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A role for nickel–iron cofactors in biological carbon monoxide and carbon dioxide utilization

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Ni–Fe containing enzymes are involved in the biological utilization of carbon monoxide, carbon dioxide, and hydrogen. Interest in these enzymes has increased in recent years due to hydrogen fuel initiatives and concerns over development of new methods for CO₂ sequestration. One Ni–Fe enzyme called carbon monoxide dehydrogenase (CODH) is a key player in the global carbon cycle and carries out the interconversion of the environmental pollutant CO and the greenhouse gas CO₂. The Ni–Fe center responsible for this important chemistry, the C-cluster, has been the source of much controversy, but several recent structural studies have helped to direct the field toward a unifying mechanism. Here we summarize the current state of understanding of this fascinating metallocluster.

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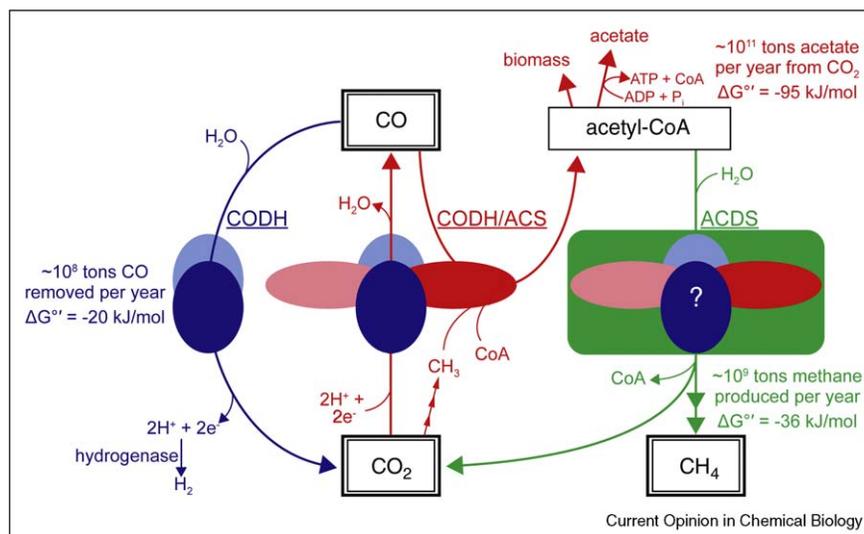
Although enzymes that utilize iron-containing active sites catalyze a wide range of well-known chemical transformations, three remarkable enzymes combine iron and nickel into complex metalloclusters that extend Nature's biochemical toolkit and lie at the heart of fundamental biological processes involving microbial hydrogen utilization and carbon fixation. [NiFe]-hydrogenase can catalyze both H₂ oxidation and evolution in anaerobic microbes to consume or produce protons and electrons, the biological equivalent of the hydrogen fuel cell anode [1,2]. Involved in carbon fixation, the enzyme acetyl-CoA synthase (ACS) contains a Ni–Fe–S active site metal center called the A-cluster that combines carbon monoxide (CO) with a methyl group and coenzyme A (CoA) to form acetyl-CoA, generating a source of carbon and energy for a variety of microbes. CO is often provided

to ACS by another Ni–Fe enzyme called carbon monoxide dehydrogenase (CODH), a dimeric enzyme which contains a distinctive Ni–Fe–S metal center termed the C-cluster that carries out the reversible reduction of carbon dioxide (CO₂) to CO, the biological equivalent of the water-gas shift reaction and a mechanism for CO₂ utilization. Interest in all three enzymes has increased dramatically in recent years due to renewed attention in the development of hydrogen fuel cells and the design of CO₂ sequestration technologies. While the first X-ray crystal structures of [NiFe]-hydrogenase [3], ACS [4[•]], and CODH [5[•],6[•]] revealed the overall architecture of these complex metallocenters, recent work has aimed to develop an understanding of the mechanisms by which these clusters catalyze their respective reactions. As major advances have been made on the C-cluster within the last three years, we have focused this review on this metallocenter. Although the CODH literature has had its share of controversies, the CODH community is now converging on a consensus mechanism, a timely achievement toward understanding one of Nature's solutions for CO₂ utilization.

