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Production and properties of enzymes that activate and produce carbon monoxide

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Abstract

The chapter focuses on the methods involved in producing and characterizing two key nickel–iron–sulfur enzymes in the Wood–Ljungdahl pathway (WLP) of anaerobic conversion of carbon dioxide fixation into acetyl–CoA: carbon monoxide dehydrogenase (CODH) and acetyl–CoA synthase (ACS). The WLP is used for biosynthesis of cell material and energy conservation by anaerobic bacteria and archaea, and it is central to several industrial biotechnology processes aimed at using syngas and waste gases for the production of fuels and chemicals. The pathway can run in reverse to allow organisms, e. g., methanogens and sulfate reducers, to grow on acetate. The CODH and ACS intertwine to form a tenacious CODH/ACS complex that converts CO₂, a methyl group, and coenzyme A into acetyl–CoA. CODH also behaves as a modular unit that can function as an independent homodimer. Besides coupling to ACS, CODH can interact with hydrogenases to couple CO oxidation to H₂ formation. These enzymes have been purified and characterized from several microbes.

1. Introduction

The chapter is focused on the methods involved in producing and characterizing the catalytic properties of the two key nickel iron–sulfur metallo-enzymes in the Wood–Ljungdahl pathway (WLP) of anaerobic conversion of CO₂ to acetyl-coenzyme A (acetyl–CoA). This pathway involves the eight-electron reduction of CO₂ to form acetyl–CoA. In the WLP, these enzymes, carbon monoxide dehydrogenase (CODH), and acetyl–CoA synthase(ACS)intwine to form a tenacious complex with a 150Å gas channel that sequesters the CO formed from CO₂ (Eq. 1) at the CODH active site and tunnels it to the A-cluster, the active site of ACS, for catalyzing acetyl–CoA synthesis (Eq. 2). The A-cluster binds and then condenses the three components of acetyl–CoA: CO, a methyl group donated by the methyl–Co(III) state of the corrinoid iron–sulfur protein (CFeSP), and coenzyme A. The WLP can operate in reverse to allow organisms to grow on acetate. Furthermore, CODH is a modular unit that can interact with other enzymes, besides ACS, to fulfill various metabolic roles. For example, it forms a CODH/ hydrogenase unit to couple CO oxidation to proton reduction. Much progress has been made on understanding the mechanisms of CODH and ACS based on spectroscopic, crystallographic, kinetic, and electrochemical studies.

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These enzymes have been purified and characterized from several microbes and are used as important inspirational models and benchmarks in energy technologies ranging from applications such as fuel cells to fundamental examples of “nearly perfect” catalytic activity.

2. Culturing the microbes

2.1 General considerations about technology for growth of anaerobic culture.

Cultivation of anaerobic cell cultures is as ancient as recorded human civilization in the form of fermentation, and it was on facultative anaerobes that the foundational work of Leeuwenhoek and Pasteur was performed, ushering in the age of modern biochemistry. Anaerobic conditions are essential for culturing and harvesting the oxygen-sensitive microbes, including *Moorella thermoacetica*, *Clostridium autoethanogenum*, *Carboxydotherrmus hydro-geoformans*, and *Methanothermobacter marburgensis*, employed in the Ragsdale Laboratory. We direct the reader to some valuable general anoxic principles and methods (Hungate, 1969; Wolfe, 2011). These principles have been important in developing the anaerobic techniques employed by the Ragsdale Laboratory as described here.

It is critical to maintain low ambient redox potentials in culture medium. As described by Hungate (1969), this requires adding a reducing agent to the culture medium (Hungate, 1969). Commonly used reductants are dithiothreitol (DTT), sodium dithionite, and Ti(III) citrate. To obtain a redox potential of 0.33V in the absence of a reductant, less than one molecule of O₂ per liter of solution must be present. That is obviously an impossible task; however, reducing agents will act as redox buffers and poise the potential at the desired value. Removal of oxygen is also required because many microbes and metalloproteins react quickly with O₂; furthermore, reducing agents are rapidly oxidized in aerobic medium. Thus, having access to an anaerobic gas chamber, described later, is highly advisable. We also use Schlenk-line technology to thoroughly degas solutions. Trace O₂ levels in compressed gases are removed using O₂ filters (Altech Oxy-trap or a SDG Purity Plus O₂-trap).

Ti(III) citrate is prepared by first mixing 1mL of 15% TiCl₃/HCl solution with 1.6mL of 1M sodium citrate and then adding 2.5mL of 2M Tris-HCl buffer at pH 8.0 (Seefeldt & Ensign, 1994). Further neutralization may be needed and can be done using sodium carbonate until no bubbles are observed.

Commonly used anaerobic containment for small liquid microbial precultures includes Hungate-type vials or Balch-type tubes with butyl rubber stoppers and screw cap or

aluminum apertures (Hungate, Smith, & Clarke, 1966), stoppered with crimpable aluminum caps (Hungate, 1969), then a bioreactor with fermenter is sufficient for large-scale growth.

2.2 Use of anaerobic chambers

Three types of chambers are used in our laboratory: a Vacuum Atmospheres Company anaerobic gas chamber (Hawthorne, CA), a refrigerated anaerobic chamber (Inert Technologies, Amesbury, MA), and a Coy chamber (Coy Laboratory Products, Grass Lake, MI). The Inert Technologies and Vacuum Atmospheres “hard” chambers are constructed of stainless steel with hard plastic windows and thick gloves and are maintained at or below 2ppm O₂. There are a total of six chambers in the laboratory varying from one to three persons in size. This allows us to equip different chambers with FPLCs and chromatography columns, spectrophotometers, a 5°C Peltier-cooled storage refrigerator, a sonicator with external cooling waterbath, a weighing scale, an isothermal calorimeter, a benchtop centrifuge, a stopped-flow absorption-fluorescence spectrophotometer, and spectroelectrochemical setup.

The Coy chamber is a soft plastic chamber with an atmosphere of 95% N₂ and 5% H₂ (note that under these conditions, H₂ levels never exceed 4.0%, its flammability limit). The atmosphere is scrubbed by pellets of a palladium catalyst surrounded by absorbent material, which convert the O₂ to H₂O. The Inert Technologies refrigerated hard chamber is equipped with an air conditioning cooling unit to allow an ambient temperature of ~15°C, which is ideal for purifying and storing enzymes from mesophilic organisms such as *C.*

autoethanogenum. The Vacuum Atmospheres hard chambers are kept in anoxic N₂, which is continually circulated through BASF catalysts consisting of 85% aluminum oxide, 12% copper oxide, with trace nickel monoxide and cobalt oxide. The N₂ is supplied by liquid nitrogen tanks, with ~2–3ppm oxygen, which is further scrubbed by chamber catalysts to working conditions.

2.3 Cultivation of the microbes

2.3.1 Growth of *M. thermoacetica*—*M. thermoacetica* (ATCC 39073) has been the model organism for study of the WLP. We routinely grow *M. thermoacetica* under anoxic cultivation conditions in batch cultures on glucose as described (Ljungdahl & Andreessen, 1978), with the following modifications. A typical preparation is described as follows: cells were grown at 55°C in a 10-L cultivation vessel (Eppendorf) using a New Brunswick Scientific BioFlo 110 bioreactor equipped with a MGW Lauda RM6 Brinkman water circulator. The 10L culture was continuously supplied with CO₂ (99.9%) or a CO₂/N₂ (20%/80%) mixture and agitated at 120rpm. Growth was monitored through the optical density at 600nm. We also measured the CO oxidation activity of whole cells using a CO-saturated assay mixture containing 20mM benzyl viologen in 50mM HEPES buffer (pH 8.0). As *M. thermoacetica* is gram (+) (Drake & Daniel, 2004) the purity of the culture during growth was established by gram staining. Cells were harvested by centrifugation under 100% N₂ and stored at 80°C for later use.

M. thermoacetica was originally isolated using glucose as carbon source (earlier). Although this has been the model organism for characterization of the autotrophic WLP, it was not

until 1983 (Kerby & Zeikus, 1983) that it was shown to be capable of autotrophic growth on CO₂/H₂ or on CO, demonstrated in a follow-up study a few years later (Daniel, Hsu, Dean, & Drake, 1990). It can grow on other sugars as well as methanol (Hsu, Daniel, Lux, & Drake, 1990), methoxylated aromatics (vanillate and syringate) (Daniel, Wu, & Drake, 1988; Kasmi, Rajasekharan, & Ragsdale, 1994), and carboxylated aromatics (proteocatechuate and *p*-hydroxybenzoate) (Hsu et al., 1990). The methyl and carboxyl groups are stoichiometrically converted to acetyl-CoA (Daniel et al., 1988; Hsu et al., 1990; Kasmi et al., 1994).

2.3.2 Growth of other microbes expressing CODH and ACS

C. hydrogenoformans: The monofunctional CODH and CODH/ACS have been isolated from the gram (+) thermophilic strict anaerobe *C. hydrogenoformans*, which was grown at 65°C on CO as the carbon source, as described (Svetlitchnyi, Peschel, Acker, & Meyer, 2001).

C. autoethanogenum: We are studying the WLP and isolating CODH and CODH/ACS from *C. autoethanogenum*. This organism will grow at 37°C on H₂/CO₂ or CO, as well as various mixes of these gases and syngas (Mock et al., 2015), producing predominantly acetate; it also produces ethanol and has been engineered to produce various industrially relevant chemicals (Kopke et al., 2011; Liew et al., 2017; Norman, Millat, Winzer, Minton, & Hodgman, 2018). *C. autoethanogenum* also grows heterotrophically on sugars (e.g., 4.5g/L fructose) (Liew et al., 2016; Mock et al., 2015).

