyme and this ligand was subsequently oxidized to CO$_2$. Bands below 2000 cm$^{-1}$ could reflect CO binding to a reduced C-cluster, most likely in the C$_{red2}$ state. Lower frequencies are to be expected for CO bound to the reduced cluster because of the ligand π-acceptor capability. Because, as mentioned above, the tunnel in the crystal structures points at an empty apical coordination site of Ni, CO should bind terminal to this ion. The observed vibration frequency of 2074 cm$^{-1}$ is compatible with CO ligation to a diamagnetic Ni(II) and it is comparable to the value of 2056 cm$^{-1}$ measured for exogenous CO binding to Ni(III) in [NiFe] hydrogenase. Some of the lower frequency bands are similar to those of an intrinsic CO ligand of Fe(II) in [NiFe] hydrogenase, compatible with CO binding to Fe$_{u}$, but they could also correspond to Ni(I)-bound CO, although no stable C-cluster with this Ni valence state has been reported.

It is not clear how many of the states corresponding to the observed IR bands play a role in catalysis. As discussed below, the two bands that were reported at 1727 and 1741 cm$^{-1}$ and were assigned to metal bound carboxylate or carboxylic acid may represent components of the catalytic cycle. These bands were still present after all CO was converted to CO$_2$, as evidenced by the disappearance of the 1996 cm$^{-1}$ band, suggesting that CO$_2$ may form a stable complex with the enzyme.

6 Effect of CO$_2$ binding on the magnetic properties of the C-cluster

The substrate/product CO$_2$ binds to both C$_{red1}$ and C$_{red2}$ states. However, the binding must be different in the two cases because CO$_2$ does not affect the g$_{av}$ = 1.86 signal (C$_{red1}$) but it does modify g$_{av}$ = 1.82 (C$_{red2}$). One possible interpretation is that CO$_2$ binds to the C$_{red1}$ state completely to the tetrahedral coordination of Ni in C$_{red2}$ (indeed, it has been shown that in the C$_{red2}$ state neither CO nor its oxidation product bind to Fe$_{u}$ and it binds non-catalytically to Ni and Fe$_{u}$ in C$_{red1}$, when Ni is square planar. However, this is more difficult to explain because the oxidative addition of CO$_2$ to Ni would require two additional electrons. Binding of CO$_2$ to a center other than the C-cluster cannot be ruled out.

An effect of CO on raising the redox potential of the C$_{red2}$/C$_{red1}$ pair at the C-cluster has already been reported. An equivalent process seems to operate at the A-cluster that cannot be reduced by dithionite unless CO is present.

7 Binding of cyanide to the C-cluster

As for other anions, CN$^-$ binds to the C$_{red2}$ (g$_{av}$ = 1.82) but not to the C$_{red1}$ (g$_{av}$ = 1.86) species. Upon binding, CN$^-$ alters the EPR signal yielding a g$_{av}$ = 1.72. Using ENDOR spectroscopy, DeRose et al. have concluded that CN$^-$ and a H$_2$O species bind to the same site of the C-cluster but only in the C$_{red2}$ state. An exchangeable proton found in C$_{red2}$ absent from C$_{red1}$ or the CN-inhibited enzyme. These authors concluded that CN$^-$ inhibits CODH by displacing OH$^-$ bound to Fe$_{u}$. Bridging of CN$^-$ between Ni and Fe$_{u}$, was predicted from IR studies of CODH$_{Ch}$ as a 2037 cm$^{-1}$ band was assigned to a CN$^-$ bound bridge connecting the Ni ion to a low-spin Fe(II). We believe that slow release of CN$^-$ upon reduction (or CO binding) of the C-cluster may be due to a square planar to tetrahedral nickel coordination transition that effectively removes the Ni–Fe$_{u}$ bridging site by moving nickel away from Fe$_{u}$ or to an excessive negative charge at the reduced C-cluster, or both. An intriguing fact is that the CN$^-$-treated C-cluster cannot be reduced by...
dithionite unless CO, CO₂, or CS₂ are also added. This argues for a combined reductive and ligand binding event as a requisite for CN⁻ dissociation from the C-cluster. However, CO₂ and CS₂ are not direct competitors of CN⁻ binding indicating that they do not bind to the same site.⁶ This is consistent with the idea that CO or CO₂ and CS₂ plus reductant bind terminally to E₁ whereas CN⁻ bridges Ni and Fe₁ at the E₂ site.