CODH and the global carbon cycle

CODH plays a central role in the global carbon cycle in anaerobic microorganisms (Figure 1). Some microbes, such as *Rhodospirillum rubrum* and *Carboxydotherrmus hydrogenoformans* depend upon a monofunctional CODH in their ability to use CO oxidation as a sole carbon and energy source [7,8]. It is estimated that CODH activity accounts for the annual removal of ~10⁸ tons of CO from the environment [9]. Acetogenic bacteria, such as *Moor-ella thermoacetica*, couple CODH-catalyzed reduction of CO₂ to CO with synthesis of acetyl-CoA in a bifunctional CODH/ACS complex [10]. Here, CO produced from CO₂ at the C-cluster is a gaseous intermediate that travels approximately 70 Å through an extraordinary hydrophobic tunnel within the enzyme complex [4[•],11–13,14[•]] to the ACS A-cluster, where it becomes the carbonyl of acetyl-CoA. Acetyl-CoA is then either converted into cellular biomass, or its high-energy thioester bond can be cleaved to drive phosphorylation of ADP to ATP in supplying energy for the cell, producing acetate as a waste product. It is estimated that ~10¹¹ tons of acetate are produced globally from CO₂ through this process every year by anaerobic acetogens [15]. Additionally, CODH and ACS components are present in the acetyl-CoA decarbonylase/synthase (ACDS) complex, a multienzyme machine that is a major route to methane production in methanogenic archaea, which

Figure 1



Schematic of NiFe-containing CODH in the microbial carbon cycle and its contributions to CO, CO₂, and methane production and consumption. CODH is shown as a dimer in light and dark blue ovals, ACS as light and dark red ovals, and the methanogenic ACDS complex as a green rectangle containing both CODH and ACS components in an unknown arrangement.

generate an estimated 10^9 tons of methane per year [16,17].

Initial structural and mechanistic studies of CODH

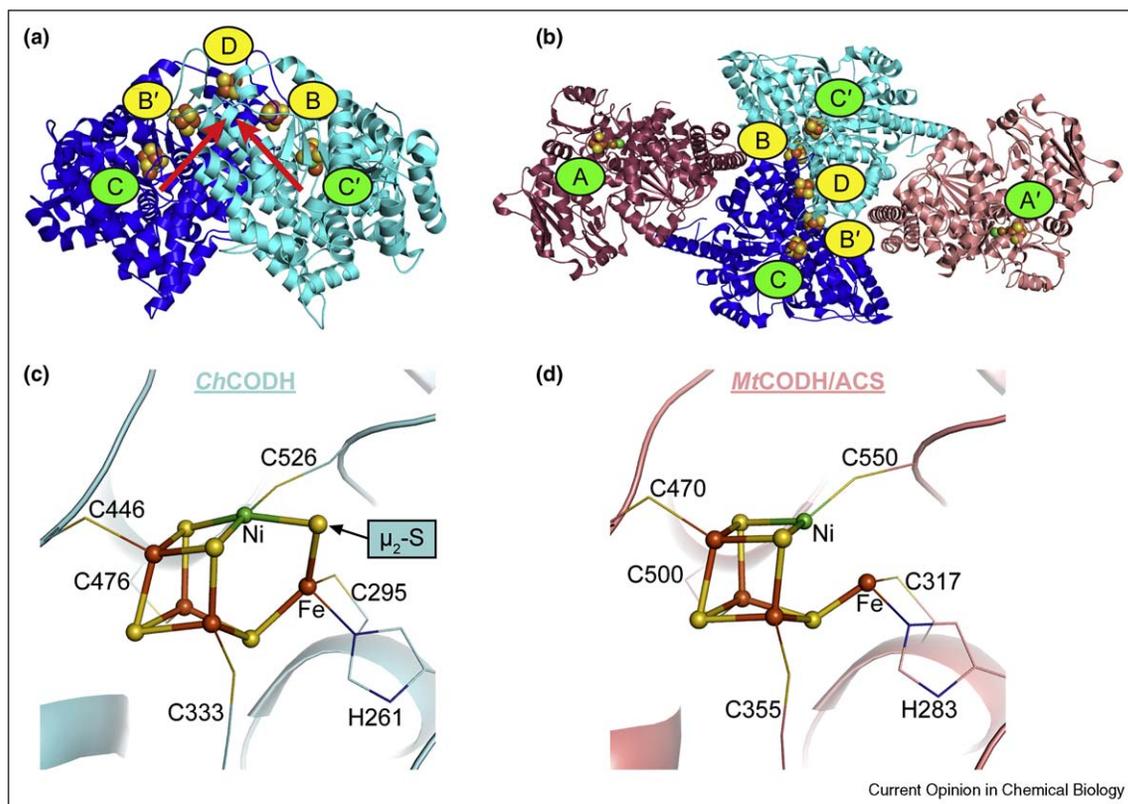
Although it had been well established that CODH harbored a Ni–Fe–S active site (see [18] for review), it was not until the initial X-ray crystal structures of CODH from *R. rubrum* (*Rr*CODH) (Figure 2a) [6], *C. hydrogeniformans* (*Ch*CODH) [5], and *M. thermoacetica* CODH/ACS (*Mt*CODH/ACS) (Figure 2b) [4,13] that the arrangement and geometry of the metals were determined. Early spectroscopic studies had suggested that the C-cluster was composed of a [4Fe–4S] cubane with a unique Ni site nearby [18,19]. However, all of these CODH structures revealed an unprecedented metallocluster that can be described as a distorted [Ni–3Fe–4S] cubane coordinated to a unique Fe site, also called ferrous component II (FCII).