Rhodospirillum rubrum: The phototropic bacterium, *R. rubrum*, grows on a variety of carbon and energy sources under aerobic, anaerobic, and fermentative conditions (Ghosh, Hardmeyer, Thoenen, & Bachofen, 1994), although it lacks the WLP. For purification of CODH, *R. rubrum* cells were grown on ammonium malate as N and C sources and then 100% CO was sparged into the culture for 24h prior to harvesting (Bonam & Ludden, 1987). Carbon monoxide is required to induce the monofunctional CODH (as well as the CO-linked hydrogenase) (Kerby et al., 1992).

Methanogens: The WLP is fundamental to the autotrophic metabolism of methanogens as well as in its growth on acetate (Ferry, 2010). To metabolize acetate, from which 2/3 of the methane in the biosphere is derived, methanogens operate the WLP in reverse. CODH and other proteins of the WLP have been isolated from *Methanosarcina barkeri* (Grahame & Stadtman, 1987), *Methanococcus vannielii* (Demoll, Grahame, Harnly, Tsai, & Stadtman, 1987), and *Methanosarcina thermophila* (Terlesky, Nelson, & Ferry, 1986), grown as described.

2.3.3 Growth of organisms and their heterologous expression of CODH—

Jeoung and Dobbek reported expression in *Escherichia coli* and purification of highly active recombinant CODH II from *C. hydrogenoformans* (Jeoung & Dobbek, 2007). As described, two plasmids, one based on the pET28a vector for apo-CODH II expression (pKS2) and the other for iron–sulfur cluster maturation (pKRISC), were transformed into the Rosetta (DE3) cell line. Growth was started aerobically in TB media with slightly high iron and nickel

concentrations; then, the culture was switched to anaerobic conditions as CODH and the Isc system were induced by adding (to final concentration) 0.2mM IPTG, 0.5mM NiCl₂, 1mM FeSO₄, 1mM Na₂S, and 50mM KNO₃. A more detailed discussion on the maturation of metal clusters of CODH has recently been published (Merrouch et al., 2018).

Expression and purification of recombinant CODH I in *E. coli* have also been reported (Inoue, Takao, Fukuyama, Yoshida, & Sako, 2014). While coexpression of CODH I along with a Ni-insertion accessory protein (*CooC3*) yielded an enzyme with an activity of 8060U/mg, expression without *CooC3* achieved a fourfold lower activity (2270U/mg).

Heterologous expression in *Clostridium acetobutylicum* of CODHs from *Clostridium carboxidivorans* and *C. ljungdahlii* was reported recently (Carlson & Papoutsakis, 2017; Fast & Papoutsakis, 2018). Characterization of these enzymes has not yet been reported.

3. Purification of CODH, ACS, and CODH/ACS

3.1 General anaerobic technology

General anaerobic protocols for culturing the microbes were explained earlier, but they are also relevant here. Here we will describe procedures for purifying and handling extremely oxygen-sensitive enzymes. All protein purification steps take place under anoxic conditions in anaerobic chambers (earlier) and reducing agents, usually DTT, Ti(III) citrate, and/or dithionite, are used in all solutions to poise and maintain the low redox potentials required for optimal enzyme activity (Wang, Can, Pierce, Ragsdale, & Armstrong, 2013).

3.2 Purification of monofunctional CODH (CooS)

3.2.1 C. hydrogenoformans CODHI and CODHII—Here we describe the purification protocol for CODH I and II described by Svetlitchnyi et al. (2001) with the revisions noted as mentioned below. While the main buffer used in the published procedure contains 50mM Tris-HCl (pH 8.0) and 2mM sodium dithionite, we include 2mM DTT (Buffer A_{Ch}). We have attempted to use methyl viologen (MV) in our columns as an oxygen scavenger; however, this leads to extreme gas formation, which disrupts the chromatography. Throughout the isolation, CO oxidation activity is determined in the various fractions by measuring the absorbance change at 578nm occurring as a few microliters of the fraction solutions are introduced to a CO-saturated 50mM HEPES buffer (pH 8.0) containing 20mM MV and 2mM DTT, essentially as described earlier (Ragsdale, Clark, Ljungdahl, Lundie, & Drake, 1983). We also examine the fractions by SDS-PAGE because CODH II runs slightly slower than CODH I.

The cell lysis buffer contains buffer A_{Ch} including 0.1mg/mL of lysozyme, 0.05mg/mL of DNase I, and 0.2mM phenyl methyl sulfonyl fluoride. While Svetlitchnyi et al. describe an osmotic lysis step with 0.6M sucrose (Svetlitchnyi et al., 2001), we achieve good results by sonication. The first step involves anion exchange chromatography (AEC) with a salt gradient of 0–1M NaCl. The second step uses a hydrophobic interaction chromatography (HIC) column, on which CODH I elutes cleanly before CODH II. In contrast, Svetlitchnyi et al. employ additional HIC, AEC, and size-exclusion chromatography columns (Svetlitchnyi et al., 2001) and found that only one additional HIC is required to obtain protein of 99%

purity. The activities of the purified proteins at 70°C are 15,756 and 13,828U/mg for CODH I and II, respectively (Svetlitchnyi et al., 2001). These enzymes are highly stable and can be stored in buffer A_{CH} under anoxic conditions at room temperature without losing any activity for more than a year; alternatively, they can be stored as frozen stocks at –80°C.

3.2.2 Rhodospirillum rubrum CooS—Purification of the fully active native CODH from *R. rubrum* can be achieved when cells are grown under CO atmosphere with elevated nickel levels in the growth medium (Ensign, Hyman, & Ludden, 1989). During the purification processes, slightly basic buffers with reductants are used; for instance, 100mM MOPS (pH 7.5) with 5mM sodium dithionite (Bonam & Ludden, 1987). Initial purification steps include sedimentation and resuspension of the chromatophore membranes after disrupting the cells using a homogenizer or a French pressure cell (Ensign, 1995). Heat-shock treatment of the resuspended chromatophores solubilizes the CODH and precipitates large amounts of impurities. After centrifugation, the supernatant is applied to DEAE-cellulose and hydroxylapatite chromatography columns. As a polishing step, preparative native gel electrophoresis or an additional high-resolution Q-sepharose column can be used. The specific activity of the purified protein for CO oxidation is 5200U/mg at 25°C and pH 7.5 (Ensign et al., 1989).

3.2.3 Recombinant CODH from E. coli—The vectors and protocols for recombinant expression of His-tagged *C. hydrogenoformans* CODH I and II were mentioned earlier. These proteins are purified in a single step on an IMAC (Ni-Sepharose) medium containing 1mM sodium dithionite (Jeoung & Dobbek, 2007). The specific activities at 70°C of the purified CODH I and II were 12,229 and 13,828U/mg, very close to the values reported for the native enzymes.

3.3 Purification of monofunctional Ni-ACS from *E. coli*

Although CODH/ACS has been studied for decades, levels of active ACS did not surpass 40% based on NiFeC spin concentration and various enzymatic assays described earlier. This shortcoming does not affect techniques like electron paramagnetic resonance (EPR) or electron nuclear double resonance (ENDOR) in which Ni_d is EPR silent, and the Ni site in the C-cluster has a different nonoverlapping signal; however, it does compromise the results of many spectroscopic studies, such as Mossbauer or X-ray absorption spectroscopy (XAS) which detect the average metal environment. For example, if the A-cluster is 30% active, the active state of Ni_p represents only 10% (0.3/3) of the total Ni. It is nearly impossible to discern the properties of this small Ni_p population, which is averaged by the remaining 90% of the Ni in the protein. Thus, it was a significant advance when the ACS of the CODH/ACS complex (the α -subunit encoded by *acsB*) was heterologously expressed in *E. coli* and reconstituted to 80%–100% activity A cluster (Can, Giles, Ragsdale, & Sarangi, 2017; Seravalli & Ragsdale, 2008). In a sample of ACS that is 80% active, Ni_p represents at least 40% of the total Ni (0.8/2), with Ni_d making up ~50% of the Ni signal. This development allowed us to obtain a high-resolution analysis of the geometry and electronic structure of the key Ni_p-CO intermediate (Can et al., 2017), providing the first direct validation of the hypothesis that the WLP operates through an organometallic mechanism involving a series of Ni–C bonds (Ragsdale & Wood, 1985).

3.3.1 Cloning and expression of ACS—The *acsB* gene encoding ACS from *M. thermoacetica* was cloned into the pET29 (Kan^R and His6-tag; *lac*-inducible) vector for heterologous expression in BL21(DE3) *E. coli* cells. Anaerobic coexpression of the monofunctional ACS with pbd1282 (*A. vinelandii* *iscS*, *iscU*, *iscA*, *hscB*, *hscA*, *fdx*; Amp^R; *ara* inducible) is used for obtaining proper construction and insertion of the ACS NiFeS cluster (Seravalli & Ragsdale, 2008). To remove the His6-tag, a C-terminal TEV-cleavage site was incorporated into the *acsB*-containing pET29 vector by inserting a GAAAACCTGTATTTCCAGTCG sequence between BamHI and XhoI restriction sites ahead of the His6 sequence, [CATCAC]₃.

