Nickel-Containing Carbon Monoxide Dehydrogenase/Acetyl-CoA Synthase[†,‡]

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I. Introduction

A. Prospectus

This article reviews an enzyme with two important catalytic activities, carbon monoxide dehydrogenase (CODH) (reaction 1) and acetyl-CoA synthase (ACS) (reaction 2). These reactions are key to an autotrophic pathway that has become known as the reductive acetyl-CoA or the Wood/Ljungdahl pathway. ACS also catalyzes two exchange reactions that have been valuable in elucidating the mechanism of acetyl-CoA synthesis: an exchange reaction between CO and the carbonyl group of acetyl-CoA (reaction 3) and an exchange reaction between free CoA and the CoA moiety of acetyl-CoA (reaction 4).

$$CO + H_2O \rightleftharpoons CO_2 + 2\,H^+ + 2\,e^- \quad (1)$$

$$CH_3\text{-}CFeSP + CO + CoA \rightleftharpoons \text{acetyl-CoA} + CFeSP \quad (2)$$

$$CH_3\text{-}{}^{14}COSCoA + {}^{12}CO \rightleftharpoons CH_3\text{-}{}^{12}COSCoA + {}^{14}CO \quad (3)$$

$$CH_3CO^*SCoA + HSCoA \rightleftharpoons CH_3COSCoA + {}^*HSCoA \quad (4)$$

Ten years ago, one of us (Ragsdale) and Harland Wood, first proposed that the CODH from acetogenic bacteria catalyzes the final steps in acetyl-CoA synthesis and, therefore, should be renamed acetyl-CoA synthase. It was previously accepted that these steps occurred on a cobalt-containing corrinoid protein. This was a controversial proposal that required the existence of carbonyl, methyl, and acetyl enzyme adducts. Proof of this postulate was nontrivial since it required the characterization of enzyme-bound intermediates. With the goal of detecting and characterizing intermediates in the pathway, my laboratory and others began to probe the enzyme in the resting state and at different stages of the catalytic cycle with sensitive spectroscopic methods. This review summarizes the fruits of the combined labor of the laboratories working on this interesting problem.

Our collective efforts have been rewarded with the discovery of new (although evolutionarily ancient) bioinorganic, enzymatic, and bioenergetic principles. Work over the past eight years has uncovered prototypical biological roles of metals, novel enzymatic mechanisms of one-carbon activation, and new ways to form carbon−carbon and carbon−sulfur bonds. "New" and "novel" are adjectives that reflect that the mechanisms were recently discovered; Nature invented these principles in the early stages of biological evolution. One of us reviewed the mechanisms of CODH and ACS in 1994.[1] From 1993 to 1996, there were several other reviews on nickel-containing enzymes that included sections on CODH/ACS.[2−6] The present article will highlight several important recent findings that have not been previously reviewed and attempt to place these discoveries in a proper perspective. (i) The ACS and CODH activities are catalyzed at unusual clusters in which nickel is bridged to $[Fe_4S_4]$ units. Once Nature chiseled the formulae for these clusters, She appears to have demanded absolute fidelity of structure and even used them to catalyze acetyl-CoA disassembly during the initial steps of methane synthesis by methano-

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‡ Abbreviations used: CODH, CO dehydrogenase; ACS, acetyl-CoA synthase; CFeSP, corrinoid iron−sulfur protein; CoA, coenzyme A; WGSR, water−gas shift reaction; ENDOR, electron−nuclear double resonance; EPR, electron paramagnetic resonance; RR, resonance raman; FTIR, Fourier-transform infrared; CH_3-H_4-MSPT, methyltetrahydrosarcinopterin.

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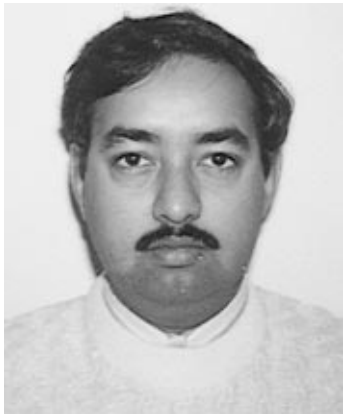
Stephen Wiley Ragsdale was born in 1952 in Rome, GA. He received his B.S. degree with a dual major in Chemistry and Biochemistry in 1979 from the University of Georgia. He then began studying electron-transfer reactions involved in acetate biosynthesis with Lars Ljungdahl at Georgia and obtained his Ph.D. degree in 1983. From January 1984 until 1987, he was a postdoctor with Harland G. Wood at Case Western Reserve University in Cleveland, OH and was an N.I.H. Postdoctoral Fellow from 1985 to 1986. In May 1987, he joined the Chemistry Department at the University of Wisconsin—Milwaukee as an Assistant Professor. He received the Shaw Scholars Award in 1987. In August of 1991, he moved to Lincoln, NE, where he joined the Biochemistry Department as an Associate Professor and in 1996 became Professor. His research interests comprise three areas, including (i) the mechanisms of enzymes that are involved in CO and CO_2 fixation, methanogenesis, and the metabolism of aromatic compounds; (ii) redox enzymology and the mechanism of reductive activation of enzymes; and (iii) Ni, iron–sulfur, and vitamin B_{12} metallobiochemistry. He serves on the editorial boards of *BioFactors* and the *Journal of Bacteriology*.



Manoj Kumar was born in India in 1962. He received his B.S. from Lucknow University and his Ph.D. in chemistry from the Indian Institute of Technology Kanpur in 1988. His Ph.D. research thesis was on heterolytic cleavage of the Co–C bond in organocobaloximes, He spent one year as a postdoctoral fellow in Professor M. J. Maroney's lab at the University of Massachusetts, Amherst, where he worked on model systems relevant to the nickel-containing enzyme, hydrogenase. He was a recipient of the Alexander von Humboldt scholarship in 1990 to design and synthesize functional model systems for the reactions of B_{12}-dependent enzymes at the University of Karlsruhe, Germany. In 1991, he joined Professor Stephen W. Ragsdale's reesarch group in the Biochemistry Department at the University of Nebraska—Lincoln to study the acetyl-coenzyme A pathway. A particular interest has been the nickel-containing metalloenzyme, carbon monoxide dehydrogenase. He is currently a research assistant professor in the same department. His research interests center on the metallobiochemistry of one-carbon metabolism and bioremediation of nitroorganic molecules.

genic *Archaea*. (ii) ACS has been shown to use these unusual metallic clusters to generate enzyme-bound organometallic intermediates. The mechanism of the acetyl-CoA synthase reaction has provided our first example of a biological organometallic reaction sequence. The resulting chemistry represents a new chapter in biochemistry that borrows much from the pages of organometallic chemistry texts. (ii) Novel biological roles for metals have been uncovered. CODH and ACS join only three other enzymes in naturally containing nickel. ACS was found to use a methylnickel intermediate, which was the first demonstrated biological example of an alkylnickel species. This discovery marks the first unambiguous and definitive assignment of a role for nickel in any enzyme. A new role for iron−sulfur clusters in the chemical steps of catalysis has been shown; an iron site in one of the Ni-X-Fe_4S_4 clusters binds CO, which is the precursor of the carbonyl group of acetyl-CoA.

B. Nomenclature

The enzymes that catalyze CO oxidation and those that catalyze acetyl-CoA synthesis often have been collectively called "carbon monoxide dehydrogenases" (CODHs). This is problematic for several reasons. CO oxidation and acetyl-CoA synthesis are entirely different in character and would fall into separate categories in the International Enzyme Commission classification scheme. In addition, some "CODHs" do not contain ACS activity. Names for each activity abound. CODH has been called "CO:acceptor oxidoreductase", "CO oxidase", and "CO_2 reductase". The ACS activity also has received numerous names including "acetyl-CoA decarbonylase synthase" and "Ni/Fe-S component". It also has been argued that both the CODH and ACS activities can be included under the umbrella "CODH" and that it is unnecessary to separately denote the ACS activity.

It is important to agree upon names that are consistent with the International Enzyme Commission classification scheme. After a two-month e-mail discussion that was organized by one of us (SWR), the nomenclature used here was overwhelmingly supported. The authors urge scientists to adopt this nomenclature in further references to CODH and ACS. The name "CO dehydrogenase" (CODH) is used as the recommended name and "CO:acceptor oxidoreductase" as the systematic name for the activity that catalyzes CO oxidation to CO_2 or its reverse. The name "acetyl-CoA synthase" (ACS) is used as the recommended name and "CO:methylated corrinoid iron−sulfur protein:CoA lyase" is the systematic name for the enzyme that assembles acetyl-CoA from enzyme-bound methyl, CO, and CoA groups. Reasons for these choices are described below. To denote the source of the enzyme, the genus and species are included as a prefix in lower-case italicized letters; i.e., for the *Clostridium thermoaceticum* CODH, *ct*CODH. The bifunctional enzyme is fully designated as "CO dehydrogenase/acetyl-CoA synthase" or "CODH/ACS".