Despite exhibiting the same arrangement of metals in the C-cluster, these initial structures possessed key differences that hindered full mechanistic understanding. Perhaps most importantly, the *Ch*CODH C-cluster contained an additional sulfide ligand in a position bridging Ni of the distorted cubane and the unique Fe (Figure 2c), a feature absent in the *Rr*CODH and *Mt*CODH/ACS structures (Figure 2d). This inconsistency led to controversy over the correct composition of the cluster, and further experiments were conducted which argued either for [20] or against [21,22] a catalytic role for the sulfide bridge.

Related to the issue of the sulfide bridge is the crucial question of where substrates bind to the C-cluster for the interconversion of CO and CO₂. In the direction of CO oxidation, CO and H₂O must bind the C-cluster, H₂O is deprotonated, and CO₂ is formed, generating two protons and two electrons that reduce the cluster. Although the pathway by which protons exchange with the bulk solvent is not firmly established, a network of histidine residues that link the buried C-cluster with the solvent exterior has been suggested as a possible route [6,23]. Electrons are passed from the C-cluster to the surface of the protein through additional [4Fe–4S] clusters that form a wire seen in all CODH structures (Figure 2a). Ferredoxin, pyruvate:ferredoxin oxidoreductase (PFOR), and hydrogenase have been proposed as ultimate electron acceptors [24,25]; intermolecular electron transfer has been suggested to be rate-limiting [26], with specific activity depending upon the electron acceptor employed [27,28]. In the direction of CO₂ reduction, CO₂ must bind the two-electron reduced C-cluster [29,30], and with the addition of two protons, H₂O and CO are formed.

Although there remained uncertainties surrounding the transfer of electrons and protons to the buried C-cluster, the most attention has been paid to binding sites for CO and H₂O on the metals of the C-cluster. Studies conducted before the *Ch*CODH and *Rr*CODH structure determinations had indicated that Ni and Fe are involved in binding the substrate CO and water molecules, respectively [19,31]. In the *Ch*CODH structure, however, the sulfide bridge fills coordination sites to complete

Figure 2



CODH and C-cluster structures. **(a)** The *Rr*CODH homodimer (PDB ID: 1JQK) and **(b)** the bifunctional *Mt*CODH/ACS complex (PDB ID: 1MJG); CODH subunits in blue and cyan ribbons, and ACS subunits in dark and light pink ribbons. Metalloclusters in spheres: Ni in green, Fe in orange, S in yellow. Active site A-clusters and C-clusters are labeled in green and B-clusters and D-clusters are labeled in yellow, with red arrows in (a) indicating the direction of electron flow during CO oxidation. **(c)** The C-cluster of *Ch*CODH (PDB ID: 1SU8, cyan ribbons), which contains a sulfido ligand (labeled μ_2 -S) that bridges Ni and the unique Fe. **(d)** The C-cluster of *Mt*CODH/ACS (pink ribbons), which does not contain the bridging sulfide. C-clusters in ball-and-stick: Ni in green, Fe in orange, and S in yellow. Protein ligands in sticks: N in blue, S in yellow, and C following protein ribbon coloring. Residue numberings follow the respective protein sequences.

square planar geometry around Ni and distorted tetrahedral geometry around the unique Fe, making the substrate binding locations unclear. Although *Rr*CODH and two independent *Mt*CODH/ACS structures contain empty coordination sites in place of the sulfide bridge, electron density for an unassigned ligand apical to Ni was present in the *Rr*CODH structure and one *Mt*CODH/ACS structure [6[•],13]. Without a clear identification of substrate binding sites, the mechanism of the C-cluster remained enigmatic.