A 100mL lysogeny broth (LB) culture of BL21(DE3) *E. coli* cells containing the ACS plasmid and the pbd1282 plasmid is grown overnight in the presence of 100µg/mL ampicillin (Amp), and 50µg/mL kanamycin (Kan) is then used to inoculate 1 L of an Amp/Kan-containing LB media on the day of growth. Once the 1L culture has reached an OD₆₀₀ of 0.7–1.0, the cells are inoculated into 10L of terrific broth (TB) media containing Kan and Amp in a BIOFLO 110 bioreactor agitated at 250rpm and 37°C. At an OD₆₀₀ of 0.3–0.4, sterile-filtered arabinose (0.5g/L) is added to induce expression. Then at an OD₆₀₀ of 0.6, the aerobic solution is vigorously bubbled with high purity N₂ (UHP grade, Cryogenic Gases, Detroit, MI) for at least 45min, an anaerobically prepared reducing solution (100 mL) consisting of 3.6g L-cysteineHCl and 3.6g Na₂S is added to the bioreactor and, 15min later, anaerobically prepared 0.5MFe^{II}(NH₄)₂(SO₄)₂ is added to give a final Fe concentration of 100µM. After 15 further min, anaerobic solutions of 0.5M isopropyl β-D-1-thiogalactopyranoside (IPTG) and 1.0M NiCl₂ are added to give final concentrations of 200µM IPTG and 200µM nickel. Cells are grown overnight at 37°C under minimal N₂ flow and are harvested 12–16h later into anaerobic, air-tight centrifuge bottles, and pelleted at 5000rpm at 4°C for 10min. Once cells are pelleted, they are transferred to anaerobic 50-mL Falcon™ tubes, flash frozen with LN₂, and stored in a –80°C freezer.

3.3.2 Lysis, purification, and characterization of ACS—Cell lysis and ACS purification are performed under strictly anaerobic conditions (3 ppm O₂). A general purification buffer, buffer A, is composed of 50mM potassium phosphate (KP_i), 300mM NaCl, 10mM imidazole, 5mM β-mercaptoethanol (βME), at pH 8.0. The lysis buffer contains 0.25mg/mL lysozyme, DNase I, and 0.5mM phenylmethane sulfonyl fluoride (PMSF) in buffer A. For each gram of cell pellet, 4mL lysis buffer is used to resuspend the cells, which are sonicated (10s “on”; 20s “off”; 80% amplitude) for 15 min in a beaker kept at 4°C. The resultant cell lysate is ultracentrifuged for 1h at 30,000rpm after which the supernatant is decanted onto a Ni-NTA column equilibrated with buffer A. After washing the Ni-NTA column with three column volumes (CVs) of buffer A, the His6-tagged ACS is eluted with a step gradient of 5% increments from 5% to 30% of buffer B (50mM KP_i, 300mM NaCl, 250mM imidazole, 5mM βME, pH 8) in buffer A. Protein concentration and purity are measured by Rose Bengal dye assay (Elliott & Brewer, 1978) calibrated to a lysozyme standard, and by SDS-PAGE, respectively. Fractions containing pure ACS are pooled and the sample is buffer exchanged into 50mM KP_i, 300mM NaCl, 5mM βME, pH 7.6 to remove imidazole. Sodium citrate is added to the protein solution at a final concentration of 1mM, and then approximately 1mg TEV protease is added to begin an

overnight cleavage reaction. The solution is then reloaded onto the reactivated Ni-NTA column equilibrated with buffer A, and the His6-tag-cleaved ACS, which elutes rapidly in the flow-through fraction, is buffer exchanged into 50mM KP_i pH 7.6, and stored anaerobically at 20°C.

3.3.3 Metal reconstitution of ACS—In order to facilitate proper metal loading in ACS, additions of 5:1 or 6:1 NiCl_2 :ACS and 1:1 $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$:ACS are added to the pooled ACS from anaerobic 100mM NiCl_2 and $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ stock solutions, βME (2mM, final) is added to the protein solution and the solution is incubated at 45°C for 72h. Extraneous nickel is then removed from the reconstituted ACS by buffer exchange into a 50mM potassium phosphate buffer containing 100 μM DTT, pH 7.5. The exchange is accomplished by passing the protein (<1mL) twice through an anaerobic 8.3mL PD-10 desalting column or by the use of Amicon® pressure filters (10⁶-fold) with a 30kDa cutoff membrane. Amicon ultrafiltration is used to concentrate the protein to 200–500 μM .

Finally, the reconstituted ACS is assayed by EPR spectroscopy to determine the morphology and spin content of the NiFeC EPR signal, metal analysis by ICP-OES/-MS, and measure catalytic activity by the CO/[1-¹⁴C]-acetyl-CoA exchange reaction (earlier).

3.4 Purification of *M. thermoacetica* CODH/ACS

The CODH and ACS enzymes form a tight complex and coelute throughout the purification. Thus, CODH/ACS fractions are identified using the CO oxidation activity assay and SDS-PAGE gels. In an early example of this preparation, heat treatment at 67°C for 30min upon cell disruption was followed with stepwise ammonium sulfate precipitation, and the CODH fractions were solubilized in a pH 7.6 Tris-HCl buffer with 2mM sodium dithionite (Ragsdale, Clark, et al., 1983). In this protocol, chromatographic purification steps included DE32-cellulose resin, hydroxylapatite resin, size exclusion, and DEAE-Sephacel, respectively.

Nowadays in our laboratory, CODH/ACS is purified by FPLC. To each gram of dry cells is added 4mL of lysis buffer consisting of buffer A_{Mf} (50mM Tris-HCl pH 7.6 with 2mM sodium dithionite and 2mM DTT), 0.5mM PMSF, 0.25mg/mL lysozyme, and 10 $\mu\text{g/mL}$ DNase I (Roche: grade II, from bovine pancreas). For 200mL of lysate, sonication is accomplished at 4°C, running 10s “on” and 20s “off” for a total of 15min. The lysate is centrifuged at 30,000rpm for 1h at 4°C. The supernatant is then applied to a 60mL Q-Sepharose Fast Flow resin (GE Healthcare) equilibrated in buffer A_{Mb} , which is developed with a salt gradient from 0 to 0.6M NaCl, with CODH/ACS eluting in fractions containing 0.3–0.4M NaCl.

The next step is hydrophobic chromatography. The CODH/ACS fractions are pooled and ammonium sulfate is added to a final concentration of 0.6M. The solution is then applied to a column containing 30mL Phenyl Sepharose 6 Fast Flow (Sigma-Aldrich) resin equilibrated with 0.6M ammonium sulfate in buffer A_{Mb} , and a decreasing gradient in ammonium sulfate, 0.6–0.0M, is applied. The CODH/ACS complex elutes in fractions containing 0.2–0.0M ammonium sulfate. The protein purity judged by SDS-PAGE is higher than 95% and CO oxidation activity varies between 200 and 300U/mg at 25°C.

4. Characterization of CODH, ACS, and CODH/ACS

4.1 Characterization of CODH

Anaerobic conditions must be used during all experiments to preserve the active states of the clusters and to poise the solutions at the optimal redox condition for the reaction. All bacterial Ni-CODHs, whether they are monofunctional or bound to ACS, form a homodimeric complex containing five iron–sulfur clusters. Each monomer contains a NiFe_3S_4 cluster (C-cluster) and a Fe_4S_4 cluster (B-cluster); in addition, a single Fe_4S_4 cluster (D-cluster) links the two monomers covalently (see earlier). Steady-state kinetic analyses of the different classes of CODH have revealed the Michaelis parameters (k_{cat} , K_{m} , $k_{\text{cat}}/K_{\text{m}}$) and transient kinetic studies have revealed the intermediates involved in the reaction (Fig. 1; a recent review is available, containing mechanistic detail, Can, Armstrong, & Ragsdale, 2014). The B- (Fe_4S_4) and C- (NiFe_3S_4) clusters of CODH have distinct spectral signatures (Can et al., 2014; Jeoung & Dobbek, 2007) allowing the use of multiple rigorous methods to dissect the redox and chemical changes that accompany this two-electron reaction. Rapid-freeze-quench (RFQ) EPR studies revealed that CO reacts with the C-cluster in the C_{red1} redox state, converting it rapidly and directly to the C_{red2} state during the two-electron oxidation of CO to CO_2 (Kumar, Lu, Liu, & Ragsdale, 1993). These studies also monitored reduction of the B-clusters and demonstrated their role in electron transfer to external acceptors, although D-cluster reduction has not been experimentally observed under catalytic conditions. Electrochemical studies revealed cooperativity, suggesting that the C_{red1} to C_{red2} conversion is concerted (Wang et al., 2013).

Methods used to dissect the details of the CODH mechanism include spectroscopy (UV–visible, EPR, FTIR, ENDOR, Mossbauer, X-ray absorption, static, and freeze-quenched EPR), kinetics (steady state and transient), electrochemistry (spectroelectrochemistry and direct enzyme immobilized), and computations. Similar methods have been used to characterize ACS. These methods exploit the variations in reduction potential, reduction kinetics, and spectral variations among the clusters due to their unique environments and roles within CODH (see Table 1) and ACS. We focus on the methods that are routinely involved in studying CODH and ACS. We refer the reader to the literature for details of methods we have used to isolate and characterize intermediates in the CODH and ACS mechanisms. We recently published a general review covering these studies (Can et al., 2014).