The term "CO dehydrogenase" or "CODH" is retained because over 100 publications have used this name and because, although there are no hydrogen atoms on CO, the oxygen donor is water which does contain hydrogen. The term "CO_2 reductase" also has been used to describe formate dehydrogenase and other proteins. "CO oxidase" is incorrect since O_2 is not involved in the reaction. The more formal name, "CO:acceptor oxidoreductase", is used as the system-

Table 1. Properties of Ni−CODHs and Ni−ACSs

organism (class[a])	subunit (gene) size	cofactor content	cluster content	catalytic activities	ref(s)
C. thermoaceticum (A)	α (*acsB*) 78 kDa; β (*acsA*) 71 kDa	2 Ni, 11−14 Fe, 14 S^{2-}, 1 Zn/149 kDa	ABC	CODH, ACS	16,23
A. woodii (A)	α, 80 kDa; β, 68 kDa	1.4 Ni, 9 Fe, 14 S^{2-}, ~1 Zn or Mg/148 kDa	ABC	CODH, ACS	12
Ms. barkerii (M)	α, 84−92 kDa; β, 63 kDa; γ, 53 kDa; δ, 51 kDa; ε, 20 kDa	0.7 Ni, 8−15 Fe, 0.9 Co, 0.5 Zn/266 kDa	ABC	CODH, ACS	25,28,33
Ms. barkerii	α, 84−92 kDa; ε, 20 kDa	0.7 Ni, 8−15 Fe, 0.5 Zn/100 kDa	ABC	CODH	35
Ms. thermophila (M)	α (*cdhA*) 89 kDa; β (*cdhC*) 71 kDa; γ (*cdhD*) 60 kDa; δ (*cdhE*) 58 kDa; ε (*cdhB*) 19 kDa	3.6 Ni, 25 Fe/297 kDa	ABC	CODH, ACS	24,26,39
Ms. thermophila	α, 79 kDa; ε, 19 kDa	0.2 Ni; 7.7 Fe; 2.7 Zn/98 kDa	ABC	CODH, ACS	24,26,39
Mt. soehngenii (M)	α (*cdhA*) 79 kDa; ε (*cdhB*), 19 kDa	2 Ni, 12.5 Fe/98 kDa	ABC	CODH, ACS	31,32,34
Mc. vannielii (M)	α (*cdhA*) 89 kDa; ε (*cdhB*), 21 kDa	1.0 Ni, 8 Fe, 0.2 Zn/110 kDa	ND	CODH	29
R. rubrum (purple nonsulfur bacterium)	62 kDa (*cooS*)	1 Ni, 8 Fe	BC	CODH	54

[a] A, acetogen; M, methanogen.

atic name that correctly denotes that this reaction is in the EC 1.2 oxidoreductase class. *The* "acceptor" cannot be further defined except probably in the case of the aerobic enzyme since CODH uses a number of physiological electron acceptors (ferredoxin, flavodoxin, cytochromes, rubredoxin, etc.) as well as dye mediators (methylene blue, thionin, methyl viologen, FMN, $FADH_2$, etc.) with high efficiency.

The name "CODH" does not adequately describe the enzyme that catalyzes the assembly or disassembly of acetyl-CoA. "Acetyl-CoA synthase" ("ACS") is the recommended name for this activity. This is the most important physiological role of the enzyme in acetogenic bacteria and methanogens and is the key activity that defines the autotrophic reductive acetyl-CoA pathway. "Acetyl-CoA synthase" has been in use since 1985. "Acetyl-CoA decarbonylase synthase" is redundant since it names the reaction in both directions. ACS should also be used to describe the methanogenic enzyme that primarily runs in the reverse direction. "Ni/Fe-S component" also is inadequate. The recommended systematic name for ACS is "CO:methylated corrinoid iron−sulfur protein:CoA lyase". This name includes the three substrates for ACS and the enzyme class that designates that the enzyme catalyzes acetyl-CoA formation by group elimination reactions without ATP hydrolysis.

To arrive at the systematic name for ACS, it was necessary to define a recommended and systematic name for the protein that donates the methyl group to CODH. This protein has been previously called the "corrinoid/iron−sulfur protein", the "corrinoid/iron−sulfur component", and the "cobalt/iron−sulfur component". The slash is awkward and unnecessary and it is important to include the name "corrinoid" to differentiate the protein from other cobalt/iron−sulfur proteins. The name "corrinoid iron−sulfur protein" (CFeSP) is suggested as both the recommended name and the systematic name.

The multiple redox centers of ACS and CODH have recommended designations. The two redox centers common to ACS and CODH will be called cluster B and cluster C. Cluster B can be simply designated B_{ox} or B_{red}, depending on its redox state. Cluster C has several states: C_{ox} (the diamagnetic fully oxidized cluster), C* (the form of cluster C that lacks Ni), C_{red1} (the paramagnetic one-electron reduced form of C_{ox} with EPR spectroscopic parameters giving values of $g_{av} \approx 1.82$), C_{red2} (another paramagnetic form of cluster C with values of $g_{av} \approx 1.86$), and C_{red3} (the form of C with $g_{av} \approx 2.18$ that is generated when the enzyme reacts with thiocyanate, azide, or isocyanide, earlier called C*). The redox cluster that is distinct to ACS is called cluster A; it apparently has two redox states, A_{ox} and A_{red}.

II. General Characteristics of ACS and CODH

Table 1 compares the properties of the CODH and ACS from *C. thermoaceticum* with those of other organisms. There are two major classes: the bifunctional enzymes that contain both ACS and CODH activity and the enzymes that contain only CODH activity. So far, no ACS has been described that lacks the CODH activity. However, since these activities occur at separate sites, discovery or generation of such an enzyme is anticipated. The bifunctional CODH/ACSs from anaerobic bacteria are involved in CO and CO_2 incorporation into cell carbon by acetogens and methanogens and in methane synthesis from acetate by methanogens (Figure 1). The latter reaction involves the disassembly of acetyl-CoA by ACS. The acetogenic and methanogenic enzymes have similar subunit sizes and share three homologous metal clusters (called clusters A, B, and C). The other class is the monofunctional CODH, which includes the Ni CODHs from phototrophic bacteria and the aerobic Mo enzymes. The phototrophic CODH shares some properties with the anaerobic bifunctional enzymes; it is oxygen sensitive and contains clusters B and C. The phototrophic enzyme is also similar to the aerobic enzymes in being induced by CO and having a high k_{cat}/K_m for CO. A review concerned primarily with the physiology and microbiology of CODHs recently appeared.[7]

A. Anaerobic CODH/ACS from Acetogens, Methanogens, and Sulfate Reducers

The anaerobic bifunctional CODH/ACSs have similar metal compositions and similar magnetic and electronic properties. Most are highly oxygen sensitive. Currently, in my laboratory, bacteria are grown and harvested anaerobically and every step in the purification and manipulation of the enzymes is

performed in an anaerobic chamber that maintains the oxygen level at ~0.2 ppm. The bifunctional CODH/ACS all have two active sites: cluster C is the site of CO oxidation and cluster A is the site of acetyl-CoA synthesis. Cluster B also is present as an electron transport site. From an evolutionary point of view, the apparent high degree of conservation of these active sites is remarkable given their high overall sequence divergence and the wide evolutionary gulf that separates the methanogens and acetogens. It is likely that these proteins descended from an organism that contained ACS and CODH and predated the split between Archaea and Bacteria. Thus, ACS and CODH must be ancient enzymes and the acetyl-CoA pathway must be an ancient pathway. Possibly it was the first CO_2 fixation pathway.[8] Wächtershäuser[9] argues that an early form of the reductive citrate cycle may have been the first carbon fixation pathway. He viewed the acetyl-CoA pathway as an anaplerotic pathway (hence, would have evolved later) for the reductive citrate cycle.