8 The nature of the two-electron acceptor/donor at the C-cluster

It has been suggested that Cₚ is two electrons more oxidized than Cₚ⁺ and that both states are involved in catalysis according to:

\[ Cₚ⁻ + CO + OH⁻ = Cₚ₂⁻ →₂⁻ + CO₂ + H⁺ \]  

(6)

Where do the two electrons go upon reduction? Mössbauer spectroscopy has shown that the unusual FCH₁ species of the C-cluster is present in both Cₚ and Cₚ⁺ so it is apparently not redox-active.⁶ In addition, there are no significant changes in either the UV/visible or the Mössbauer spectrum in going from Cₚ to Cₚ⁺, suggesting that the two electrons are not located in the Fe-containing part of the C-cluster.¹²,²⁹ We have recently proposed that the proximal Ni of the A-cluster oscillates between distorted tetrahedral Ni(δ) and square planar Ni(δ) during acetyl-CoA synthesis based on the crystal structure of ACS/CODHₚ₂ₐ, that displayed open and closed α subunit conformations. A similar mechanism could operate at the C-cluster. The ligand environment of Ni at this site shares attributes with the proximal Ni of the A-cluster, both are three-coordinate with all sulfur ligands and Ni coordination oscillates between tetrahedral and square planar. However, there are two apparent problems with the Ni(δ)/Ni(δ) assignment for the C-cluster: 1) the EXAFS experiment mentioned in the previous section suggests that Ni is 2⁺ in both Cₚ and Cₚ⁺ (but the charge could be delocalized, as suggested above) and 2) the requirement for the Ni ligands to stabilize Ni(0).

Another possibility for a redox-active species is the Cys₃₁₆ cluster labile sulfide couple that we detected as forming a minor persulfide component of the C-cluster in ACS/CODHₚ₂ₐ. Recent results from Lindahl’s laboratory indicate that mutating Cys₃₁₆ into Ala completely abolished the C-cluster activity. Unfortunately, this was due to the complete loss of the cluster.³⁹ This result shows that CODH can fold without the C-cluster and that Cys₃₁₆ is essential for cluster integrity. It does not, however, tell us anything about a possible mechanistic role for this residue. One argument against the persulfide hypothesis is its absence from all the other C-cluster structures determined so far. For instance, the initial structure of CODHCh is central to any postulate of active intermediates. For instance, in our 1.9 Å resolution CODH/ACSₚₐ structure, where the crystals were exposed to a high-pressure CO atmosphere prior to immediate flash-cooling for X-ray data collection, there is no visible Ni–Fe₁ bridging ligand. However, the Ni ion seems to bind CO. Janke et al.⁴⁶ has shown that we will argue below that CO-treated CODH discussed in the previous section (eqn. (7)). As already mentioned, in the latter case the shift of the cysteine residue to a bridging position (Cys₅₃₁ in Rz and Cys₅₅₀ in Mi) (Fig. 2). In the former case, the bridging site could be occupied by hydride, a proposition that is consistent with the decay of CO-treated CODH discussed in the previous section. (eqn. (7)). As already mentioned, in the latter case the shift of the cysteine residue to a bridging position (Fig. 2a and 2i) may be required to complete the coordination sphere of Fe₁ in the absence of any exogenous bridging ligand.

Extensively CO-reduced C-clusters may represent non-physiological species. For instance, in our 1.9 Å resolution CODH/ACSₚₐ structure, where the crystals were exposed to a high-pressure CO atmosphere prior to immediate flash-cooling for X-ray data collection, there is no visible Ni–Fe₁ bridging ligand. However, the Ni ion seems to bind CO. Janke et al.⁴⁶ has shown that we will argue below that CO-treated CODH discussed in the previous section (eqn. (7)). As already mentioned, in the latter case the shift of the cysteine residue to a bridging position (Cys₅₃₁ in Rz and Cys₅₅₀ in Mi) (Fig. 2). In the former case, the bridging site could be occupied by hydride, a proposition that is consistent with the decay of CO-treated CODH discussed in the previous section. (eqn. (7)). As already mentioned, in the latter case the shift of the cysteine residue to a bridging position (Fig. 2a and 2i) may be required to complete the coordination sphere of Fe₁ in the absence of any exogenous bridging ligand.