4.1.1 Steady-state kinetic measurements of CODH—The steady-state reaction has been monitored spectrophotometrically by observing the products and turnover of mediators in the enzyme reaction, by electrochemistry, and by NMR methods, monitoring the CO– CO_2 exchange (see a recent review, Can et al., 2014). Such studies have established values of k_{cat} , K_{m} (and hence $k_{\text{cat}}/K_{\text{m}}$) for the various substrates in CO oxidation and CO_2 reduction, as well as identified inhibitors and their mode of inhibition. By comparing values of $k_{\text{cat}}/K_{\text{m}}$, it has been established which physiological (e.g., ferredoxin, flavodoxin, rubredoxin, pyruvate ferredoxin oxidoreductase) and nonphysiological (e.g., methyl/benzyl viologen) redox partners couple most effectively to the enzyme.

MV-based assays are conducted by monitoring the time-dependent increase in absorption at 568nm indicative of one-electron reduced MV; thus two cycles of MV reduction are required for each turnover of CO or CO₂. The assay (Ragsdale, Clark, et al., 1983; Ragsdale, Ljungdahl, & DerVartanian, 1983) is performed in a 1mL reaction mixture containing 50mM HEPES pH 8.0, 2mM DTT, 0.02mM sodium dithionite, and 10mM MV. The solution is sparged with CO in an anaerobic cuvette sealed with a rubber septum stopper, then the reaction is initiated by injection of CODH.

4.1.2 EPR and ENDOR measurements—EPR is a critical technique for studying electron transfer between the B- and C-clusters, which have discrete signals, and for measuring the metal cluster spin intensity, which reflects the percentage activity of the protein (Table 1). Our group uses a Bruker (Billerica, MA) Model EMX X-band EPR instrument with a Cold Edge closed cycle helium cryostat capable of achieving temperatures as low as 8K. The instrument is equipped with a Hewlett-Packard model 5340 automatic frequency counter and a Bruker gaussmeter.

Samples are either frozen in-chamber with liquid nitrogen, or frozen outside the chamber after capping with a septum prior to removal. The time course of reactions on timescales of 30s and above can be studied by manual freeze quench—running the reaction in an EPR tube and inserting the tube into liquid nitrogen at specified time points.

Pulsed EPR (electron nuclear double resonance, ENDOR) measurements (DeRose, Telser, Anderson, Lindahl, & Hoffman, 1998) using isotopically labeled enzyme and substrates (¹H, ²H, ¹⁴N, ¹³C, and ⁵⁷Fe) indicated that a nucleophilic iron-bound hydroxide ion at the C-cluster reacts with a Ni–CO intermediate to form a Ni–Fe-bridged carboxylate product (Fig. 1). This bound carboxylate further evolves to give CO₂, releasing two electrons and two protons. This description is consistent with other spectroscopic (Can et al., 2014) and crystallographic studies (Jeoung & Dobbek, 2007), although the formation and decay of the Ni–CO species at catalytically relevant rates have never been established by spectroscopic studies.

4.1.3 Transient kinetic experiments

Stopped flow: Rapid kinetics methods enable the reactions as they occur on the enzyme to be monitored and allow the identification of intermediates. Stopped-flow spectrophotometry, with the instrument housed in the anaerobic chamber, is a powerful method to study the reduction of the FeS clusters of CODH. Reduction from the 2+ to the 1+ state results in a significant bleaching in absorbance, with a ϵ_{420} (monomer) of $\sim 20\text{mM}^{-1}\text{cm}^{-1}$ (Svetlitchnyi et al., 2001), the changes stemming predominantly from reduction of the B-cluster. As with any transient kinetic experiments on CODH, the fast rates of reduction challenge the dead time of the instrument given that most mixing experiments are subject to a dead time of 1.0 ms. Therefore, low temperatures or subsaturating substrate concentrations are often used to follow the enzyme-monitored CO oxidation reaction (reviewed in Can et al., 2014). Our stopped-flow instrument is housed within an anaerobic chamber.

Rapid freeze-quench (RFQ) EPR: Rapid freeze quench (RFQ) involves rapidly (5ms) mixing two solutions and quenching the reaction by low-temperature freezing (Ballou,

1978). Solutions are placed in syringes; then the reagents are pushed by a ram through a mixing chamber and into a single tubing (of varying lengths) connected to a nozzle that sprays the reaction mixture into an EPR tube immersed in an isopentane bath kept at 140°C. RFQ-EPR methods can assay the rate of substrate binding to the CODH or ACS active site and assess the overall catalytic competence of any species detected. Our RFQ experiments are performed in an anaerobic chamber with a Bio-Logic SFM-2000 rapid mixing unit employing an in-chamber cryobath and an external liquid nitrogen pump. The major limitation of this method is that the CODH reaction is very fast. The steady-state k_{cat} value for the *M. thermoacetica* CODH/ACS is as high as 500s^{-1} at pH 7.6 and 55°C, the organism's growth temperature (Ragsdale, Clark, et al., 1983; Ragsdale, Ljungdahl, et al., 1983); thus, all kinetically relevant reactions have to occur within this timescale. For the CODH/ACS from *M. thermoacetica*, the rate constant for reduction of the C-cluster was 400s^{-1} at 5°C, equating to a $t_{1/2}$ of 1.8ms, closer to the dead time of the instrument (Kumar et al., 1993). Any earlier steps, such as binding of CO would have occurred within the mixing time and evade detection. Furthermore, the rate constant for this reaction, if extrapolated to the optimum growth temperature of the organism, would approximate to $17,200\text{s}^{-1}$ at 55°C ($t_{1/2}=40\text{ }\mu\text{s}$!).

4.1.4 Spectroelectrochemical and direct electrochemical experiments—

Various electrochemical experiments have been performed on CODH. EPR (Lindahl, Munck, et al., 1990) and Mössbauer (Lindahl, Ragsdale, & Münck, 1990) spectroelectrochemical studies revealed the reduction potentials for the B- (440mV) and C- (530mV) clusters (Lindahl, Munck, et al., 1990; Lindahl, Ragsdale, et al., 1990). We have used two types of specialized EPR spectroelectrochemical cells for titrating oxygen-sensitive proteins with low-potential metal centers like CODH (Harder, Feinberg, & Ragsdale, 1989; Lindahl, Munck, et al., 1990; Lindahl, Ragsdale, et al., 1990). For EPR spectroelectrochemical studies of this highly O₂-sensitive protein, is important to use a cell (Harder et al., 1989) in which the EPR tube is integrated with the electrochemical titration cell, so that the sample does not need to be transferred from the electrochemical to the spectroscopic cell/tube.

While spectroelectrochemical studies are ideal for examining the properties of specific chromophoric redox centers, it is also possible to examine (Leger et al., 2003) catalytic properties of enzymes with the enzyme directly attached to an electrode surface (also reviewed recently in Canetal.,2014). Over the past decade, we have also described various CODH-catalyzed nanoparticle systems that can rapidly and selectively reduce CO₂ to CO using visible light, most recently in 2018 (Zhang, Can, Ragsdale, & Armstrong, 2018). The studies demonstrate that CODH is remarkably reversible, showing catalytic activity close to the equilibrium redox potential for the CO₂/CO couple (0.52V), i.e., operating selectively without any significant overpotential.

4.2 Characterization of ACS

Steady-state kinetic methods were central to identifying the ACS activity of the enzyme formerly known as CODH as well as characterizing the mechanism shown in Fig. 2. These experiments revealed the existence of enzyme-bound CO, and methyl, acetyl, and CoA-

bound intermediates formed during the ACS reaction (Fig. 2). Transient kinetic, spectroscopic, and computational experiments have led to identification of the intermediates, established their kinetic competence, and revealed the changes in Ni_p geometry and electronic structure stemming from these interactions.

4.2.1 Steady-state kinetics—Steady-state kinetic experiments offer some of the best insight into the capability of ACS to produce acetyl-CoA:

Total synthesis of acetyl-CoA: The total synthesis of acetyl-CoA involves the reaction of CO, CH_3 - H_4 folate, and CoA to form acetyl-CoA using an assay developed by Hu, Drake, and Wood (1982). This reaction requires CODH/ACS, the CFeSP, and methyltransferase (MeTr); however, when this assay was developed in 1982, enriched fractions containing these enzymes instead of purified proteins were used. The revised method, which uses the purified proteins are described here.

An anaerobic buffer composed of 10mM Tris HCl, 0.1M NaCl, at pH 7.6 is prepared with 1mM Ti^{III} citrate and 1mM CoA. After adding CO to the buffer to obtain concentrations of 0%–100% saturation, 50μM ACS (or CODH/ACS), 1mM CH_3 - H_4 folate, and methyltransferase (MeTr) are added, the reaction is initiated with CFeSP and 100μL samples are taken and quenched with 100μL 1M HCl at specified time points. The amount of acetyl-CoA produced is determined by HPLC analysis. Alternatively, the CFeSP can be present in the assay mix prior to adding CODH/ACS (ACS) to initiate the reaction.

CO/acetyl-CoA exchange: The reversibility of the ACS reaction offers a direct way to assay acetyl-CoA synthesis. In 1982, Hu et al. found that $[1-^{14}C]$ -acetyl-CoA can exchange with a headspace of CO (Hu et al., 1982). This assay is now a routine procedure for measuring ACS activity and is convenient because it requires only ACS; thus, it was key to the discovery that ACS (not the CFeSP) is the enzyme that catalyzes acetyl-CoA synthesis (Ragsdale & Wood, 1985). In this reaction (Eq. 5), unlabeled CO exchanges with the ^{14}C -carbonyl group in acetyl-CoA, forming volatile ^{14}CO and leading to a decrease in solution-phase radioactive counts, as quantified by scintillation counting. The $[1-^{14}C]$ -acetyl-CoA method is an excellent way to assess ACS catalytic ability (Raybuck, Bastian, OrmeJohnson, & Walsh, 1988; Raybuck et al., 1991).