1. Characteristics of the Acetogenic CODH/ACS

The best studied bifunctional CODH/ACS is from *C. thermoaceticum*. The enzyme behaved as a hexamer of 440 kDa on chromatography columns; however, recent electron microscopic[10] and analytical ultracentrifugation studies[11] indicate that actually it is a tetramer. The confusion resulted from the large Stokes radius of the enzyme that caused aberrant behavior in gel filtration studies. On the basis of the EM studies, a model for the quaternary structure of CODH/ACS has been proposed (Figure 2). The β subunits (71 kDa) form a central elongated core with the α subunits (81 kDa) attached to each end. It is expected that a similar arrangement exists for the *Acetobacterium woodii* enzyme since its physical properties, including subunit composition, size, and Stokes radius are nearly identical to those of the *C. thermoaceticum* enzyme.[12]

CODH/ACS is one of the four nickel-containing proteins so far discovered in nature. As discussed below, nickel plays a key role in the ACS mechanism. It was first suggested that CODH contained nickel when its addition to the growth medium was found to stimulate CODH activity.[13,14] Stronger evidence was provided when CODH activity and radioactivity were shown to comigrate in polyacrylamide gels of *C. thermoaceticum* cell extracts prepared from cells grown in medium containing ^{63}Ni.[15] Then, the *C. thermoaceticum* CODH was purified to homogeneity and shown to contain 2 mol of nickel per mol of $\alpha\beta$ dimeric enzyme.[16] CODH from *C. thermoaceticum* also was purified to near homogeneity by Diekert and Ritter and shown to contain nickel.[17] Besides two nickel, CODH contains ~12 iron, ~1 zinc, and ~14 acid-labile inorganic sulfide per $\alpha\beta$ dimeric unit.[16] The metals are assembled into three separate iron−sulfur clusters. Although clusters A and C have discrete activities, they share similar compositions with one Ni bridged to a Fe_4-S_4 cluster. Cluster B is a standard $[Fe_4\text{-}S_4]^{2+/1+}$ cluster that is involved in electron transfer reactions. The only genes (*cooCTJ*) so far shown to be involved in assembly of the metal clusters of CODH/ACS have been located in *Rhodospirillim rubrum* and are located adjacent to the structural genes for both CODH and a CO induced hydrogenase.[18,19] These genes apparently function in nickel insertion.

2. Subunit Functions of the Acetogenic CODH/ACS

Several results indicate that CODH activity resides on the β subunit (AcsA). First, AcsA has 46% identity (75% homology) with the *R. rubrum* CooS protein (67 kDa) that contains CODH activity. There is no significant homology between the *C. thermoaceticum* α subunit and CooS. Additionally, Xia and Lindahl have shown that, by mild treatment with SDS, they can partially dissociate CODH into an isolated α subunit and an $\alpha\beta_2$ form.[20] The $\alpha\beta_2$ form has the same level of CO oxidation activity as the native protein, indicating that the α subunit is not involved in CO oxidation and that the β subunit must contain the clusters required for CO oxidation, as depicted in Figure 1.[20] Although the α subunit isolated by the above-mentioned procedure has no ACS activity, it contains one Ni and four Fe and has spectroscopic properties[21] similar to those of cluster A, the active site of acetyl-CoA synthesis.[22] Therefore, ACS activity may reside in the α subunit or it may require both the α and the β subunits. If clusters B and/or C of the β subunit are involved in acetyl-CoA synthesis, one possible role could be in electron transfer. Although acetyl-CoA synthesis and the CO/exchange reactions do not involve net electron transfer, both of these reactions are stimulated by ferredoxin, indicating that internal electron transfer within CODH/ACS may be required during the reaction.[23] Further studies with the isolated subunits and the reconstituted enzyme are required to resolve the roles of the two subunits in acetyl-CoA synthesis.

3. Characteristics of the Methanogenic CODH/ACS

Methanogens also contain a bifunctional CODH/ACS (Table 2). The properties of the methanogenic CODH/ACS and its role in methanogenesis have been reviewed recently.[7] General characteristics are covered here. Mechanistic information on the methanogenic enzyme is described in the pertinent sections below. The bifunctional methanogenic CODH is found as part of a five-subunit complex with the following components: α (~90 kDa), β (60−70 kDa), γ (60 kDa), δ (58 kDa), and ϵ (~20 kDa).[24,25] This complex could be dissociated by a cationic detergent into a dimeric protein containing the 89 and 19 kDa subunits; a dimeric CFeSP containing the 60 and 58 kDa subunits;[26] and a 71 kDa component (whose actual molecular mass is 52 kDa[27]). In some cases, the CODH activity has been isolated as a dimeric protein consisting of the 89 and 19 kDa subunits without the detergent treatment.[28−32] The 71 kDa component appears to be quite labile and its function is part of the controversy surrounding the minimum composition required for ACS activity. Several results indicate that CODH activity resides in the α subunit. Since 1984, when the first methanogenic CODH was isolated from *Methanosarcina barkeri*,[33] it has been clear that CODH activity resides in a complex of the α and ϵ subunits, with an apparent molecular mass of 232 kDa. It is likely that CODH

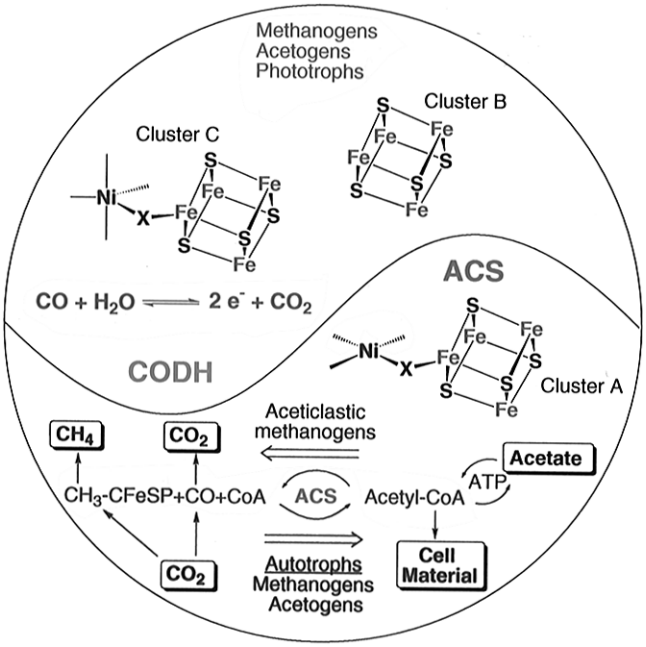


Figure 1. The Ni-CODH/ACS. Phototrophs contain only the CODH subunit. Autotrophic anaerobes like acetogens and methanogens contain a bifunctional CODH/ACS. ACS catalyzes the synthesis of acetyl-CoA to allow autotrophic growth on CO_2 or CO by methanogens and acetogens; it catalyzes the disassembly of acetyl-CoA to allow the utilization of acetate by methanogens and sulfate reducers.

activity is in the α subunit, since the ϵ subunit lacks cysteine residues and, therefore, could not be involved in ligation of any of the metal clusters.[34] In addition, there is reasonable homology, especially in blocks of cysteine and histidine residues, among the α subunit of the methanogenic CODH, the β subunit of the *C. thermoaceticum* enzyme, and the *R. rubrum* CODH (cooS).[18]

It is not clear which subunit(s) of the methanogenic enzyme is responsible for acetyl-CoA cleavage or synthesis. The five-subunit compex described above from *Ms. barkeri* clearly has ACS activity since it has been shown to catalyze the cleavage of acetyl-CoA and transfer the methyl group to tetrahydrosarcinapterin.[35] The complex responsible for acetyl-CoA synthesis and cleavage in methanogens could contain as many as 30 subunits, containing six copies of each of the five subunits.[35−37] Ferry's group showed that the complex could synthesize acetyl-CoA from methyl iodide, CoA, and CO.[38] It has been thought that the $\alpha_2\epsilon_2$ subunits contain this activity since both CODH and ACS activities were shown to exist in this form of the *Methanothrix soehngenii* enzyme[39] that contained two Ni and 19 Fe[31] and a similar form of the *Methanosarcina thermophila*[39] enzyme. In addition, an EPR signal characteristic of cluster A was found in the $\alpha_2\epsilon_2$ form of the *Ms. thermophila* enzyme. However, the ACS activity (∼7 min^{-1}) and the NiFeC EPR signal described for this form of the enzyme were much lower than observed for the five-subunit complex.[39] Possibly the ACS activity and EPR signal are characteristics of the α subunit and are highly labile. Another possibility is that another subunit is required for ACS activity. Evidence for this possibility is provided by DNA sequence analysis. There is 42% identity and 68% functional similarity between the N-terminal 397 amino acids of *CdhC* (the β subunit of the methanogenic five-enzyme complex) and residues 317−729 of the α subunit (harbors ACS activity, see above) of the *C. thermoaceticum* enzyme.[27] This homology includes two cysteine-rich motifs and several tryptophan and arginine residues thought to be important in CoA binding. Evidence that β subunit of the methanogenic complex assembles acetyl-CoA was provided by recent experiments involving limited proteolysis of the five-subunit complex from *Ms. barkeri*. This treatment resulted in partial subunit dissociation.[40] One of the components purified from the proteolytic