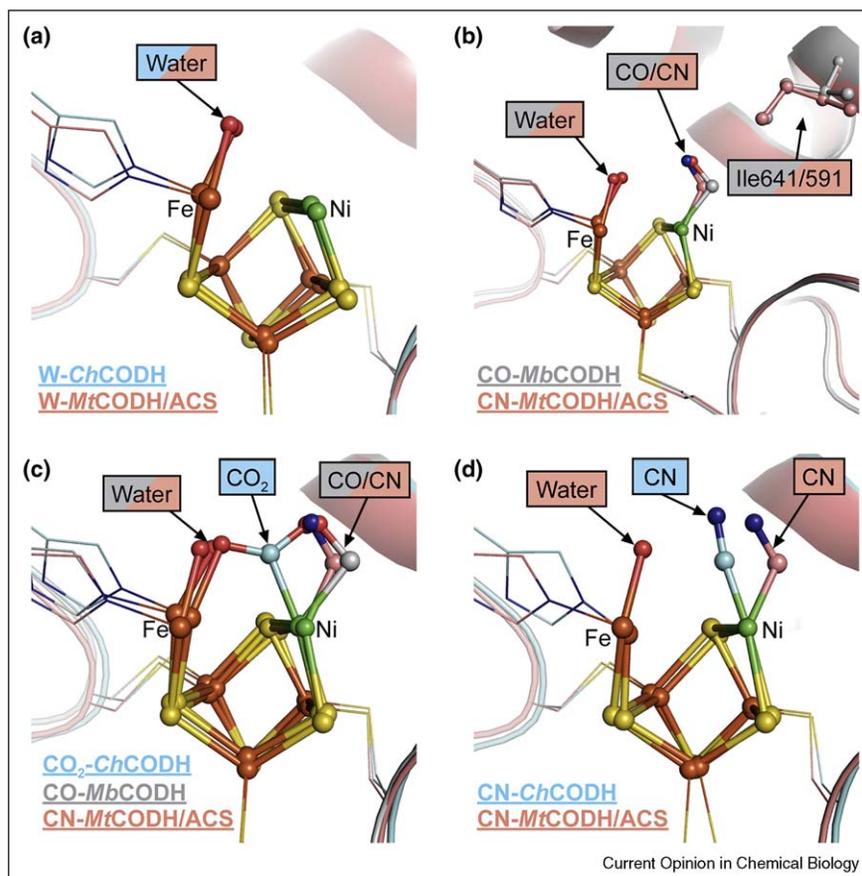
Substrate-bound and inhibitor-bound C-cluster structures identify the active site

Over the past three years, many crystal structures have been solved that depict substrates bound to the C-cluster. Structures of *Ch*CODH [32^{••}] and *Mt*CODH/ACS [33^{••}] show the substrate water molecule bound to the unique Fe site in an identical fashion, completing a distorted tetrahedral geometry (Figure 3a). These observations are

consistent with previous studies which also suggest that water binds Fe [31]. None of these structures contain the sulfide bridge, as the water molecule occupies the sulfide coordination site on Fe.

A structure of the CODH component of the ACDS complex from *Methanosarcina barkeri* (*Mb*CODH) depicts CO bound to Ni of the C-cluster in a position adjacent the water molecule, which remains bound to the unique Fe (Figure 3b) [34^{••}]. With both substrates bound to the cluster, it was hypothesized that the low pH of the crystallization condition (4.6) prevented turnover by disfavoring deprotonation of water to the active hydroxide nucleophile, allowing the capture of the C-cluster state immediately before catalysis. CO is bound to Ni in an unexpected bent conformation, with a Ni–C–O bond angle of 103°, completing a distorted tetrahedral geometry. Interestingly, a structure of cyanide, a CODH inhibitor, bound to the *Mt*CODH/ACS C-cluster illustrates