To conduct the CO/acetyl-CoA exchange assay, a 1mL anaerobic reaction solution is prepared in a 10–15mL vial containing 300mM MES, pH 6.5, 20mM KCl, 200μM MV,

2mM Ti^{III} citrate, and 200μM unlabeled acetyl-CoA. The solution headspace is purged with pure CO for 5–10min, 5μL of a 1mg/mL ACS sample (5μg ACS) is added, and the reaction solution is incubated at 55°C for 5min. The assay is initiated by adding 0.05μCi of [1-¹⁴C]-acetyl-CoA solution; 40μL samples are quenched into scintillation vials containing 100μL 1M HCl at various time points, 4mL scintillation liquid is added to the samples, and the radioactivity remaining in the [1-¹⁴C]-acetyl-CoA is determined by scintillation counting.

The CO exchange rate is calculated as a function of exchange velocity for a given concentration of unlabeled acetyl-CoA, or AcCoA, as shown in Eq. (6) (Grahame, 2011; Raybuck et al., 1988), where F is the isotopic fraction exchanged at a given time, t . Mathematically, $F = (([A_0^*] - [A^*])/([A_0^*] - [A_\infty^*]))$, and A^* is the remaining 1-[¹⁴C]-acetyl-CoA in solution at time t ; A_0^* the initial cpm at time=0min; and A_∞^* the cpm at time = ∞ , or the count at isotopic equilibrium (Grahame, 2011; Raybuck et al., 1988). Assuming the reaction proceeds to 100% completion, the isotopic equilibrium for [¹⁴CO] is expected to be far greater than the isotopic equilibrium for [1-¹⁴C]-acetyl-CoA, simplifying Eq. (6) to Eq. (7). Rearranging Eq. (7), the CO exchange rate can be assessed (Eq. 8) by plotting the natural log of the fraction as a function of time.

$$v^* = - ([CO][AcCoA]/([CO] + [AcCoA])) * (1/t) * [\ln(1 - F)] \quad (6)$$

$$v^* = - [AcCoA] * (1/t) * [\ln(1 - F)] \quad (7)$$

$$-((t) * (v^*)/[AcCoA]) = [\ln(1 - F)] \quad (8)$$

Methylation of ACS using CH₃-CFeSP: The only known direct methyl donors to ACS are methyl-CFeSP and methylated corrinoids. Both methyl iodide and methyl-H₄folate can generate active methyl-Co(III); however, they do not generate the active methylated form of ACS. Thus, when methyl iodide or methyl-H₄folate is used as the methyl donor for acetyl-CoA synthesis or ACS, the reaction involves: (1) transfer of a methyl group from methyl iodide or methyl-H₄folate to the Co(I)-state of the protein bound or free corrinoid (usually achieved using reduction by Ti(III) citrate), followed by (2) transfer of the methyl group from CH₃-Co(III) to ACS (Seravalli, Brown, & Ragsdale, 2001). Thus, use of methyl-H₄folate or methyl iodide as methyl donor, as described (Menon & Ragsdale, 1998), requires only catalytic amounts of the CFeSP, which undergoes turnover at each cycle of the reaction. For use in these reactions, the CFeSP is purified essentially as described (Ragsdale, Lindahl, & Münck, 1987), except that FPLC chromatography is now used. To determine the fractions containing CFeSP, samples from the chromatographic column are mixed with cyanide and proteins are precipitated by heating at 95°C to release the cyanide-bound cofactor. After centrifugation, the pink cyanocobalamin-containing fractions are revealed by absorption bands at 367, 540, and 580nm.

When the CH₃-CFeSP is used directly as the methyl donor, the reaction is performed with near- or above-stoichiometric levels of CH₃-CFeSP relative to those of ACS (Menon & Ragsdale, 1999). This reaction, which is fast and requires transient kinetic methods, is described later.

Methylation of ACS using methyl-cobinamide: We have also used free methyl-cobalamin, methyl-cobinamide, and methyl-(Me₃-benzimidazolyl)cobamide to methylate ACS (Seravalli et al., 2001). To synthesize cobinamide, dried cyanocobalamin is mixed with anhydrous CF₃SO₃H (triflic acid). After overnight incubation, this extremely acidic solution is neutralized by mixing with 1 M dibasic sodium phosphate solution containing a trace amount of cyanide (Zou, Evans, & Brown, 1995). The final mixture is applied to a reverse phase chromatography column (Porapak Rxn) and the cobinamide is purified using an acetonitrile gradient. Cobinamide is lyophilized and then solubilized in water to a final 20 mM concentration. Methyl-cobinamide is generated by reducing cobinamide with a fivefold excess of Ti(III) citrate and reacting with excess methyl iodide (40% in methanol) for 1 h; after which a pure product is obtained by gel filtration on a BioGel P3 (BioRad) column. Similar to methylation by CH₃-CFeSP, methylation of ACS can be achieved by reducing ACS with a threefold excess of Ti(III) citrate and mixing with methylcobinamide. The base-off methyl-Co species are significantly more reactive than methyl-cobalamin (Seravalli et al., 2001).

Reaction of ACS with CoASH: ACS can catalyze an exchange reaction between CoA and acetyl-CoA. This reaction was first followed using [3'-³²P]-coenzyme A (Ramer, Raybuck, Orme-Johnson, & Walsh, 1989) and involves cleavage of the C-S bond of acetyl-CoA to form the Ni-acyl intermediate, which rebinds to the radiolabeled CoA. Samples from the assay solution at various time points are separated by isocratic solvent HPLC, and radioactive counts measured for the corresponding CoA peaks. Similar to the CO/[1-¹⁴C]-AcCoA to ¹⁴CO exchange assay, the acetyl-CoA/[3'-³²P]-CoA exchange assay continues until the reaction reaches isotopic equilibrium. In place of ³²P, the CoA/acetyl-CoA exchange reaction can also be followed using ³²P-dephospho-CoA (Bhaskar, Demoll, & Grahame, 1998), instead of the radiolabeled substrate. The *K_m* for dephospho-CoA is similar to that of CoA.

4.2.2 Reaction of ACS with CO—EPR spectroscopy is one of the most powerful methods for studying ACS. While the A-cluster of as-isolated ACS is EPR silent, exposure of the protein to CO in the presence of a reductant, e.g., dithionite, Ti(III)-citrate, DTT, reveals a unique EPR spectrum with *g* values of 2.074 and 2.028 (Fig. 3). Many experiments have shown this to be a key intermediate in the ACS mechanism (Can et al., 2014). By measuring the splitting of the lines in the EPR spectrum observed upon substitutions with ⁵⁷Fe, ⁶¹Ni, and ¹³CO, this signal was proposed to originate from a Ni-CO bond formed by the complex of CO with this spin-coupled cluster and became known as the “NiFeC signal” (Ragsdale & Kumar, 1996; Ragsdale, Ljungdahl, et al., 1983; Ragsdale, Wood, & Antholine, 1985). Pulsed EPR (ENDOR) (Fan, Gorst, Ragsdale, & Hoffman, 1991) and Mössbauer (Lindahl, Munck, et al., 1990; Lindahl, Ragsdale, et al., 1990) methods have also been used to describe the electronic state of ACS and its interactions with substrates. The catalytic

competency of ACS samples can be measured quantitatively by determining the double integrated signal intensity (the spin concentration) of the NiFeC signal in comparison to that of a Cu^{II} perchlorate standard measured under the same conditions (Fee, 1978). The amount of the NiFeC EPR signal is a direct measure of enzyme activity. The form of ACS that binds CO was proposed to be Ni_p^{I} , based on EPR and FTIR spectroscopy following low-temperature photolysis of the NiFeC species (Bender et al., 2010). This Ni(I) species is unstable and immediately converts back to the NiFeC species with an activation energy of only 1kJ/mol, indicating the existence of an alcove that sequesters CO near Ni_p , a hypothesis consistent with crystallographic studies of CODH/ACS (Doukov et al., 2008).

Although FTIR is a commonly used technique to study small molecules, it is less commonly used in the study of proteins because of the strong and broad absorbance bands of water in the 1645cm^{-1} region, which overlap with the amide I band of proteins and some side chain bands. However, metal–CO bands have been studied in various proteins like hydrogenase, myoglobin, CODH, and ACS because the CO stretch is discretely located in the 2000cm^{-1} region, significantly away from other contaminating bands. As shown in Fig. 3, the CO stretching frequency at 1995cm^{-1} allowed us to demonstrate that CO was bound to the A-cluster, validate photolysis of the CO, and determine the energy barrier of CO rebinding to the ACS A-cluster, as described earlier (Bender et al., 2010; George, Seravalli, & Ragsdale, 2005; Kumar & Ragsdale, 1992). Preparation of 300 μL FTIR samples is accomplished by buffer exchanging ACS sample into an anaerobic D_2O buffer of 50mM KP_i , pH 7.7 by Amicon pressure filtration. The ACS sample is then concentrated to 1mM, 300 μL of 2mM sodium dithionite is added to reduce the protein, and the sample is purged with CO gas for 10min. The ACS sample is then anaerobically transferred to an FTIR CaF_2 window setup, which is immediately subjected to FTIR data acquisition.

XAS has been used to probe the Ni and Fe sites in CODH and ACS. The first direct demonstration of the Ni–CO bond in ACS was obtained through XAS studies (Can et al., 2017), with DFT calculations providing a powerful complement to the experimental studies.

