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Surprising cofactors in metalloenzymes

Catherine L Drennan* and John W Peters†

Transition metal complexes are located at the active sites of a number of enzymes involved in intriguing biochemical reactions. These complexes can exhibit a wide variety of chemical reactivity due to the ease at which transition metals can adopt different coordination environments and oxidation states. Crystallography has been a powerful technique for examining the structure and conformational variability of complex biological metalcenters. In particular, the past ten years have provided a wealth of structural information and several surprises concerning the metalcenters at the active sites of nitrogenase, hydrogenase and carbon monoxide dehydrogenase/acetyl-coenzyme A synthase.

Addresses

*Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
e-mail: cdrennan@mit.edu

†Department of Chemistry and Biochemistry, Montana State University, Bozeman, MT 59717, USA
e-mail: john.peters@chemistry.montana.edu

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Abbreviations

ACS acetyl-coenzyme A synthase
CODH carbon monoxide dehydrogenase

Introduction

The application of X-ray diffraction methods to the determination of the structures of complex metal clusters is a fascinating endeavor. Over the past decade, the structures of several proteins containing complex metal clusters have been determined, revealing several unanticipated features. The nitrogenase ‘P-cluster’ and ‘FeMo-cofactor’ [1–4,5**], the hydrogenase ‘H-cluster’ [6,7], and the ‘A- and C-clusters’ of carbon monoxide dehydrogenase/acetyl-coenzyme A synthase (CODH/ACS) [8**–10**] are all what Holm and others [11,12] have classified as ‘bridged biological metal assemblies’. The common feature of this class of metal clusters is that they all contain an [Fe₄S₄] cubane, or fragment thereof, as a fundamental unit. Another common thread amongst these enzymes is that, for the most part, they are found only in microorganisms and are key components of the global cycling of

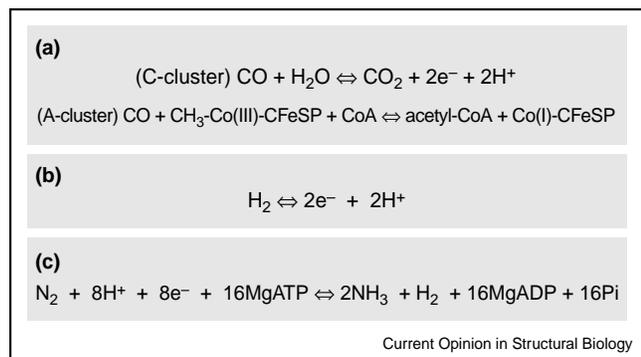
nitrogen, hydrogen and carbon (Figure 1). For the structural biologist, the pursuit of the structures of these complex metal-containing proteins is quite fascinating because the rules governing the bonding of transition metal complexes are not hard and fast. In the case of each of the aforementioned metal clusters, although their composition, and catalytic and electronic properties had been extensively characterized, their overall architecture as described by crystallographic analysis revealed surprises, which, in some cases, challenged prevailing paradigms. The structures serve as a fundamental basis for a better understanding of the available biochemical and biophysical data that have amassed on these systems, and provide the framework for generating hypotheses for catalysis that can be approached experimentally.

Carbon monoxide dehydrogenase/acetyl-coenzyme A synthase

CODH/ACSs are key components of numerous energy-yielding pathways in microorganisms. Phototrophic anaerobes such as *Rhodospirillum rubrum* use a monofunctional CODH to oxidize CO, allowing growth in the dark with CO as the sole carbon and energy source. Acetogens such as *Moorrella thermoacetica* use a bifunctional CODH/ACS as part of a pathway by which greenhouse gas CO₂ is fixed to produce acetyl-coenzyme A. By contrast, methanogens can use CODH/ACS to degrade acetyl-coenzyme A in the production of greenhouse gas methane. At the heart of this unusual one-carbon chemistry are two metallocofactors: the C-cluster of CODH, responsible for the reversible reduction of CO₂ (Figure 1); and the A-cluster of ACS, responsible for the synthesis of acetyl-coenzyme A from CO, a methyl group donated by the corrinoid-iron-sulfur protein (CFeSP) and coenzyme A (Figure 1). The mechanism of CO oxidation at the C-cluster is thought to involve the binding and deprotonation of a water molecule to form iron-bound hydroxide [13], and the binding of CO to a site adjacent to the hydroxide. The next step is metal-assisted hydroxide attack on the metal-bound CO. The resulting metal–COOH intermediate is then deprotonated and CO₂ is lost to yield a two-electron reduced C-cluster (reviewed in [14,15]). The synthesis of acetyl-coenzyme A involves a carbonylation reaction, a methyl group transfer reaction and the synthesis of a high-energy thioester bond (Figure 1).