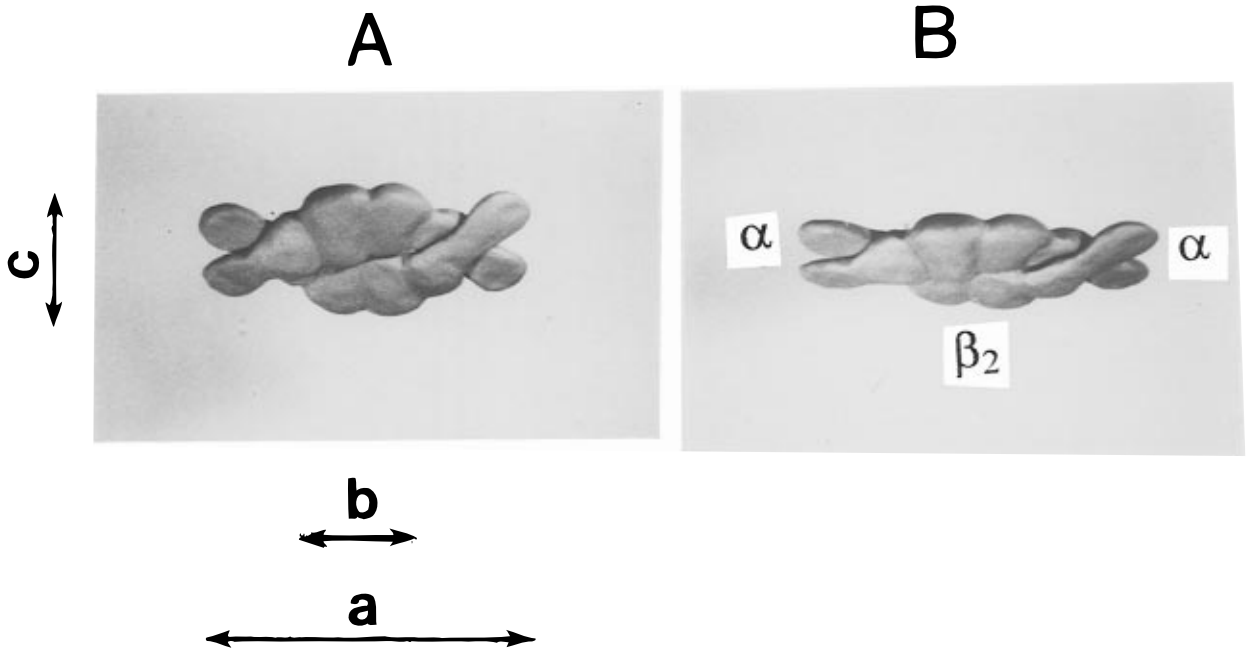


Figure 2. Electron microscopic analysis of the *C. thermoaceticum* CODH/ACS. Two small subunits form a core to which two large subunits are appended forming an elongated elipsoid. Top (A) and edge (B) views of idealized model for the quaternary structure of CO dehydrogenase from *C. thermoaceticum*: (a) the length of the tripartite structure and (b and c) sides of near rhomboidal, central core; *a*, ∼216 Å, *b*, ∼100 Å, and *c*, ∼115 Å. From ref 10.

Table 2. Comparison of the Metal Centers in CODHs/ACSs

	center A−CO adduct		center B		center C		
source of CODH/ACS	*g* values	E_0' (mV)	*g* values	E_0' (mV)	*g* values	E_0' (mV)	ref(s)
C. thermoaceticum	2.08, 2.07, 2.03		2.04,1.94,1.90	−440	2.01,1.81.1.65	−220	84
	2.06, 2.05, 2.03	−530			1.97, 1.87, 1.75	−530[b]	
						−360[c]	
Ms. thermophila	2.06,2.05,2.03	ND[a]	2.04,1.93,1.89	−444	2.02,1.87,1.72	−154	39
Ms. barkeri	NO[a]		2.05,1.94,1.90	−390	2.005,1.91,1.76	−35	28
Mt. soehngenii	NO		2.05,1.93,1.86	−410	2.005,1.89,1.73	−230	30
R. rubrum	NO		2.04, 1.94, 1.89	−418	2.03, 1.88, 1.71	−110	55,85,88,90
					1.97, 1.87, 1.75		

[a] ND = not determined; NO = not observed. [b] Determined in the presence of argon. [c] Determined in the presence of CO_2.

digest was a truncated form of the β subunit that retained full ability to cleave and resynthesize the C−S bond of acetyl-CoA. Therefore, there is a possibility that the ACS activity resides in the β subunit or requires a complex association of components ($\alpha\epsilon$ or $\alpha\epsilon\beta$).

B. Properties of Other CODHs

1. Properties of the Mo-CODH from Aerobic Carboxydotrophic Bacteria

The CODH from carboxydotrophic bacteria is a monofunctional CODH without ACS activity. Since this enzyme lacks nickel, its properties will not be discussed in detail here. CODH activity allows these aerobic bacteria to grow on CO as their sole source of carbon and energy. The carboxydobacteria are well suited for a role in CO detoxification in the environment because they have a high propensity for uptake of this trace gas. The mesophilic and thermophilic enzymes have K_m values for CO of 53−63 and 0.6 μM, respectively.[41] The Mo-CODH shares most of its properties with the Mo hydroxylases, such as sulfite oxidase and xanthine oxidase. Although little mechanistic information is available, the Mo-CODH will likely be similar to the Mo oxo-transfer enzymes. The carboxydotrophic bacteria are aerobic and (reaction 1), transfer the electrons derived from CO oxidation through an electron transport chain that finally reduces oxygen according to eq 5.[42]

$$O_2 + 2.19CO \rightarrow 1.83CO_2 + 0.36\text{cell carbon} \quad (5)$$

$$NADH + H^+ + \text{acceptor (ox)} \rightarrow NAD^+ + \text{acceptor (red)} \quad (6)$$

Although the E_0' of the CO/CO_2 couple (−540 mV) is very negative, the two electrons released during CO oxidation by the Mo-CODH can only be transferred to electron acceptors with $E'_{1/2}$ values between +0.011 and 0.043 V. This property is quite different from the Ni-CODHs which reduce a variety of high and low potential electron acceptors. The CODH from carboxydotrophs also can oxidize NADH, an activity associated with all known Mo hydroxylases (eq 6). Unlike the Ni-containing CODHs, the Mo enzymes are not oxygen sensitive.

The mesophilic enzyme from *Oligotropha carboxydovorans* and the thermophilic enzyme from *Oligotropha thermocarboxydovorans* have been purified and the CODH from *O. carboxydovorans* has been best studied. The Mo is present as molybdopterin cytosine dinucleotide.[43] The *O. carboxydovorans* and *O. thermocarboxydovorans* enzymes have similar molecular masses (230000−310000) and $(\alpha\beta\gamma)_2$ structures.[44] Both enzymes also contain 2 mol of FAD, eight Fe, eight acid-labile sulfide, and two Mo per mol of enzyme,[41] with copper (0.7−1.54 atom/mol) and zinc (2−3 atoms/mol) also being present in most preparations.[45] The clusters are present as [2Fe-2S] centers.[46] It is believed that Mo represents the substrate binding site; however, incubation of CODH with ^{13}CO did not result in detectable hyperfine broadening of Mo(V) EPR signal.[46] Evidence for a crucial role for Mo in the mechanism comes from (a) inhibition of enzyme activity by methanol which traps Mo in the V state, (b) Mo requirement for growth on CO, but not for heterotrophic growth, and (c) inhibition of growth on CO by the Mo antagonist, tungstate.[47]

The carboxydotrophic CODH is encoded by plasmid-borne genes,[48−50] whereas the Ni-enzymes are chromosomally encoded. The genes for both the mesophilic and thermophilic CODH enzymes have been cloned and sequenced.[51,52]

2. Properties of the Monofunctional CODH from R. rubrum

The CODH from purple non-sulfur bacteria is a 61.8 kDa peripheral membrane protein that allows these phototrophs to grow on CO in the dark as a sole carbon and energy source.[53] Electrons released from CO oxidation by CODH are passed to a 22 kDa Fe−S protein called CooF[54] and then through an electron transfer chain to a membrane-bound hydrogenase that reduces protons to H_2.[54] The *R. rubrum* CODH contains one Ni and seven to nine Fe and lacks ACS activity.[55] Referring to Figure 1, this enzyme contains only the CODH subunit with clusters B and C, and lacks cluster A.

The gene encoding the *R. rubrum* enzyme has been cloned and sequenced.[18] Genes encoding the CO oxidation system of *R. rubrum* are part of a gene cluster that includes the CODH (cooS), the Fe−S electron-transfer protein (cooF), and several genes involved in nickel insertion.[18] This cluster also contains the genes encoding a CO-induced hydrogenase (CooH)[19] and a regulatory protein (CooA) that is required for CO-induced activation of expression of the CO oxidation system.[56] When cells are grown in the absence of CO and then exposed to CO, CODH activity is induced ~200-fold.[54] When *R. rubrum* is grown in the absence of Ni, CODH can be prepared in a form that lacks Ni yet retains all the iron

atoms.[57,58] Ni can be added to this enzyme to restore the native activity and spectroscopic properties.[57,58]

III. Mechanism of CO Oxidation

The next two sections of this review focus on the mechanisms of CO oxidation/CO_2 reduction and acetyl-CoA synthesis/disassembly. Both of these activities influence the earth's ecology as well as the microbial ecology of many organisms' digestive systems. The enzymatic mechanisms are discussed followed by the relevant model chemistry for each reaction.