4.2.3 Single turnover and transient kinetics to study ACS—General aspects of stopped-flow and RFQ-EPR methods are described earlier in the CODH section. Using RFQ-EPR, rates of CO binding to ACS to form $\text{Ni}_p\text{-CO}$ and its condensation with the methylated CFeSP to form Ni_p acetyl have been measured by growth and decay of the paramagnetic NiFeC EPR signal over time (Seravalli, Kumar, & Ragsdale, 2002). Exponential curves are plotted based on the intensity at NiFeC as a function of time, and the kinetic rate constants derived from the exponential curves.

RFQ has been used in conjunction with other spectroscopic methods, such as Mossbauer or ENDOR. The same unit can also be used for rapid chemical quench. In this case, the reaction is quenched by spraying the solution into a chemical denaturant (generally base or acid).

Reaction of ACS with CO: The binding of CO to ACS is studied by RFQ-EPR by measuring the rate of formation of the NiFeC signal. In a typical experiment (Seravalli et al.,

2002), a solution containing 100 μ M ACS is rapidly mixed with a buffered solution of CO and the reaction is quenched by spraying the mixture into liquid isopentane.

Stopped-flow FTIR has also been used to measure the rate of CO binding to ACS, providing further evidence that the NiFeC species is a catalytically competent intermediate in the ACS mechanism. The reaction is performed as described (Bender et al., 2010; George et al., 2005).

Reaction of ACS with CH₃-CFeSP: This reaction is fast and must be monitored by rapid kinetic methods. The most convenient option is stopped-flow UV–visible spectrophotometry by monitoring the spectral changes at 390nm (formation of Co¹⁺) and 450nm (depletion of methyl-Co³⁺). First, the CFeSP needs to be methylated, and a convenient methyl donor is methyl iodide used as described (Lu, Harder, & Ragsdale, 1990). Approximately 50 μ M CFeSP in 100mM Tris–HCl (pH 7.6) buffer is reduced by addition of 0.625mM Ti(III) citrate. Methyl iodide is added (250 μ M, final) at 55°C in the dark, then after about 15min, CH₃-CFeSP is buffer exchanged into 100mM Tris–HCl (pH 7.6) buffer. Reduced Co(I)- and CH₃-Co(III) states of the CFeSP can be distinguished by UV–visible spectroscopy since reduced CFeSP shows a sharp band at 390nm (ϵ =40cm⁻¹ mM⁻¹) and CH₃-CFeSP shows a broad band at 450nm (ϵ =16.2cm⁻¹ mM⁻¹). Alternatively, if ¹⁴CH₃I is used to prepare CH₃-CFeSP, the methylation can be followed by measuring the radioactivity after separating the ACS and CFeSP by phenyl-sepharose chromatography and measuring the ¹⁴CH₃ group associated with each protein (Lu et al., 1990). Methylation of the CFeSP with methyl-H₄folate is performed as described (Ragsdale et al., 1987).

ACS is methylated by adding Ti(III) citrate and rapidly mixing with CH₃-CFeSP in the stopped-flow instrument. The reaction is monitored by following the changes in the absorption spectrum associated with demethylation of CH₃-CFeSP (Seravalli et al., 2002). In a typical reaction, 10–100 μ M CODH/ACS (or ACS) is mixed with 10–100 μ M CH₃-CFeSP and the reaction is monitored for a few seconds. Additionally, the impact of other ACS substrates can be studied by mixing 100 μ M ACS including CO and/or CoA in different concentrations with 100 μ M CH₃-CFeSP.

Methylation of ACS-CO: Because binding of CO and the methyl group follow a random mechanism, enzyme-monitored acetyl-CoA synthesis can be followed by measuring the depletion of the NiFeC signal. In a typical freeze-quench experiment (Seravalli et al., 2002), a solution containing 200 μ M ACS incubated with CO is rapidly mixed with 100 μ M CH₃-CFeSP (or other cobalamin-based methyl donor). CoA (1mM) can be included in this reaction. The sample is sprayed at different reaction times over a 10s time frame into a freezing bath of isopentane as described earlier. Most of the NiFeC decay occurs within the first 0.5s. When CoA is included, the amount of synthesized acetyl-CoA can be measured by HPLC.

5. Closing and future directions

This is an opportune time to review the methods used to characterize the two key enzymes in the WLP. We now have heterologous and homologous genetic systems for expressing

CODH and ACS. Strains of acetogens (*Clostridium ljungdahlii*, *Clostridium acetogenum*, *Clostridium ragsdalei*, etc.) have been developed for biotechnological applications. Combining mutagenesis and directed evolution with mechanistic studies offer a powerful toolbox for gaining a better understanding of the mechanisms of these two enzymes as well as for developing more powerful biotechnological platforms to activate CO₂ and to convert syngas and various waste gas mixtures into desired fuels and chemicals.

In terms of mechanism, we can look forward to the use of mutagenesis coupled to the methods described in this chapter to understand the early steps in the mechanisms of CO oxidation and CO₂ reduction that have yet not been detected. We have recently obtained validation of the organometallic ACS mechanism by XAS analysis of the Ni(I)–CO complex (Can et al., 2017); the metal-CH₃ and metal-Ni intermediates still require characterization. Photoactive Ru and Ni bimetallic models have been developed that selectively reduce CO₂, i.e., do not reduce H⁺ to evolve H₂ (Schneider, Manesis, Stevenson, & Shafaat, 2018). Similarly, effective biomimetic ACS models have been developed, allowing comparison of the properties of the model with those of the enzyme (Manesis, O'Connor, Schneider, & Shafaat, 2017; Manesis & Shafaat, 2015). Recent studies coupling mutagenesis with spectroscopic and kinetic studies are also uncovering the importance of the intermolecular channel in enzymatic activity. We hope that the methods used in characterizing CODH and ACS described here will spur researchers to discover more of the unique important properties of these proteins.

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References

- Ballou DP (1978). Freeze-quench and chemical-quench techniques. *Methods in Enzymology*, 54, 85–93. [PubMed: 215881]
- Bender G, Stitch TA, Yan L, Britt RD, Cramer SP, & Ragsdale SW (2010). Infrared and EPR spectroscopic characterization of a Ni(I) species formed by photolysis of a catalytically competent Ni(I)–CO intermediate in the acetyl–CoA synthase reaction. *Biochemistry*, 49, 7.
- Bhaskar B, DeMoll E, & Grahame DA (1998). Redox-dependent acetyl transfer partial reaction of the acetyl–CoA decarbonylase/synthase complex: Kinetics and mechanism. *Biochemistry*, 37, 14491–14499. [PubMed: 9772177]
- Bonam D, & Ludden PW (1987). Purification and characterization of carbon monoxide dehydrogenase, a nickel, zinc, iron–sulfur protein, from *Rhodospirillum rubrum*. *Journal of Biological Chemistry*, 262, 2980–2987. [PubMed: 3029096]
- Can M, Armstrong FA, & Ragsdale SW (2014). Structure, function, and mechanism of the nickel metalloenzymes, CO dehydrogenase, and acetyl–CoA synthase. *Chemical Reviews*, 114, 4149–4174. [PubMed: 24521136]
- Can M, Giles LJ, Ragsdale SW, & Sarangi R (2017). X-ray absorption Spectroscopy reveals an organometallic Ni–C bond in the CO-treated form of acetyl–CoA synthase. *Biochemistry*, 56, 1248–1260. [PubMed: 28186407]
- Carlson ED, & Papoutsakis ET (2017). Heterologous expression of the *Clostridium carboxidivorans* CO dehydrogenase alone or together with the acetyl coenzyme A synthase enables both reduction of CO₂ and oxidation of CO by *Clostridium acetobutylicum*. *Applied and Environmental Microbiology*, 83, e00829–17. [PubMed: 28625981]