To aid mechanistic understanding of the C-cluster activity, two CODH structures have been determined: the structure of the well-characterized *R. rubrum* CODH (CODH_R) at 2.8 Å resolution [9**] and the structure of *Carboxydotherrmus hydrogenoformans* CODH (CODH_C) at 1.6 Å resolution [8**]. The X-ray analyses revealed that

Figure 1

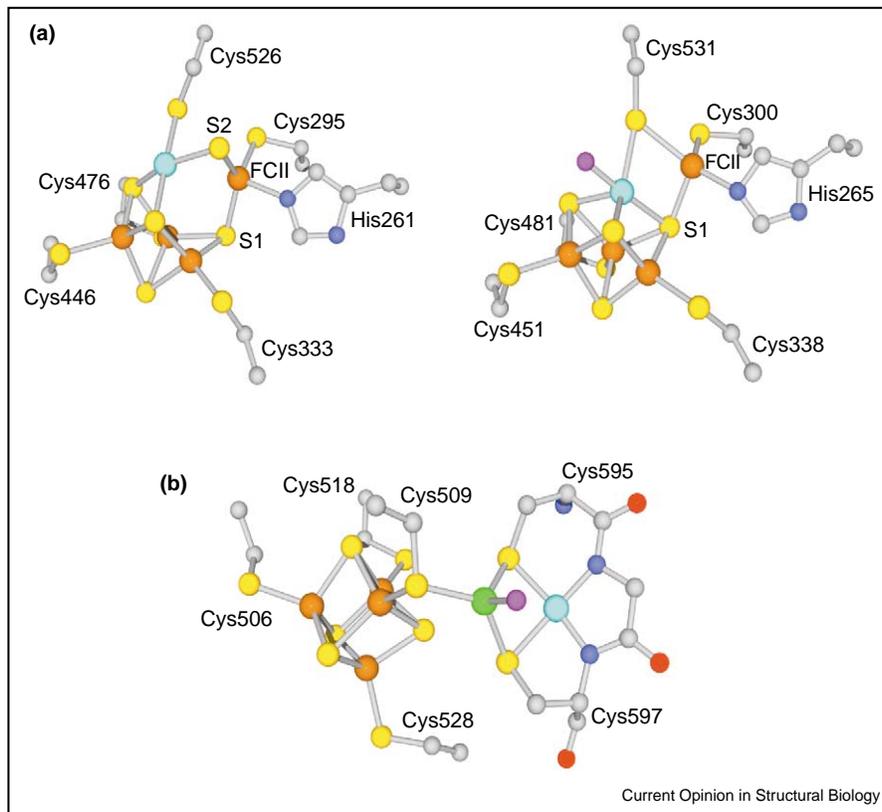


The overall stoichiometry of the reactions catalyzed by the enzymes **(a)** CODH/ACS, **(b)** hydrogenase and **(c)** nitrogenase. CFeSP, corrinoid-iron-sulfur protein.

the C-cluster was not as expected from spectroscopic analyses [16]. Instead of an $[\text{Fe}_4\text{S}_4]$ cubane bridged to a mononuclear Ni site, the Ni is part of a highly unusual $\text{FeNi-}[\text{Fe}_3\text{S}_4]$ cluster (Figure 2). The two C-cluster structures are very similar in terms of metal positioning and

protein ligand identity. They differ, however, in terms of the coordination geometries of the Ni and the unique Fe (called FCII), and the identity of the small-molecule ligands. Some of these differences, such as the position of sulfide S1 and the variation in sidechain orientation of Cys531 (*R. rubrum* numbering), may be due to the different resolution of the data [9^{••}]. Others, such as the presence of a bridging S (S2) in the CODH_{Ch} structure and the presence of electron density for an unknown ligand in the CODH_{Rr} structure, are probably due to variation in protein handling and/or oxidation state. Ideally, both X-ray structures would have revealed the same two open coordination sites, one open site for binding CO and one open site for binding water. However, the two structures do not have any open coordination sites in common, leaving the question of where the substrates bind unanswered. One means of rationalizing the existence of these two different forms of the C-cluster is that CODH_{Ch} may represent a precatalytic state and CODH_{Rr} may represent the catalytic state, in analogy with the NiFe hydrogenase literature [17]. For NiFe hydrogenase from *Desulfovibrio vulgaris*, a sulfide that bridges the active site Ni and Fe is displaced before catalysis, resulting in the release of H_2S and open coordination sites on Ni and Fe [17]. For