A plausible structure-based catalytic cycle is depicted in Scheme 1. The different intermediates are numbered in the following discussion. The C-cluster in state (2) could correspond to either Ni–CO₂⁻ or Ni–COOH (the latter is shown). These two states may give rise to the IR bands at 1724 and 1741 cm⁻¹ respectively.

The two-electron acceptor reservoir is Cₚ and the C-cluster state (1) is depicted as a hydride based on the similar WGSR: alternatives are a Ni⁺/Ni⁺₀ couple or a CysSH/Cys₅₅₀ persulfide couple formed between Cys₃₁₆ and one of the labile sulfufides (not shown). Oxidative addition of CO, leads to either a Ni–CO₂⁻ or a Ni–COOH species (2). The C–OH bond is broken (3) and a pentacoordinated Ni(δ) carbonyl (4) is generated. As discussed by Lu and Crabtree,⁶¹ Ni(δ) carbonyls are rare, except when the Ni ion is penta-coordinated. Subsequently, CO dissociates from the C-cluster and the C-cluster state (3), and a bridging OH⁻ appears. One-electron reduction of Cₚ⁺ gives rise to Cₚ, an intermediate that does not accumulate.⁶⁸

The recipient of this electron remains ill-defined but possible species are either a Ni(δ) with the bridging OH⁻ still bound, or a Ni(δ)–H⁻ if the water molecule has already dissociated from the C-cluster. An additional one-electron reduction regenerates...
the $C_{\text{red}}$ (1) species. This cycle is similar to the one proposed by Feng and Lindahl. In fact, as these authors indicate in the acknowledgements paragraph, it emerged from discussions between our two groups.

The same intermediates and possibly others would be involved in the decay of CO IR bands observed by Chen et al.\textsuperscript{30} in the absence of electron acceptors other than protons: starting with CO-bound (4), CO$_2$ would be generated through (3) and (2). The reductive elimination of CO$_2$ would then lead to the appearance of (1). Finally, protonation of this species would evolve H$_2$ and the oxidized C-cluster could bind OH$^-$ and CO to regenerate (4).

10 Conclusions

In spite of the observed structural heterogeneity of the various C-clusters some key points can be made: 1) as in the A-cluster,\textsuperscript{11} the Ni ion displays two coordination sites that can be occupied by exogenous ligands. This appears to be a requirement, as both CO and OH$^-$ should bind simultaneously to the C-cluster. Previous models that proposed that CO would terminally bind to Ni and OH$^-$ would terminally bind to Fe$_1$ (Fe1)$^{36}$ can now be ruled out given the proximity of Ni to Fe$_1$ that indicates that both ligands should bind Ni; 2) the crystal structure of the S$_2$-bound C-cluster of CODH$_{\text{C}}$ is most likely an inhibited version of the $C_{\text{red}}$ state. This structure that shows the binding of an anionic SH$^-$ species that bridges the Ni and Fe1 ion is a very good model for the binding of other anions, such as the substrate OH$^-$ and the inhibitor CN$^-$ at the same site.

Although the nature of the species accepting the two electrons when going from $C_{\text{red}}$ to $C_{\text{ox}}$ remains elusive, we tend to favor a proton. Besides the obvious similarities with the WGSR and the results by Chen et al.,\textsuperscript{30} several reduced structures show that Fe1 is only coordinated by three protein ligands. Consequently, it is tempting to propose that the fourth ligand, invisible in the crystal structures, is a hydride.

Both the structure and the catalytic mechanism of the C-cluster is clearly one of the most complex systems in biology. Although great progress has been made in understanding the subtleties of the CODH reaction, further advancements will require the study of better-defined, more homogenous C-clusters.

References

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