- Daniel SL, Hsu T, Dean SI, & Drake HL (1990). Characterization of the H₂- and CO-dependent chemolithotrophic potentials of the acetogens *Clostridium thermoaceticum* and *Acetogenium kivui*. *Journal of Bacteriology*, 172, 4464–4471. [PubMed: 2376565]
- Daniel SL, Wu Z, & Drake HL (1988). Growth of thermophilic acetogenic bacteria on methoxylated aromatic acids. *FEMS Microbiology Letters*, 52, 25–28.
- DeMoll E, Grahame DA, Harnly JM, Tsai L, & Stadtman TC (1987). Purification and properties of carbon monoxide dehydrogenase from *Methanococcus vannielii*. *Journal of Bacteriology*, 169, 3916–3920. [PubMed: 3624199]
- DeRose VJ, Telser J, Anderson ME, Lindahl PA, & Hoffman BM (1998). A multinuclear ENDOR study of the C-cluster in CO dehydrogenase from *Clostridium thermoaceticum*: Evidence for HxO and histidine coordination to the [Fe₄S₄] center. *Journal of the American Chemical Society*, 120, 8767–8776.
- Doukov TI, Blasiak LC, Seravalli J, Ragsdale SW, & Drennan CL (2008). Xenon in and at the end of the tunnel of bifunctional carbon monoxide dehydrogenase/ acetyl-CoA synthase. *Biochemistry*, 47, 3474–3483. [PubMed: 18293927]
- Drake HL, & Daniel SL (2004). Physiology of the thermophilic acetogen *Moorella thermoacetica*. *Research in Microbiology*, 155, 869–883. [PubMed: 15630808]
- Elliott JJ, & Brewer JM (1978). The inactivation of yeast enolase by 2,3-butanedione. *Archives of Biochemistry and Biophysics*, 190, 351–357. [PubMed: 360996]
- Ensign SA (1995). Reactivity of carbon monoxide dehydrogenase from *Rhodospirillum rubrum* with carbon dioxide, carbonyl sulfide, and carbon disulfide. *Biochemistry*, 34, 5372–5381. [PubMed: 7727395]
- Ensign SA, Hyman MR, & Ludden PW (1989). Nickel-specific, slow-binding inhibition of carbon monoxide dehydrogenase from *Rhodospirillum rubrum* by cyanide. *Biochemistry*, 28, 4973–4979. [PubMed: 2504285]
- Fan CL, Gorst CM, Ragsdale SW, & Hoffman BM (1991). Characterization of the Ni–Fe–C complex formed by reaction of carbon monoxide with the carbon monoxide dehydrogenase from *Clostridium thermoaceticum* by Q-band ENDOR. *Biochemistry*, 30, 431–435. [PubMed: 1846295]
- Fast AG, & Papoutsakis ET (2018). Functional expression of the *Clostridium ljungdahlii* acetyl-CoA synthase in *Clostridium acetobutylicum* as demonstrated by a novel in vivo CO exchange activity, on the way to heterologous installation of a functional Wood–Ljungdahl pathway. *Applied and Environmental Microbiology*, 84, e02307–17.
- Fee JA (1978). Transition metal electron paramagnetic resonance related to proteins *Methods in Enzymology*, 49, 512–528 Academic Press. [PubMed: 206805]
- Ferry JG (2010). How to make a living by exhaling methane. *Annual Review of Microbiology*, 64, 453–473.
- George SJ, Seravalli J, & Ragsdale SW (2005). EPR and infrared spectroscopic evidence that a kinetically competent paramagnetic intermediate is formed when acetyl-coenzyme A synthase reacts with CO. *Journal of the American Chemical Society*, 127, 1.
- Ghosh R, Hardmeyer A, Thoenen I, & Bachofen R (1994). Optimization of the stirred culture medium for large-scale batch cultivation of *Rhodospirillum rubrum* under semiaerobic conditions with maximal yield of photosynthetic membranes. *Applied and Environmental Microbiology*, 60, 1698–1700. [PubMed: 16349265]
- Grahame DA (2011). Methods for analysis of acetyl-CoA synthase: Applications to bacterial and archaeal systems. *Methods in Enzymology*, 494, 189–217. Elsevier. [PubMed: 21402216]
- Grahame DA, & Stadtman TC (1987). Carbon monoxide dehydrogenase from *Methanosarcina barkeri*. Disaggregation, purification, and physicochemical properties of the enzyme. *Journal of Biological Chemistry*, 262, 3706–3712. [PubMed: 3818661]
- Harder SR, Feinberg BA, & Ragsdale SW (1989). A spectroelectrochemical cell designed for low temperature electron paramagnetic resonance titration of oxygen sensitive proteins. *Analytical Biochemistry*, 181, 283–287. [PubMed: 2554761]
- Hsu T, Daniel SL, Lux MF, & Drake HL (1990). Biotransformations of carboxylated aromatic compounds by the acetogen *Clostridium thermoaceticum*: Generation of growth-supportive CO₂

- equivalents under CO₂-limited conditions. *Journal of Bacteriology*, 172, 212–217. [PubMed: 2104603]
- Hu S-I, Drake HL, & Wood HG (1982). Synthesis of acetyl coenzyme A from carbon monoxide, methyltetrahydrofolate, and coenzyme A by enzymes from *Clostridium thermoaceticum*. *Journal of Bacteriology*, 149, 440–448. [PubMed: 6895749]
- Hungate RE (1969). A roll tube method for cultivation of strict anaerobes. *Methods in Microbiology*, 3B, 117–132.
- Hungate RE, Smith W, & Clarke RT (1966). Suitability of butyl rubber stoppers for closing anaerobic roll culture tubes. *Journal of Bacteriology*, 91, 908–909. [PubMed: 5327378]
- Inoue T, Takao K, Fukuyama Y, Yoshida T, & Sako Y (2014). Over-expression of carbon monoxide dehydrogenase-I with an accessory protein co-expression: A key enzyme for carbon dioxide reduction. *Bioscience, Biotechnology, and Biochemistry*, 78, 582–587.
- Jeoung JH, & Dobbek H (2007). Carbon dioxide activation at the Ni,Fe-cluster of anaerobic carbon monoxide dehydrogenase. *Science*, 318, 1461–1464. [PubMed: 18048691]
- Kasmi AE, Rajasekharan S, & Ragsdale SW (1994). Anaerobic pathway for the conversion of the methyl group of aromatic methyl ethers to acetic acid by *clostridium thermoaceticum*. *Biochemistry*, 33, 11217–11224. [PubMed: 7727373]
- Kerby RL, Hong SS, Ensign SA, Coppoc LJ, Ludden PW, & Roberts GP (1992). Genetic and physiological characterization of the *Rhodospirillum rubrum* carbon monoxide dehydrogenase system. *Journal of Bacteriology*, 174, 5284–5294. [PubMed: 1644755]
- Kerby R, & Zeikus JG (1983). Growth of *Clostridium thermoaceticum* on H₂/CO₂ or CO as energy source. *Current Microbiology*, 8, 27–30.
- Kopke M, Mihalcea C, Liew F, Tizard JH, Ali MS, Conolly JJ, et al. (2011). 2,3-Butanediol production by acetogenic bacteria, an alternative route to chemical synthesis, using industrial waste gas. *Applied and Environmental Microbiology*, 77, 5467–5475. [PubMed: 21685168]
- Kumar M, Lu W-P, Liu L, & Ragsdale SW (1993). Kinetic evidence that CO dehydrogenase catalyzes the oxidation of CO and the synthesis of acetyl-CoA at separate metal centers. *Journal of the American Chemical Society*, 115, 11646–11647.
- Kumar M, & Ragsdale SW (1992). Characterization of the carbon monoxide binding site of carbon monoxide dehydrogenase from *Clostridium thermoaceticum* by infrared spectroscopy. *Journal of the American Chemical Society*, 114, 8713–8715.
- Léger C, Elliott SJ, Hoke KR, Jeuken LJ, Jones AK, & Armstrong FA (2003). Enzyme electrokinetics: Using protein film voltammetry to investigate redox enzymes and their mechanisms. *Biochemistry*, 42, 8653–8662. [PubMed: 12873124]
- Liéw F, Henstra AM, Kpke M, Winzer K, Simpson SD, & Minton NP (2017). Metabolic engineering of *Clostridium autoethanogenum* for selective alcohol production. *Metabolic Engineering*, 40, 104–114. [PubMed: 28111249]
- Liew F, Martin ME, Tappel RC, Heijstra BD, Mihalcea C, & Kopke M (2016). Gas fermentation-A flexible platform for commercial scale production of low-carbonfuels and chemicals from waste and renewable feedstocks. *Frontiers in Microbiology*, 7, 694. [PubMed: 27242719]
- Lindahl PA, Munck E, & Ragsdale SW (1990). CO dehydrogenase from *Clostridium thermoaceticum*. EPR and electrochemical studies in CO₂ and argon atmospheres. *Journal of Biological Chemistry*, 265, 3873–3879. [PubMed: 2154491]
- Lindahl PA, Ragsdale SW, & Munck E (1990). Mossbauer studies of CO dehydrogenase from *Clostridium thermoaceticum*. *Journal of Biological Chemistry*, 265, 3880–3888. [PubMed: 2303484]
- Ljungdahl LG, & Andreessen JR (1978). Formate dehydrogenase, a selenium-tungsten enzyme from *Clostridium thermoaceticum*. *Methods in Enzymology*, 53, 360–372. [PubMed: 713844]
- Lu WP, Harder SR, & Ragsdale SW (1990). Controlled potential enzymology of methyl transfer reactions involved in acetyl-CoA synthesis by CO dehydrogenase and the corrinoid/iron-sulfur protein from *Clostridium thermoaceticum*. *Journal of Biological Chemistry*, 265, 3124–3133. [PubMed: 2303444]