Figure 3



Substrate-bound and inhibitor-bound C-cluster structures. **(a)** Superposition of water-bound C-clusters from *ChCODH* and *MtCODH/ACS*. **(b)** Superposition of CO/water-bound and cyanide/water-bound C-clusters from *MbCODH* and *MtCODH/ACS*, respectively. A conserved isoleucine residue (Ile641 in *MbCODH* and Ile591 in *MtCODH/ACS*) that is believed to sterically impede linear CO/CN-binding is shown in ball-and-stick. **(c)** Superposition of the structures in (b) plus the CO₂-bound C-cluster from *ChCODH*. **(d)** Superposition of the cyanide-bound C-clusters from *MtCODH/ACS* and *ChCODH*. Protein chains in ribbons: *ChCODH* in cyan, *MtCODH/ACS* in pink, and *MbCODH* in gray. C-clusters in ball-and-stick, and protein ligands to the cluster in sticks: Ni in green, Fe in orange, S in yellow, N in blue, O in red, and C following protein ribbon coloring. The Ni and unique Fe sites are labeled 'Ni' and 'Fe', respectively. For clarity, not all protein ligands to the cluster are shown.

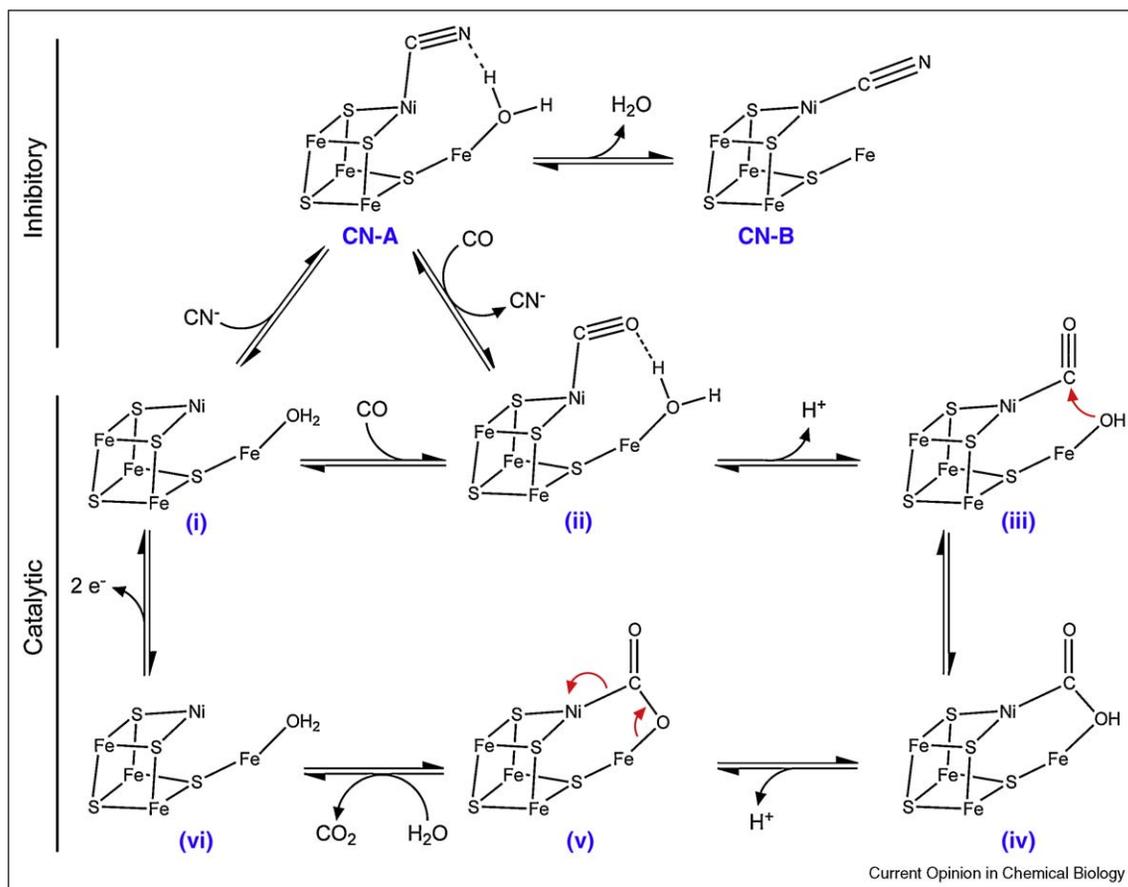
analogous bent geometry (Figure 3b), with a Ni–C–N bond angle of $\sim 114^\circ$ [33^{••}]. The substrate water molecule in this structure also remains bound to the unique Fe. A conserved isoleucine residue is seen in both structures to sterically block linear binding of CO and cyanide to Ni (Figure 3b). Such bent coordination is not likely to be stable; indeed, infrared spectroscopy had suggested that there is no single, stable site for CO binding to the C-cluster [35]. Thus, it is possible that binding of CO to give bent geometry is mechanistically important, as the enhanced stability of a linear binding mode may impede turnover. In this model, isoleucine would contribute to ground state destabilization, lowering the activation barrier to catalysis by preventing linear substrate binding.