An important role of CODH is to remove CO from the environment helping to maintain this toxic gas at subhazardous levels. Annual CO removal from lower atmosphere and earth by bacteria is estimated to be about $\sim 1 \times 10^8$ tons.[59] CO is a colorless, odorless gas that is very toxic because it can bind to metalloproteins required in life-sustaining activities, notably cytochrome oxidase. Atmospheric concentrations range from about 0.1 ppm in rural to 200 ppm in urban settings.[45] The CO_2 is then further metabolized by one of several CO_2 fixation pathways—the Calvin Cycle, the reductive tricarboxylic acid cycle, or the reductive acetyl-CoA pathway. The net result is to convert CO into cell carbon. We think that a proper understanding of the mechanism of CO oxidation could lead to the development of biomimetic catalysts that could be used on a large scale to lower the concentration of CO in heavily polluted areas. A better understanding of CODH could also help in the development of CO sensors. It could also benefit industry and agriculture. Since CO_2 is so abundant, many chemical processes could be achieved if an effective method of activating CO_2 to more reactive compounds like CO or formate. CODH rapidly performs CO_2 reduction ($\sim 11\ s^{-1}$)[60] and CO oxidation ($\sim 2000\ s^{-1}$)[61] under mild conditions and with virtually no overpotential.

The study of enzymatic CO oxidation began around 1903, when Beijerinck and van Delden tried to grow algae on a purely mineral medium.[62] Although they failed, they discovered thin films of bacteria that grew slowly and named the organism *Bacillus oligocarbophilus*. Apparently the carbon source was coal gas, a mixture of H_2 and CO that was used as the major source of fuel at the turn of the century. Kaserer disovered a similar organism and concluded that the bacterium was growing on a carbon-containing component of the atmosphere.[63] Lantzsch isolated the first bacterial strain that definitely grew on CO.[64] It is now known that CO can serve as a carbon and electron source for many bacteria[47,65−67] including acetogenic bacteria such as *C. thermoaceticum*.[68,69]

The discovery of an enzymatic activity responsible for the oxidation of CO to CO_2 was first reported in 1959 in sulfate-reducing bacteria.[70] Although a CODH has been highly purified from *Desulfovibrio desulfuricans*,[71] ironically CODH has never been purified to homogeneity and characterized from a sulfate reducer. It was 25 years before a CODH was purified to homogeneity; this occurred simultaneously in two laboratories from the same organism, *Clostridium thermoaceticum*.[16,17] Diekert and Thauer had identified this activity in acetogenic bacteria approximately five years earlier.[72] At that time, no one could have predicted that the discovery and purification of this interesting enzyme was the prelude to a rich and exciting research area that has significantly enriched and modified our concepts of the role of metals in enzymes and the reaction mechanisms of enzymes. It has been exciting to have participated in the overture as well as the main symphony. It is likely that the finale will not be played for many years.

A. Chemistry of CO and CO_2

1. CO Chemistry

The first step in enzymatic CO oxidation is binding of CO by an active site transition metal center. The chemistry of CO with transition metals is wide ranging.[73] Although it is an exceedingly weak Lewis base, CO is an unsaturated soft ligand due to the C≡O multiple bond and can accept metal dπ electrons by a process called back-bonding. Thus, CO is a strong ligand for transition metals because it acts both as a σ donor and π acceptor. The metal carbonyls that are produced undergo a variety of reactions such as ligand substitutions, insertions, eliminations, and nucleophilic additions.[74,75] All these reactions depend on the polarization of CO by binding the metal. The polarizability of metal-bound CO is, in turn, affected by the properties of the coligands and the net charge of the metal complex. For example, nucleophilic addition reactions and migratory insertion reactions are promoted by increasing the electrophilicity of the CO carbon, whereas reactions involving electrophilic attack at oxygen is enhanced by increasing the nucleophilicity at the CO oxygen.

After binding to the metal, CO becomes subject to a variety of reactions, some of which play a key role in the chemical industry.[76] Important industrial organometallic catalysis reactions include catalytic carbonylation, the Fischer−Tropsch reaction, reactions with syn Gas, hydroformylation reactions, homologation reactions, the water−gas shift reaction, and hydrogenation reactions using water as the hydrogen source.

2. CO_2 Chemistry

CO_2, the product of CO oxidation, is the most oxidized form of carbon and is often classified as a stable, almost inert compound. Reactions with CO_2 often have a high activation energy. Metal complexes act as catalysts by lowering this energy barrier. Introduction of a carboxylate group into an organic molecule using the Grignard reaction, by inserting CO_2 in the Mg−C bond, is a classic example.[77] The first transition metal−CO_2 chemistry was the reaction of Wilkinson's complex with CO_2.[78] Nature also activates CO_2 by complexation to metal centers; examples include carbonic anhydrase, formate dehydrogenase, carbamate synthases and kinases, ribulose bisphosphate carboxylase, biotin-dependent carboxylases, acetyl-CoA synthase, and carbon monoxide dehydrogenase.

CO_2 has several potential coordination sites. The carbon is a Lewis acid and the oxygens are weak

Lewis bases. Thus, the carbon is an electrophilic center and the oxygens are mildly nucleophilic. In addition, CO_2 in its ground state possesses two equivalent π bonds that could also play a role in bonding to transition metals. Since CO_2 has three possible bonding modes, a wide variety of transition metal−CO_2 complexes are possible. Transition metal−CO_2 complexes can be synthesized by different possible methods.[79] These include substitution of labile ligands, reduction of complexes, *in situ* synthesis, and addition to the electron deficient transition metal complexes.

B. Discovery of the CODH Active Site

Kumar et al.[80] first proposed that the active site of CO oxidation is cluster C based on a rapid freeze−quench EPR study (Figure 3).[80] This technique combines pre-steady-state kinetics with EPR spectroscopy.[81] This study showed that cluster C of CODH/ACS undergoes changes in its EPR signal as it binds CO ($k = 2 \times 10^8$ M^{-1} s^{-1}) and delivers electrons to cluster B (3000 s^{-1}) at rates that are catalytically relevant for CO oxidation ($k_{cat} = 2000$ s^{-1} and k_{cat}/K_m^{co} is 2×10^7 M^{-1} s^{-1} at 55 °C[61]). Almost simultaneously, the Lindahl group published the proposal that cluster C is the CO oxidation site based on EPR and ENDOR studies of the cyanide adduct of the enzyme.[82] That proposal was based on the premise that CO and cyanide compete for the same binding site. The mode of cyanide inhibition of CODH is discussed in more detail below.

Once the importance of cluster C became evident, determining its properties became of great interest. One question was, what type of iron does cluster C contain? The EPR spectra were unusual; in the CO-

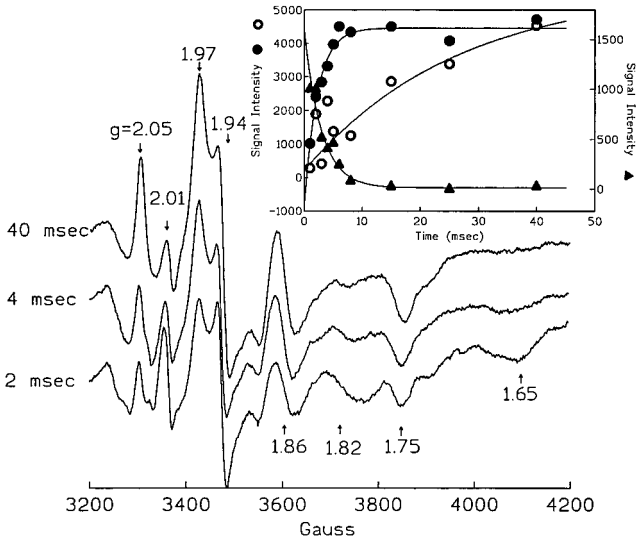


Figure 3. Freeze−quench and low-temperature EPR spectra of the reaction of CODH with CO. CODH (30 μM) was mixed at 5 °C with a solution containing a CO/N_2 (20%/80%) gas mixture. (a) Selected EPR spectra at the delay times shown. (b) Plot of signal intensities of the $g = 1.86$ (●), $g = 1.65$ (▲), and $g = 2.05$ (○) resonances versus time. The best fit lines shown are based on calculated rate constants of ~440 s^{-1} (17 200 s^{-1} at 55 °C) for the formation of the C_{red2} signal and for decay of the C_{red1} signal, and 64 s^{-1} (2600−3900 s^{-1} at 55 °C) for the reduction of cluster B. (Reprinted from ref 80. Copyright 1993 American Chemical Society.)