- Manesis AC, O'Connor MJ, Schneider CR, & Shafaat HS (2017). Multielectron chemistry within a model nickel metalloprotein: Mechanistic implications for acetylCoA synthase. *Journal of the American Chemical Society*, 139, 10328–10338. [PubMed: 28675928]
- Manesis AC, & Shafaat HS (2015). Electrochemical, spectroscopic, and density functional theory characterization of redox activity in nickel-substituted azurin: A model for acetyl-CoA synthase. *Inorganic Chemistry*, 54, 7959–7967. [PubMed: 26234790]
- Menon S, & Ragsdale SW (1998). Role of the [4Fe-4S] cluster in reductive activation of the cobalt center of the corrinoid iron-sulfur protein from *Clostridium thermoaceticum* during acetate biosynthesis. *Biochemistry*, 37, 5689–5698. [PubMed: 9548955]
- Menon S, & Ragsdale SW (1999). The role of an iron–sulfur cluster in an enzymatic methylation reaction. Methylation of CO dehydrogenase/acetyl-CoA synthase by the methylated corrinoid iron–sulfur protein. *Journal of Biological Chemistry*, 274, 11513–11518. [PubMed: 10206956]
- Merrouch M, Benvenuti M, Lorenzi M, Léger C, Fourmond V, & Dementin S (2018). Maturation of the [Ni-4Fe-4S] active site of carbon monoxide dehydrogenases. *Journal of Biological Inorganic Chemistry*, 23, 613–620. [PubMed: 29445873]
- Mock J, Zheng Y, Mueller AP, Ly S, Tran L, Segovia S, et al. (2015). Energy conservation associated with ethanol formation from H₂ and CO₂ in *Clostridium autoethanogenum* involving electron bifurcation. *Journal of Bacteriology*, 197, 2965–2980. [PubMed: 26148714]
- Norman RO, Millat T, Winzer K, Minton NP, & Hodgman C (2018). Progress towards platform chemical production using *Clostridium autoethanogenum*. *Biochemical Society Transactions*, 46, 523–535. [PubMed: 29666216]
- Ragsdale SW, Clark JE, Ljungdahl LG, Lundie LL, & Drake HL (1983). Properties of purified carbon monoxide dehydrogenase from *Clostridium thermoaceticum*, a nickel, iron–sulfur protein. *Journal of Biological Chemistry*, 258, 2364–2369. [PubMed: 6687389]
- Ragsdale SW, & Kumar M (1996). Nickel-containing carbon monoxide dehydrogenase/ acetyl-CoA synthase. *Chemical Reviews*, 96, 25.
- Ragsdale SW, Lindahl PA, & Munck E (1987). Mössbauer, EPR, and optical studies of the corrinoid/Fe-S protein involved in the synthesis of acetyl-CoA by *Clostridium thermoaceticum*. *Journal of Biological Chemistry*, 262, 14289–14297. [PubMed: 2821001]
- Ragsdale SW, Ljungdahl LG, & DerVartanian DV (1983). ¹³C and ⁶¹Ni isotope substitutions confirm the presence of a nickel(III)–carbon species in acetogenic CO dehydrogenases. *Biochemical and Biophysical Research Communications*, 115, 7.
- Ragsdale SW, & Wood HG (1985). Acetate biosynthesis by acetogenic bacteria: Evidence that carbon monoxide dehydrogenase is the condensing enzyme that catalyzes the final steps of the synthesis. *Journal of Biological Chemistry*, 260, 3970–3977. [PubMed: 2984190]
- Ragsdale SW, Wood HG, & Antholine WE (1985). Evidence that an iron–nickel– carbon complex is formed by reaction of CO with the CO dehydrogenase from *Clostridium thermoaceticum*. *Proceedings of the National Academy of Sciences of the United States of America*, 82, 6811–6814. [PubMed: 2995986]
- Ramer SE, Raybuck SA, Orme-Johnson WH, & Walsh CT (1989). Kinetic characterization of the [3'-³²P]-coenzyme A/acetyl coenzyme A exchange catalyzed by a three-subunit form of the carbon monoxide dehydrogenase/acetyl-CoA synthase from *Clostridium thermoaceticum*. *Biochemistry*, 28, 4675–4680. [PubMed: 2569891]
- Raybuck SA, Bastian NR, Orme-Johnson WH, & Walsh CT (1988). Kinetic characterization of the carbon monoxide–acetyl-CoA (carbonyl group) exchange activity of the acetyl-CoA synthesizing CO dehydrogenase from *Clostridium thermoaceticum*. *Biochemistry*, 27, 7698–7702. [PubMed: 2905170]
- Raybuck SA, Ramer SE, Abbanat DR, Peters JW, Orme-Johnson WH, Ferry JG, et al. (1991). Demonstration of carbon–carbon bond cleavage of acetyl coenzyme A by using isotopic exchange catalyzed by the CO dehydrogenase complex from acetate-grown *Methanosarcina thermophila*. *Journal of Bacteriology*, 173, 3.
- Schneider CR, Manesis AC, Stevenson MJ, & Shafaat HS (2018). A photoactive semisynthetic metalloenzyme exhibits complete selectivity for CO₂ reduction in water. *Chemical Communications (Cambridge)*, 54, 4681–4684.

- Seefeldt LC, & Ensign SA (1994). A continuous, spectrophotometric activity assay for nitrogenase using the reductant titanium(III) citrate. *Analytical Biochemistry*, 221, 379–386. [PubMed: 7810881]
- Seravalli J, Brown KL, & Ragsdale SW (2001). Acetyl coenzyme A synthesis from unnatural methylated corrinoids: Requirement for “Base-Off” coordination at cobalt. *Journal of the American Chemical Society*, 123, 1786–1787. [PubMed: 11456791]
- Seravalli J, Kumar M, & Ragsdale SW (2002). Rapid kinetic studies of acetyl-CoA synthesis: Evidence supporting the catalytic intermediacy of a paramagnetic NiFeC species in the autotrophic Wood–Ljungdahl pathway. *Biochemistry*, 41, 1807–1819. [PubMed: 11827525]
- Seravalli J, & Ragsdale SW (2008). Pulse-chase studies of the synthesis of acetyl-CoA by carbon monoxide dehydrogenase/acetyl-CoA synthase: Evidence for a random mechanism of methyl and carbonyl addition. *Journal of Biological Chemistry*, 283, 8384–8394. [PubMed: 18203715]
- Svetlitchnyi V, Peschel C, Acker G, & Meyer O (2001). Two membrane-associated NiFeS-carbon monoxide dehydrogenases from the anaerobic carbon-monoxideutilizing eubacterium *Carboxydotherrmus hydrogenoformans*. *Journal of Bacteriology*, 183, 5134–5144. [PubMed: 11489867]
- Terlesky KC, Nelson MJ, & Ferry JG (1986). Isolation of an enzyme complex with carbon monoxide dehydrogenase activity containing corrinoid and nickel from acetate-grown *Methanosarcina thermophila*. *Journal of Bacteriology*, 168, 1053–1058. [PubMed: 3023296]
- Wang VC, Can M, Pierce E, Ragsdale SW, & Armstrong FA (2013). A unified electrocatalytic description of the action of inhibitors of nickel carbon monoxide dehydrogenase. *Journal of the American Chemical Society*, 135, 2198–2206. [PubMed: 23368960]
- Wolfe RS (2011). Techniques for cultivating methanogens In Ragsdale SW & Rosenzweig AC (Eds.), *Methods in enzymology: Vol. 494* (pp. 1–22); Elsevier 2011/03/16 ed. [PubMed: 21402207]
- Zhang L, Can M, Ragsdale S, & Armstrong F (2018). Fast and selective photoreduction of CO₂ to CO catalyzed by a complex of carbon monoxide dehydrogenase, TiO₂ and Ag nanoclusters. *ACS Catalysis*, 8, 2789–2795.
- Zou XA, Evans DR, & Brown KL (1995). Efficient and convenient method for axial nucleotide removal from vitamin-B-12 and its derivatives. *Inorganic Chemistry*, 34, 1634–1635.

Fig. 1.

The CODH mechanism, focusing on reactions at the C-cluster. The two electrons are transferred to the B- and D-clusters en route to external electron acceptors, e.g., ferredoxin.

Fig. 2.

Proposed organometallic mechanism of anaerobic acetyl-CoA synthesis catalyzed by the A-cluster of ACS. This mechanism, which is supported by recently reviewed kinetic and spectroscopic studies (Can et al., 2014), invokes the formation of the Ni-CO state, which undergoes reaction with a methyl group donated by the methylated Cys to form an acetyl-Ni species. Nucleophilic attack by CoA generates acetyl-CoA to regenerate the active state of CODH, ready to undergo another catalytic cycle. This is a simplified mechanism, given that pulse-chase studies demonstrate that CO and the methyl group bind randomly, followed by ordered binding of CoA (Seravalli & Ragsdale, 2008).

Fig. 3.

Binding of CO to the Ni(I) form of ACS to form the catalytically active Ni(I)-CO state. Included are EPR and FTIR spectral details for the Ni-CO state of the A-cluster and the EPR spectrum of the Ni(I) species which is also generated upon photolysis.

Table 1

EPR spectra and electrochemical parameters of CODH/ACS

Metal centers	Cluster type	EPR parameters <i>g</i> values	Electron transfer kinetics	Reduction potential	Literature
B _{red}	[4Fe4S]	<i>g</i> =2.04, 1.93, 1.89	90s ⁻¹ , 5°C 3000s ⁻¹ , 55°C	B _{ox} /B _{red} -440mV	Lindahl, Munck, and Ragsdale (1990)
C _{ox}	[Ni-3Fe4S]	EPR silent	Reductive activation; C _{ox} inactive	C _{ox} /C _{red1} -200mV	Lindahl, Munck, et al. (1990)
C _{red1}	[Ni-3Fe4S]	<i>g</i> =2.01, 1.81, 1.65	C _{red1} /C _{red2} 400s ⁻¹ , 5°C 17,200s ⁻¹ , 55°C 2 × 10 ⁸ M ⁻¹ s ⁻¹	C _{red1} /C _{int} -530mV	Kumar et al. (1993) and Lindahl, Munck, et al. (1990)
C _{red2}	[Ni-3Fe4S]	<i>g</i> =1.97, 1.87, 1.75	Above	Above	Lindahl, Munck, et al. (1990)
D-cluster	[4Fe4S] bridging subunits	Not observed spectroscopically		D _{ox} /D _{red} ?	Doukov, Blasiak, Seravalli, Ragsdale, and Drennan (2008)
A-cluster	[2Ni-4Fe4S]	2.074, 2.028	A _{ox} /A _{red} -CO 1.1s ⁻¹ , 55°C	<-600mV	Kumar et al. (1993)