The structures of CO and cyanide bound to the C-cluster place the carbon atom and water molecule too far apart for catalysis; a shift in coordination must occur during the

reaction. The crystal structure of the product CO₂ bound to the *ChCODH* C-cluster [32^{••}] provides a unique perspective on how such a shift may occur. Here, the CO₂ carbon is bound to Ni, while one CO₂ oxygen is bound to the unique Fe. A superposition of CO₂-bound *ChCODH* with CO-bound *MbCODH* and cyanide-bound *MtCODH/ACS* structures exhibits a nearly identical position of all substrate atoms except for the carbon atom (Figure 3c). While the oxygen/nitrogen atoms remain stationary across the structures, the carbon atom has shifted closer to the water molecule in the CO₂-bound structure. This 'carbon shift' has been proposed [33^{••}] to alter the Ni coordination geometry from distorted tetrahedral in the CO-bound and CN-bound forms to square planar in the CO₂-bound form.

Taken together, these structures reveal the active site on the C-cluster of CODH. In the direction of CO oxidation,

Figure 4



Proposed catalytic and inhibitory mechanisms of the CODH C-cluster, consistent with crystal structures containing bound substrate and inhibitor molecules. To begin the reaction, a water molecule binds the unique Fe of the C-cluster (state i), as seen in *Ch*CODH and *Mt*CODH/ACS structures. CO binds Ni with bent geometry (state ii), as seen in CO-bound *Mb*CODH and CN-bound *Mt*CODH/ACS. Upon deprotonation of water, a 'carbon shift' may occur to position the carbon in proximity to the resulting hydroxide (state iii), promoting nucleophilic attack to yield a Ni-COOH intermediate (state iv). A second deprotonation gives a Ni-COO⁻ species (state v). The crystal structure of CO₂-bound *Ch*CODH resembles states iv and v, which differ only in protonation state. CO₂ may then be released, with the C-cluster becoming reduced by two electrons (state vi). Electrons are then passed to external electron acceptor proteins through a wire of [4Fe-4S] clusters within the CODH dimer, as seen in Figure 2a, completing the catalytic cycle. Protons generated during CO oxidation may access the external solvent through a histidine-lined channel. In the proposed inhibitory mechanism, cyanide competes with CO for binding to Ni of the C-cluster with bent geometry, as seen in the structure of cyanide-bound *Mt*CODH/ACS (state CN-A). Following displacement of Fe-bound water, cyanide may relax to a tighter linear binding mode, as seen in the structure of cyanide-bound *Ch*CODH (state CN-B).

crystal structures from *Ch*CODH, *Mb*CODH, and *Mt*CODH/ACS depict water bound to the unique Fe, while structures of *Mb*CODH and *Mt*CODH/ACS show CO or an inhibitor CN⁻ bound to Ni, respectively. Meanwhile, one structure of *Ch*CODH shows CO₂, the substrate in the direction of CO₂ reduction, bound to the cluster. In all structures, regardless of the organism, no sulfide bridge is present when a substrate molecule is bound. Consistent with these structures, a catalytic mechanism is proposed (Figure 4).

The complexity of cyanide inhibition

A distinguishing feature of all Ni-containing CODHs is potent inhibition by cyanide [24,27,36–40]. Although direct binding to the C-cluster has been implicated as

the root of cyanide inhibition, years of study have not led to a clear inhibitory mechanism. On the one hand, cyanide has been described as a competitive inhibitor that binds Ni: Ni-deficient *Rr*CODH does not bind cyanide [37,38], and X-ray absorption spectroscopy (XAS) indicated that cyanide shares with CO a binding site on Ni with a Ni–C distance of 1.81–1.84 Å [40]. In contrast, other studies have suggested that cyanide binds Fe: electron-nuclear double resonance (ENDOR) spectroscopy indicated that cyanide displaces the Fe-bound water molecule [31], and Mössbauer spectroscopy showed a change in quadrupole splitting (ΔE_Q) of the unique Fe signal upon cyanide treatment [19]. It has also been suggested that cyanide may bind at multiple sites [26].