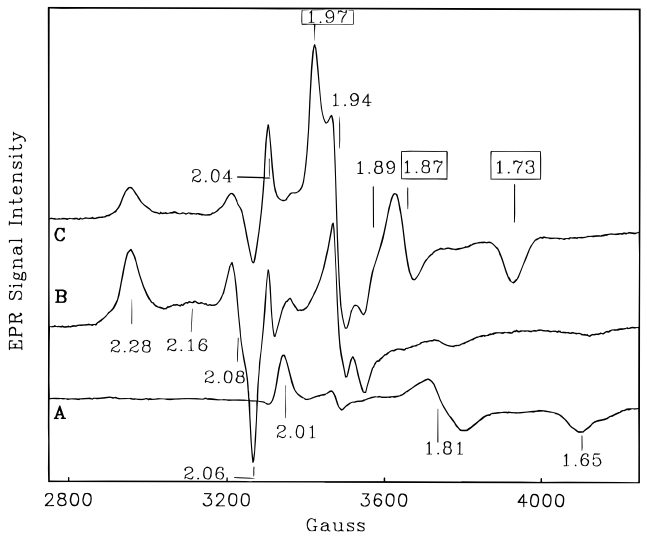


Figure 4. EPR spectra of cluster C: (A) CODH as-isolated, C_{red1}; (B) CODH after reaction with *n*-butyl isocyanide, $C_{red1} + C_{red3} + B_{red}$; (C) CODH after extended reaction with *n*-butyl isocyanide, $C_{red2} + C_{red3} + B_{red}$. (Modified from ref 114.)

reduced or dithionite-reduced state, called C_{red2}, the spectrum is highly rhombic with all three *g* values below 2.0 (1.97, 1.86, 1.75) (Figure 4).[83,84] There are at least three more stable states of cluster C: a diamagnetic form called C_{ox}; C_{red1},which is a one-electron reduced state with *g* values at 2.01, 1.81, and 1.65;[84] and a state called C_{red3} with all three *g* values above 2 (*g* values ~2.28, 2.08, 2.06 and $g_{av} \sim 2.18$) that is generated when the enzyme reacts with thiocyanate, azide, or isocyanide (earlier called C*). The midpoint potential for the C_{ox}/C_{red1} couple was relatively oxidizing for a $[Fe_4\text{-}S_4]^{2+/1+}$ cluster, −220 mV in the *C. thermoaceticum* enzyme[84] and −110 mV for the *R. rubrum enzyme*.[85] In addition, C_{red1} was found to be replaced by C_{red2} as the redox potential was lowered according to a midpoint potential of −530 mV.[85] It still is not clear if C_{red1} and C_{red2} are at the same oxidation state or if they differ by two electrons. EPR spectroscopy and electrochemistry alone were not able to define the structure of cluster C because they resembled those of three types of iron-containing clusters: the mixed-valent state of hemerythrin (a μ-oxo bridged iron dimer), the substrate bound form of aconitase (a $[Fe_4\text{-}S_4]^{2+/1+}$ cluster in which one of the irons is six coordinate), and the Rieske $[Fe_2\text{-}S_2]$ cluster. Mössbauer spectroscopy of cluster C were consistent with an Fe−S cluster; however, it was difficult to provide a detailed analysis because cluster C could not be fully converted into a single paramagnetic state and because of the overlap of the spectra of cluster C with that of the other iron−sulfur components in the enzyme.[86]

Another unresolved question was, is Ni present in cluster C? Clearly Ni was important in the enzyme. Several experiments on the *R. rubrum* enzyme indicated that it was: (i) By growing the cells in Ni-deficient medium, the *R. rubrum* enzyme was isolated in a form (called the C* state) that lacked nickel, had no CODH activity, and could not bind cyanide.[58,87] When Ni was added, the CO oxidation activity was reconstituted and cyanide could bind. (ii) The EPR spectrum of cluster C_{red1} in the ^{61}Ni-

substituted enzyme exhibits an 8 G broadening. And (iii) the EPR spectrum of cluster C_{red1} is absent in the Ni-depleted enzyme and reappears when Ni is replaced.[88] However, since the observed broadening was small in the *R. rubrum* enzyme and is absent in the ^{61}Ni-substituted *C. thermoaceticum* CODH enzyme, the question of a Ni component remained open. Therefore, until recently, we were unable to define the structure of cluster C except to state that it was likely to be an Fe-S cluster that probably contained nickel.

EPR and resonance raman (RR) experiments have provided strong evidence that nickel is present and that there is a bridge between Ni and the Fe-S cluster components of cluster C. (i) EPR studies of the thiocyanate adduct of cluster C provided evidence that Ni is bridged to an iron site in the C cluster from *C. thermoaceticum*. The *g* values of the adduct are far from that of a $[Fe_4\text{-}S_4]^{2+/1+}$ cluster and more closely resemble the spectra of paramagnetic Ni(I) complexes with a g_{av} of ~2.17 (Figure 4).[61,89] However, the spectra show hyperfine broadening from substitution with ^{57}Fe and not ^{61}Ni. The unusual *g* values and the small ^{61}Ni coupling constant for the different states of cluster C were recently explained by assuming that a high-spin Ni(II) site is weakly coupled ($J \approx 2$ cm^{-1}) to the $S = {}^1/_2$ state of the $[Fe_4\text{-}S_4]^{2+/1+}$ cluster.[90] This weak exchange interaction explains the lack of observable ^{61}Ni hyperfine interactions on the EPR spectra of cluster C. In addition, the change in the EPR spectra when anions like azide and thiocyanate bind was proposed to result from a change in the sign of *J* which moves g_{av} above 2 by approximately the same amount that g_{av} was below 2.0 in the C_{red1} state. (ii) We had learned earlier by freeze−quench EPR studies that when CO is reacted with CODH, reactions at clusters A, B, and C occur at vastly different time scales.[80] At 25 °C, cluster C binds CO and completes its reaction within 10 ms. Next, cluster B is reduced within 100 ms. Reduction and binding of CO to cluster A requires 10−50 ms. Therefore, one can distinguish between spectroscopic signals from each of these clusters by following the signals as they evolve with time. The freeze−quench method was modified for Resonance Raman (RR) spectroscopy to assign various Raman bands to the different clusters. Bands at 333, 353, and 365 cm^{-1} were associated with cluster C because of their kinetics.[91] RR spectroscopy is valuable because metal−ligand bond vibrations can be assigned by performing isotopic substitutions; for example, one can distinguish between a Ni−S and a Fe−S vibration by comparing the RR spectra of ^{64}Ni- and ^{58}Fe- or ^{54}Fe-substituted enzyme with the natural abundance enzyme. The 365 cm^{-1} band was shown by isotopic substitution to be associated with Ni and the 333 and 353 cm^{-1} bands to be associated with iron, most likely in an Fe−S cluster because Fe−S stretching modes are commonly observed at these positions. Thus, the FQ-RR spectrum of the *C. thermoaceticum* CODH showed that Ni is a component of cluster C and is probably bridged to an Fe in the Fe-S cluster. (iii) Study of the cyanide-inhibited *C. thermoaceticum* CODH by RR spectroscopy provided strong evidence that the Ni and iron are bridged in the active enzyme. Reaction of the enzyme with cyanide resulted in a bridged complex best described by a model including an Fe−CN−Ni species. Detailed analysis of the various isotope shifts (using ^{13}CN, C^{15}N, ^{13}C^{15}N, ^{58}Fe, ^{54}Fe, ^{64}Ni, and double substituted samples) by normal-mode calculations indicate that the Fe−CN bond is nearly linear with a 140° angle between the Fe−CN and Ni.[92] It is not clear whether cyanide replaced an inherent bridge between the cluster or if it provided an additional bridge.

EXAFS studies of the *R. rubrum* CODH indicated that the nickel site adopts a five-coordinate high-spin state with three N/O and two sulfur ligands.[93] In addition, iron could not be observed from the Ni EXAFS indicating that if Ni and Fe are part of the same cluster, they could not be present in a $[Ni\text{-}Fe_3\text{-}S_4]$ cluster. In combination with the results described above, recent EPR and Mössbauer studies of the *R. rubrum* enzyme indicate that cluster C contains high-spin Ni(II) ion that is bridged to a cubane $[Fe_4\text{-}S_4]$ cluster.[90] The Ni-deficient enzyme contains a form of cluster C, called cluster C* that is diamagnetic in its oxidized state and is a relatively standard $S = {}^3/_2$ $[Fe_4\text{-}S_4]$ cluster when reduced. In the reduced state, EPR signals are observed in the low field ($g = 6-3$) region of the spectrum. The redox potential for the 2+/1+ couple is −418 mV. Incorporation of nickel into the active site activates the enzyme and profoundly affects the properties of the cluster. The cluster appears to be a $S = {}^1/_2$ $[Fe_4\text{-}S_4]^{2+/1+}$ cluster in which one of the iron sites, called ferrous component II (FCII), becomes similar to the special iron site in aconitase[94,95] or the site-subsituted clusters synthesized and studied by Holm.[96] These properties suggest that this Fe becomes five- or six-coordinate. In addition, the midpoint potential for the C_{ox}/C_{red1} couple is unusually positive (−110 mV) for the $[Fe_4\text{-}S_4]^{2+/1+}$ couple of the cluster.