Figure 2

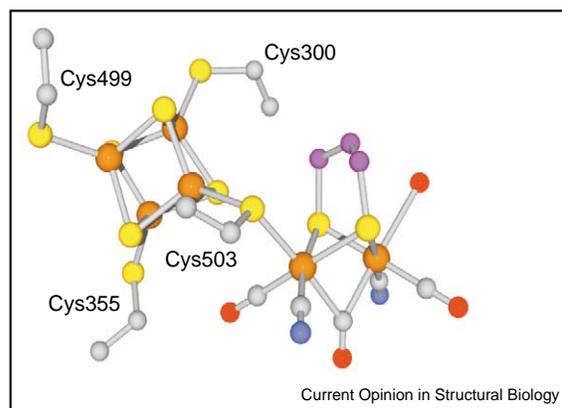


Structures of the C- and A-clusters of CODH and ACS enzymes. **(a)** Structures of the C-clusters of CODH from *C. hydrogenoformans* (left) and *R. rubrum* (right). **(b)** Structure of the A-cluster of CODH/ACS from *M. thermoacetica*. The color scheme is Fe orange, Ni cyan, S yellow, C gray, N blue, O red and Cu green. Positions of unknown ligands are marked by magenta spheres. All the figures were made in SWISS-Pdb [40].

CODH, the presence of sulfide (S₂) bridging Ni and FCII results in a structure for which there are no open coordination sites close enough to each other for a reaction to occur without a structural rearrangement [8^{••}]. On the other hand, in the absence of sulfide S₂, the C-cluster has an open coordination site on Ni and another on FCII that are near each other. Reaction at the site bridging Ni and FCII has the added value of being in close proximity to the best candidate for a catalytic base (His95). Thus, in analogy with NiFe hydrogenase from *D. vulgaris*, the C-cluster structure with S₂ may represent a precatalytic state and the C-cluster structure without S₂ may represent the catalytic state. Elucidation of additional structures of CODH C-clusters will be valuable in resolving these crystallographic issues.

Before the X-ray studies, the C- and A-clusters of CODH/ACS enzymes were both thought to have the form Ni-X-[Fe₄S₄] [15,16], and it was difficult to understand how these clusters catalyzed such distinct reactions. Now it is clear that the clusters are, in fact, quite different (Figure 2a,b). For the A-cluster, the first surprise from the crystallographic analysis was that it contains a binuclear metal site in addition to the [Fe₄S₄] cubane. The second surprise was that dispersive difference electron density map calculations showed that one of the metals in this crystal structure is Cu [10^{••}]. The third surprise was the coordination of the Ni by backbone N atoms. Although there are several examples of backbone coordination of Ni and Cu by peptides [18], it has not been observed in structures of any Ni-dependent enzymes. In fact, the coordination of metals by backbone N atoms in proteins is uncommon, with serum albumin [18] and nitrile hydratase being the best-characterized examples [19,20]. Interestingly, both nitrile hydratase and ACS use a Cys-X-Cys motif (Figure 2b), in which the two cysteine sidechains and two backbone N atoms coordinate the metal with square planar geometry. In nitrile hydratase, the metal is Fe and the pseudo-ring system created by the protein has been compared to a heme. In ACS, the pseudo-ring system created by the protein for Ni is reminiscent of the Ni corphinoid ring system of methyl-coenzyme M reductase. The latter similarity is interesting because the chemistry performed by both enzymes involves a methyl transfer reaction and the formation of a carbon-metal bond. Although the Ni site resembles nitrile hydratase and methyl-coenzyme M reductase, the overall architecture of the A-cluster is similar to the H-cluster of Fe-only hydrogenase, which also contains an [Fe₄S₄] bridged by a cysteine residue to a binuclear metal site (compare Figures 2b and 3). The A-cluster is thus a montage of themes found in other structures of complex metalloenzymes. The presence of Cu was unanticipated and is currently being investigated [21]. Interestingly, aerobic CODH, which was known to contain Mo, Fe-S and flavin cofactors, was recently found to contain Cu as well [22]. For ACS, there