However, these data appear to be in conflict only if it is assumed that cyanide must adopt a single binding mode. Indeed, two crystal structures of cyanide bound to the C-cluster show that the same inhibitor can actually adopt multiple binding modes. As mentioned above, the cyanide-bound *Mt*CODH/ACS structure [33^{••}] shows cyanide bound to Ni in a bent conformation, in an analogous fashion as CO in the *Mt*CODH structure, with water still bound to Fe (Figure 3b). The cyanide carbon completes distorted tetrahedral geometry around Ni and mimics how CO binds to the active C-cluster. On the other hand, a cyanide-bound *Ch*CODH structure [41^{••}] shows CN⁻ bound to Ni with effectively linear geometry (Ni–C–N bond angle of 175°), conferring square planar geometry around Ni. Notably, the substrate water molecule is absent in this structure, which represents an inhibited form where neither substrate is bound to the cluster. A superposition of the two cyanide-bound C-clusters is shown in Figure 3d.

These dissimilar crystal structures depicting cyanide binding mirror the seemingly contradictory spectroscopic results described above. It has been recently suggested that a rapid, reversible cyanide binding step is followed by a slow rearrangement step to achieve tighter binding [26,41^{••}], explaining how cyanide could be both a rapid, reversible inhibitor under some conditions and a slow-binding inhibitor under others [38]. Here, we reason that the *Mt*CODH/ACS cyanide structure illustrates an ‘easily reversible’ cyanide binding mode, where cyanide binds Ni in the same bent manner as CO. This bent, Ni-bound cyanide structure is consistent with studies indicating that cyanide is a competitive inhibitor of CO and, like CO, binds Ni. In a subsequent, slow rearrangement step, the substrate water molecule bound to the unique Fe may then be displaced, freeing space to allow cyanide to relax into a more favored linear binding mode, represented by the *Ch*CODH cyanide structure. One would expect this structure to represent cyanide in a ‘tight binding’ mode. The displacement of the Fe-bound water molecule upon cyanide treatment seen in the *Ch*CODH structure is consistent with the ENDOR experiments [31] and further clarifies that water displacement is not a result of direct binding of cyanide to Fe, an assumption made in the ENDOR study. Water displacement and linear cyanide binding can also explain why Mössbauer spectroscopy showed a change in ΔE_Q of the unique Fe signal upon cyanide treatment [19]. Therefore, while neither cyanide-bound C-cluster structure can alone reconcile all of the spectroscopic studies, both structures together can provide an explanation for the seemingly inconsistent data on CODH cyanide inhibition, a 20-year mystery in the field.

In asking why the *Ch*CODH and *Mt*CODH/ACS structures revealed different cyanide binding modes, the most likely answer lies in the dissimilar cyanide crystal soaking

protocols. *Ch*CODH crystals were soaked for 30 min in 70 mM KCN [41^{••}], while *Mt*CODH/ACS crystals were soaked for 1 hour in only 100 μ M KCN [33^{••}]. Because *Ch*CODH crystals were exposed to cyanide concentrations a few orders of magnitude higher than *Mt*CODH/ACS crystals, it is possible that the equilibrium was shifted toward displacement of Fe-bound water and linear cyanide binding. Regardless, these structures have offered a clearer picture of the mechanism of cyanide inhibition (Figure 4).

Conclusions

The truly distinctive NiFe₄S₄ CODH C-cluster has inspired decades of biochemical research; however, the literature has been fraught with controversy and contradictions regarding the C-cluster’s structure, mechanism, and mode of inhibition. Crystal structures of CODHs from several organisms have brought the C-cluster into focus and allowed us to rationalize the abundance of seemingly inconsistent biochemical data. From these structural studies, a unified view of the C-cluster has emerged, presenting key insights into the function of this remarkable and environmentally important metallocluster.

Acknowledgements

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Three structures of *ChCODH* presented here identified both the water and CO₂ binding sites of the C-cluster and provided the first images of substrates bound to the cluster. Water was bound in the same fashion to the unique Fe of the C-cluster in two structures (1.40 and 1.48 Å resolution) at differing redox states. The redox state was modulated by soaking crystals in solutions at different reduction potentials. CO₂ binding was observed in one structure (1.50 Å resolution) after soaking crystals in sodium bicarbonate.
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