C. Mechanistic Studies of Enzymatic CO Oxidation

The first issue to be resolved was how to correctly write the balanced equation for the reaction. Thus, it was important to determine whether hydroxide or water is the substrate and whether the product of the reaction is CO_2 or bicarbonate. Earlier experiments using cell extracts indicated that water, not hydroxide, is the substrate and CO_2, not bicarbonate, is the product of this reaction[97] as shown by eq 7.

$$CO + H_2O + \text{acceptor}_{ox} \rightleftharpoons CO_2 + 2\,H^+ + \text{acceptor}_{red} \quad (7)$$

That two protons were released per molecule of CO oxidized was confirmed recently using rapid reaction methods with a pH indicator (Seravalli and Ragsdale, unpublished). This was a difficult experiment because almost all pH indicators available served also as electron acceptors and many electron acceptors take up protons upon reduction. Identity of CO_2 as the product was based on the pH dependence of the reverse reaction. The reaction rate was dependent upon the CO_2 concentration and was optimum at pH values where CO_2, not bicarbonate, was predominant.

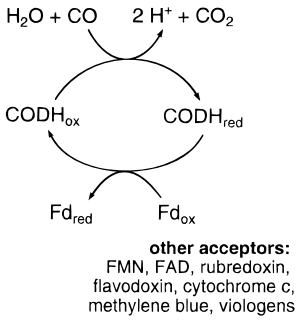


Figure 5. Ping-pong mechanism of CODH catalysis. The two electrons are shown here to be transferred to the C. thermoaceticum ferredoxin II, which has two Fe_4-S_4 clusters.

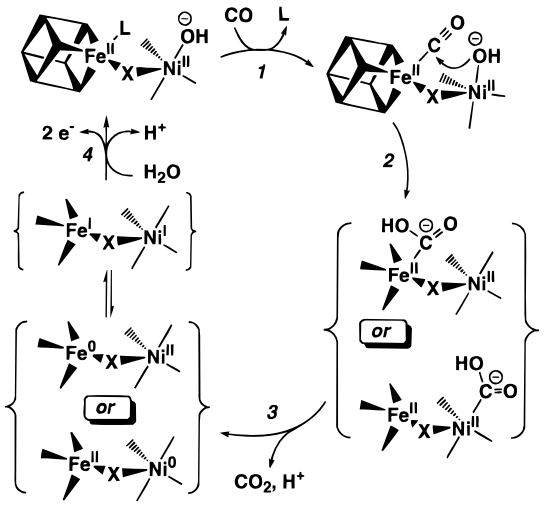


Figure 6. Mechanism of CO oxidation by CODH. (Modified from ref 92.)

There was no observable reaction above pH 6.6, where the ratio of $NaHCO_3/CO_2$ is high.[60]

Figure 5 describes a ping-pong mechanism for CO oxidation in which CO_2 is released before the electron acceptor binds and the two reducing equivalents are transferred. This was proposed first on the basis of studies with cell extracts from *C. thermoaceticum*[72] and *C. pasteurianum*[98] and was recently confirmed by steady-state kinetic analyses of the purified *C. thermoaceticum* enzyme using methyl viologen,[61] ferredoxin, and cytochrome *c* as electron acceptors (Seravalli and Ragsdale, unpublished). We also found that the k_{cat}/K_m for CO did not increase over a 50-fold range in concentration of methyl viologen[61] or ferredoxin (manuscript in preparation). In ping-pong mechanisms, the k_{cat}/K_m for one substrate is independent of the concentration of the other substrate. A ping-pong mechanism also is consistent with the results of a pre-steady-state study of the first half-reaction of CO oxidation.[80] If the acceptor is the eight Fe clostridial ferredoxin, one electron would be transferred to each of its two Fe_4-S_4 clusters.

The two half-reactions in CO oxidation are (i) oxidation of CO to CO_2 generating the two-electron reduced enzyme and (ii) reoxidation of the enzyme by the electron acceptor. This is described in more detail in a recently proposed mechanistic scheme (Figure 6).[61]

Step 1. The first step in the mechanism involves binding of CO to the Ni−X−Fe_4-S_4 cluster. We presume that one of the ligands undergoes dissociation. Binding of CO is associated with changes in the EPR spectrum of cluster C and occurs with a rate constant of 2×10^8 M^{-1} s^{-1}.[80] We proposed that water binds to the Ni[II] site and CO to an Fe[II] site (FCII) based on RR studies of cyanide binding to cluster C.[92] Since CN is a selective and competitive inhibitor for CO oxidation and has similar properties to CO, we assumed that cyanide binds to the enzyme in a manner similar to CO. RR spectroscopy reveals that CN^- binds simultaneously to Fe and Ni; three bands, at 719, 384 and 342 cm^{-1}, whose ^{13}C and ^{15}N shifts identify them as cyanide−metal stretching and bending modes, are sensitive to incorporation of both ^{54}Fe and ^{64}Ni into the enzyme.[92] Vibrational modeling indicates an off-axis Fe−CN−Ni bridging geometry for the adduct. Thus, we have considered that CO binds in a similar orientation as CN, except that, since CO is not as strong a π acceptor as CN, it is not likely that CO could bridge the two components.

The enzyme is assumed to already be in the state with the hydroxide nucleophile bound. Evidence for the deprotonation of enzyme-bound water comes from several studies. The EPR spectrum of cluster C was pH dependent ($pK_a = 7.2$).[61] The *g* values for the protonated and unprotonated forms of cluster C were 2.005, 1.815, 1.651 (low pH) and 2.015, 1.800, 1.638 (high pH). The pH dependence of the cluster C EPR spectrum is similar to that obtained for the value of k_{cat}/K_m for CO, which reflects the protonation/deprotonation of free enzyme, determined by steady-state kinetics.

Step 2. It is proposed that preorganization of the metal ions by the enzyme is important in promoting the next step which is the Ni[II]-assisted hydroxide attack on Fe(II)-bound CO to form enzyme-bound carboxyl (or carbohydroxy). Ni is also proposed to play a Lewis acid role in polarizing bound CO to make the CO carbon more electrophilic.

Step 3. Next, the resulting ^-COOH would undergo deprotonation and decarboxylation to form CO_2 and the reduced form of cluster C. This would be expected to transiently produce Ni(0) or Fe(0), which would be expected to rapidly transfer electrons to the other metal clusters in the protein. Presumably, the ligand dissociated by CO in step 1 would reassociate as the carboxyl group leaves.

Step 4. In the second half-reaction, the reduced enzyme is reoxidized by an electron acceptor. A promiscuous electron donor, CODH can use a variety of proteins including flavodoxin, both ferredoxins in *C. thermoaceticum*, cytochrome *c*, and rubredoxin as well as a variety of cofactors and dye mediators including FAD, FMN, methylene blue, and methyl and benzyl viologen. CODH cannot, however, reduce NAD or NADP.[12,16] It also can directly transfer electrons to the CFeSP[99] and to hydrogenase[100] although ferredoxin stimulates this reaction by approximately 4-fold. A proton transfer step is apparently associated with the second half-reaction. Steady-state kinetics with ferredoxin were recently performed (Seravalli and Ragsdale, unpublished). The k_{cat}/K_m for ferredoxin was pH dependent and followed a single titration curve with a pK_a of 5.0 which probably reflects transfer of the second proton in the

mechanism. This could be either from ionization of bound carbohydroxy in step 3 or proton transfer from the reduced enzyme to solvent.