Figure 3



Structure of the H-cluster of the Fe-only hydrogenase from *C. pasteurianum* in its oxidized state. The color scheme is as described in Figure 2, with the unknown atoms of the dithiolate linkage indicated in magenta.

are reports to suggest that the 'Cu' or proximal site can be occupied by other metals, such as Ni [23] and Zn [21]. The next step is to fully resolve the metal dependence of A-cluster activity and to test the mechanistic proposals suggested by the structural analysis.

Hydrogenase

The metal-containing hydrogenases catalyze reversible hydrogen oxidation (Figure 1) and exist in either NiFe or Ni-independent, or what has been referred to as Fe-only, forms. The structures of NiFe hydrogenase from the sulfate-reducing bacteria *Desulfovibrio gigas* and *D. vulgaris* have been determined [24,25]. In these microorganisms, the hydrogenase generates reducing equivalents for the energy-yielding process of sulfate reduction through the oxidation of hydrogen. The active site is heterobimetallic, consisting of a Ni ion bridged to an Fe atom via two bridging thiolates supplied by cysteine residues of the protein. Before the structure determination, Fe was not anticipated to be a component of the active site. Another surprise, which was experimentally realized through Fourier transform IR spectroscopy, was the presence of CO and cyanide ligands to Fe [26]. In addition, the presence of a sulfoxo ligand to Fe in the *D. vulgaris* NiFe hydrogenase has been reported [25].

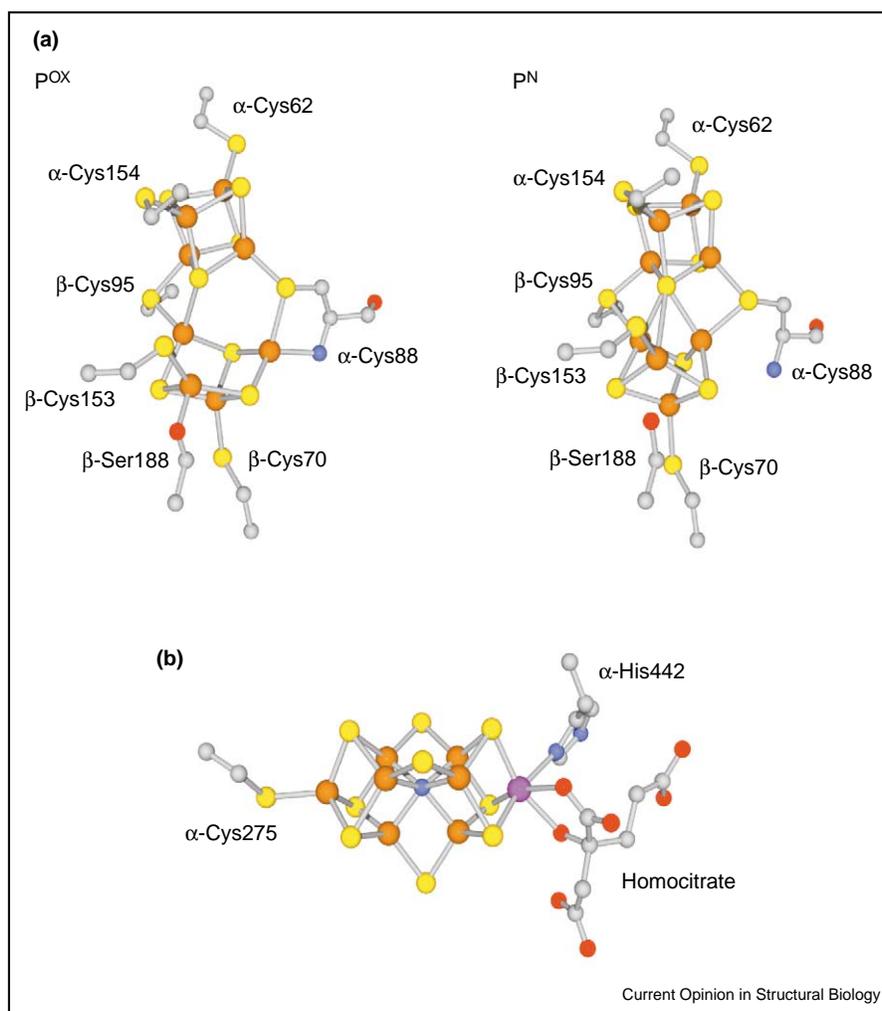
The structure of the Fe-only hydrogenase and its active site metal cluster, termed the H-cluster, has been determined from two sources [27], *Clostridium pasteurianum* [6] and *Desulfovibrio desulfuricans* [7]. In the case of the latter enzyme, the physiological role is presumed to be analogous to that of the aforementioned NiFe hydrogenases from the sulfate-reducing bacteria. In *C. pasteurianum*, the enzyme functions in proton reduction (hydrogen evolution), oxidizing reduced electron carriers that accumulate during the anaerobic fermentation of sugars. The H-cluster

of the Fe-only hydrogenase consists of a regular $[\text{Fe}_4\text{S}_4]$ cubane bridged to a unique 2Fe-containing subcluster through a single bridging cysteine thiolate. In addition to this bridging thiolate sulfur, the 2Fe cluster is coordinated by five diatomic ligands, a water molecule (in the cluster's oxidized state) and a nonprotein bridging dithiolate ligand for which the precise composition has not been unambiguously determined (Figure 3). The presence of CO and cyanide ligands was anticipated from the results of Fourier transform IR studies [28]. The bridging dithiolate ligand is of significant interest and it has been suggested to be either 1,3-propanedithiol (PDT) or dithiomethyl amine (DTN) [7,29]. The latter has some very interesting mechanistic possibilities for this ligand involving a role as a proton donor/acceptor group during catalysis [29].

Within the framework of the structure of the H-cluster, it was suggested that hydrogen binding and subsequent

oxidation, or hydride formation and subsequent hydrogen formation, could occur at the site of the terminal Fe via the displacement of the weakly bound water molecule [6]. Consistent with this line of thinking, the structure of the enzyme from *D. desulfuricans*, presumed to be a mixture of oxidized and reduced molecules in the crystal, reveals an open coordination site at this position [27,29]. Subsequent to the initial structural characterization of the Fe-only hydrogenases from *C. pasteurianum* and *D. desulfuricans* [6,7], there have been several structural studies focused on the characterization of defined states. The structural characterization of the fully reduced state indicates that, upon reduction, the bridging CO ligand observed in the oxidized state changes to a terminal mode in which it is bound solely to the distal Fe atom of the subcluster [29]. Interestingly, although CO is a component of the active H-cluster, the addition of exogenous CO reversibly inhibits both the hydrogen oxidation and proton reduction activities of the enzyme [30]. The

Figure 4



Structures of the P-cluster and FeMo-cofactor of nitrogenase. (a) Structure of the nitrogenase P-cluster in the oxidized (P^{OX}) and reduced (P^{N}) states. (b) Structure of the nitrogenase FeMo-cofactor. The Mo atom is shown in magenta, with the remainder of the color scheme as in Figure 2.

CO-inhibited form of the enzyme unambiguously reveals the presence of inhibitory CO at the aforementioned site on the distal Fe atom [31], providing additional support for the involvement of this site in catalysis.

Nitrogenase

Nitrogenase is responsible for the enzymatic conversion of dinitrogen gas to ammonia (Figure 1). It consists of two separable protein components termed the Fe protein and the MoFe protein. During nitrogenase catalysis, the Fe protein and MoFe protein associate to form a complex in which MgATP is hydrolyzed and an electron is transferred from the $[\text{Fe}_4\text{S}_4]$ cluster of the Fe protein to the MoFe protein, where the substrate is bound and reduced. Subsequent to MgATP hydrolysis and electron transfer, the complex dissociates and the free Fe protein must then be rereduced and bind MgATP before the next cycle can begin. Multiple cycles of Fe protein–MoFe protein association and dissociation are required for the complete reduction of a single molecule of dinitrogen to ammonia.

The nitrogenase MoFe protein contains two types of complex metal clusters: the P-cluster and the FeMo-cofactor (Figure 4) [1–4,5**]. The nitrogenase P-cluster is an $[\text{Fe}_8\text{S}_7]$ cluster that can be described in its reduced state as the covalent attachment of two regular $[\text{Fe}_4\text{S}_4]$ clusters through a shared corner S atom (Figure 4a) [1,3,4]. These two cubanes are also bridged by two cysteine residues that bind Fe atoms of each cubane. Additional cysteine residues provide terminal coordination to the remaining Fe atoms of the cluster. The P-cluster serves as an intermediate in the transfer of electrons from the $[\text{Fe}_4\text{S}_4]$ cluster of the Fe protein to the FeMo-cofactor substrate-reduction sites. Crystallographic analysis of defined oxidation states has shown that the P-cluster undergoes a redox-dependent structural rearrangement [3,4]. This structural rearrangement involves the exchange of two Fe atom ligands from the shared S atom (in the reduced state) to the polypeptide (in the oxidized state). In the oxidized state, these Fe atoms are coordinated by the sidechain oxygen atom of a serine and an amide nitrogen atom of the peptide backbone. The mechanistic relevance of the structural rearrangement is unknown; however, it has been suggested that these structural changes could provide a means to couple proton uptake to electron transfer [4].

The FeMo-cofactor is the site of nitrogen binding and reduction in nitrogenase. The structure of the nitrogenase FeMo-cofactor can be described as two bridged partial cubanes (Figure 4b) [1,2,5**]. The cluster consists of an $[\text{Mo-Fe}_3\text{S}_3]$ partial cubane bridged to an $[\text{Fe}_4\text{S}_3]$ partial cubane via three sulfur atoms that bind to Fe atoms of each cluster fragment. Recent high-resolution X-ray diffraction studies of the MoFe protein have revealed that the two cluster fragments are bridged by a light atom that

is hexacoordinately bound to three Fe atoms of each partial cubane [5**]. The atom has been tentatively assigned as nitrogen. The FeMo-cofactor also possesses an organic constituent, homocitrate, which is bound to the Mo atom via two oxygen atoms. The cluster is covalently attached to the protein by only two protein ligands: a cysteine residue to the terminal Fe atom of the cluster and a histidine via its imidazole N δ atom to the Mo. Although the structure has been defined to near 1 Å resolution, it is still unknown how nitrogen is reduced or even how it initially binds the FeMo-cofactor, although a prevailing viewpoint that the binding and reduction of nitrogen occurs at one of the central 4Fe faces of the cluster has some very attractive features [32,33].

Conclusions

Crystallography has made significant contributions to our understanding of complex metallocluster structure and mechanism. Because metalloclusters can undergo conformational changes upon ligand binding and/or variation in oxidation state, multiple structures are often necessary to understand the structural dynamics of these centers. Last year's discovery of a light atom in the middle of the nitrogenase MoFe-cofactor is an excellent example of the surprises that are unveiled by continued structural studies. The first crystallographic view of a metallocluster typically inspires new efforts in biomimicry. The resulting models provide valuable insights into the properties of metals with the observed geometries and ligand sets [34–39]. It is a combination of crystallography, model studies, spectroscopy and biochemical characterization that, in the end, is necessary for a detailed understanding of these amazing metallocofactors.

Update

A recent paper by Fontecilla-Camps, Lindahl and co-workers [41**] reports an X-ray structure of CODH/ACS in which each of the two A-clusters of the heterotetrameric enzyme have metal compositions that are different from each other and different from the previously observed $[\text{Fe}_4\text{S}_4]$ -Cu-Ni cluster. In contrast to the first structure of CODH/ACS [10**], which had uniform metal composition, the recent structure contains one ACS subunit with Zn in place of Cu in the proximal site and the other ACS subunit with Ni in this site. This heterogeneity within one structure of a CODH/ACS is fascinating. Thus, three forms of the A-cluster are now confirmed to exist: $[\text{Fe}_4\text{S}_4]$ -Cu-Ni, $[\text{Fe}_4\text{S}_4]$ -Zn-Ni and $[\text{Fe}_4\text{S}_4]$ -Ni-Ni. The next question is which form or forms of the A-cluster are catalytically active. In terms of the C-cluster, the authors report an X-ray structure that does not contain a bridging sulfide S2 and thus more closely resembles CODH_R [41**]. This new CODH/ACS structure [41**], in addition to the other recent CODH and CODH/ACS structure determinations [8**–10**], suggests new mechanistic possibilities that can now be tested.

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