Ferredoxin is likely to be the natural electron acceptor for the *C. thermoaceticum* enzyme and has been shown to accept electrons from CO.[12,16] Ferredoxin also significantly stimulates the rate of the CO/acetyl-CoA exchange reaction[23] and the synthesis of acetyl-CoA from methyl-H_4folate, CO, and CoA.[101] The mode of activation involves formation of an electrostatically stabilized complex with CODH and a complex between ferredoxin and residues 229−239 of the large subunit was isolated by crosslinking the two proteins with a carbodiimide.[102] In *R. rubrum*, a 22 kDa iron−sulfur protein was isolated which coupled CO oxidation to H_2 evolution via a CO-induced membrane bound hydrogenase.[54] The gene encoding this Fe-S protein, *cooF*, has been sequenced and is part of the gene cluster containing CODH and the hydrogenase.[18]

Besides its ability to assemble acetyl-CoA and to oxidize CO or reduce CO_2, CODH can perform redox reactions with CO and CO_2 analogues. In the presence of a suitable reductant, it can convert nitrous oxide to N_2 at a rate approaching those of the copper-containing nitrous oxide reductases from denitrifying bacteria.[103] The E_0' of the N_2O/N_2 redox couple is +1175 mV.[104] Interestingly, the nitrous oxide reductase from *Pseudomonas stutzerii* also oxidizes CO to CO_2 and CO binding was proposed to occur at the copper site.[105] The *R. rubrum*[106] and *C. thermoaceticum*[107] CODHs reduce carbonyl sulfide (COS) to CO and H_2S. For the *R. rubrum* enzyme, the K_m is quite low −2.2 μM.[106] COS also serves as a competitive inhibitor (with respect to CO, $K_i = 2.3$ μM) of the *R. rubrum* CODH.[108] CS_2 was also found to serve as a reversible, rapid-equilibrium inhibitor of CO_2 reduction that was competitive with respect to CO_2[106] and to reverse cyanide inhibition of the enzyme.[108,109]

D. Anions and Their Reactivity with Cluster C

A variety of anions bind to cluster C, including cyanide, azide, thiocyanate, cyanate, and isocyanides. Among the anions, cyanide binds most tightly to the enzyme. It is a specific and potent inhibitor (K_i < 10 μM)[110] of CO oxidation with little effect on acetyl-CoA synthesis.[16,23,111] Early studies of cyanide inhibition of CO oxidation led to some interesting concepts regarding the role and nature of CODH. In whole-cell studies, CO production from CO_2, but not CO incorporation into acetate, was found to be sensitive to cyanide; it was, thus, incorrectly concluded that the acetogenic CODH functions to reduce CO_2 to CO but is not involved in the incorporation of the carbonyl group into acetyl-CoA.[112] This was a reasonable conclusion; however, it was not recognized at the time that CODH/ACS is a bifunctional enzyme that oxidizes CO and assembles acetyl-CoA at separate active sites. That CODH was sensitive to both cyanide and propyl iodide led to the incorrect postulate that CODH was a B_{12} enzyme.[72] This was a reasonable assumption since cobamides are extraordinarily sensitive to these reagents. However, the CO oxidation site has proven to be an unusual metal cluster with inhibition characteristics similar to those of cobamides.

Cyanide is a potent slow binding inhibitor for the CODHs from *R. rubrum*[87] and *C. thermoaceticum*.[110] CO also protects against cyanide inhibition and relieves cyanide inhibition.[87,109,110] How to interpret these results has led to two apparently conflicting explanations. Classification of an inhibitor as a "slow binding inhibitor" can result from at least two types of mechanisms: (i) the inhibitor binds slowly or (ii) the inhibitor binds rapidly and the inhibitor-bound enzyme isomerizes into a state from which release of the inhibitor is slow. Extensive kinetic studies by Ludden's group on the *R. rubrum* CODH were interpreted by the former model (i) including competition between CO and cyanide for the active site. They stated, "The protection against cyanide inhibition and the requirement of CO for reactivation of cyanide inhibited enzyme indicate that binding of CO and cyanide are mutually exclusive and suggest a common binding site."[87] It was proposed that CO and cyanide bind to the nickel site in cluster C of the *R. rubrum* CODH because (i) the Ni-deficient enzyme was unable to bind CN and (ii) Ni-deficient CODH could be activated by Ni even if the enzyme was previously incubated with CN.[58,87] Tom Morton in Ljungdahl's group extensively studied cyanide inhibition of the *C. thermoaceticum* enzyme and developed a model that supports competition between CO and CN.[110] CO_2, COS, and CS_2 have also been shown to reverse cyanide inhibition.[108,109]

Binding of cyanide to cluster C of the *C. thermoaceticum* enzyme was demonstrated by EPR and ENDOR spectroscopies.[113] The EPR spectrum of the CN adduct has *g* values at 1.87, 1.78, and 1.55. The Lindahl group proposed that center C is the CO oxidation site based on the premise that CO and cyanide compete for the CO oxidation site.[82] Later, Anderson and Lindahl proposed[109] that CO and cyanide bind at separate sites because CO protects against CN inhibition and accelerates the dissociation of CN from both the *R. rubrum*[87] and *C. thermoaceticum* enzymes.[109] Then, it was shown that cyanide binding to the *R. rubrum* CODH perturbs the Mössbauer signal of a Fe(II) site (apparently a five-coordinate site called ferrous component II or Fe_A) of cluster C, suggesting that CN^- does indeed bind to cluster C (the site of CO oxidation) at the Fe_A site.[90] Recent RR spectroscopic results indicate that CN^- binds to both Fe_A via its C end and to Ni at the N end.[92] Vibrational modeling indicates that the Ni is bound at an angle of about 140° to the end of an essentially linear FeCN unit.

The observations that CN is a slow-binding inhibitor and that CO protects against and relieves CN inhibition does not require that CO and cyanide bind at separate sites as proposed;[109] they are also consistent with a common binding site, but different binding *modes* for the two ligands. On the basis of the RR spectroscopic results performed in collaboration with Spiro's group, it was proposed that CO and CN^- bind to Fe_A; however, CO does not bind simultaneously to the nearby Ni^{2+}, as CN^- does.[92] The additional interaction with Ni^{2+} in the bridged complex would explain the slow inhibition of CO oxidation by CN^-. The initial binding of CN^- to Fe_A would be rapid and reversible by CO; however, a slow

subsequent rearrangement to the bridged complex would be more difficult to reverse. CO could protect against CN^- inhibition by occupying the Fe^{2+} site and accelerate CN^- dissociation by producing a transient mixed complex, with CO bound to Fe^{2+} and CN^- bound to Ni^{2+}. This model of the inhibited active site also provided the basis for a mechanistic proposal regarding CO oxidation (described above).

Azide, thiocyanate, and cyanate also have been shown to bind to cluster C.[89] They are very weak inhibitors of the enzyme and cause marked changes in the EPR spectrum of cluster C.[61] Reaction with thiocyanate, azide, or cyanate converts the EPR spectrum from $g_{av} = 1.82$ ($g = 2.01, 1.81, 1.65$) to a two-component spectrum with $g_{av} = 2.15$ ($g = 2.34, 2.067, 2.03$) and $g_{av} = 2.17$ ($g = 2.34, 2.115, 2.047$). Thiocyanate acted as a mixed partial inhibitor with respect to CO with pH- and temperature-dependent inhibition constants. The pH dependence of the inhibition constant ($pK_a \approx 7.7$) is the same as the value measured for the k_{cat}/K_m for CO. Binding of thiocyanate to the oxidized form of Center C appears to be favored by a negative enthalpy that is offset by a decrease in entropy yielding a slightly unfavorable free energy of association.

n-Butyl isocyanide (*n*-BIC) was found to behave as a CO analog at both the CODH and ACS active sites.[114] Isocyanides [RNC][115,116] are isoelectronic with CO and have used for many years as CO analogs for heme proteins.[117−121] They are stable organic compounds that formally possess a divalent carbon and can behave as strong carbon ligands for transition metals. Since the negative charge is on the terminal carbon, isocyanides have a tendency to form cationic species and can stabilize high oxidation states of metal ions. Conversely, CO characteristically stabilizes the lower oxidation states of metal ions. *n*-BIC was found to compete with CO in the CO oxidation reaction ($K_i = 1.66$ mM) and to act as a slow substrate in a *n*-BIC:acceptor oxidoreductase activity. *n*-BIC caused EPR spectral changes at clusters A, B, and C similar to those elicited by CO, albeit at greatly reduced rates. An early intermediate in the *n*-BIC reaction with cluster C called the C_{red3} state (with *g* values of 2.27, 2.14, and 2.081, originally called C*) was observed by EPR spectroscopy that is similar to the spectrum obtained on reaction with azide and other anions. We think that this intermediate may also be important in CO oxidation because its decay mirrored the rate of reduction of cluster B (Kumar and Ragsdale, manuscript in preparation).

E. Modeling CODH

1. Inorganic Models for the CODH and Related Reactions

a. Structural Models of Cluster C. It would be extremely interesting to test the above hypotheses concerning the advantage of a bimetallic system for CO oxidation to CO_2 or the reverse reaction by studying structural models of cluster C of CODH. Strong efforts have been made toward the synthesis of such models.[122] Clusters that contain Ni and Fe have been synthesized including $[NiFe_3Y_4(SR)_4]^{3-}$ and

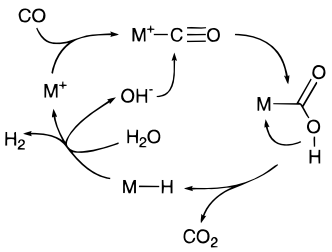


Figure 7. Mechanism of the water−gas shift reaction.

$[NiFe_3Y_4(SR)_3(PPh_3)]^{2-}$, where Y = S or Se and R = ethyl or mesityl.[123] These complexes were studied by X-ray diffraction, EXAFS, EPR, Mössbauer, and cyclic voltammetry.[124] Ni K-edge and EXAFs studies indicated that the Ni was in the 2+ oxidation state and the Mössbauer data implied that electron density was significantly delocalized from the cluster to the nickel site.[125] The phosphine ligand on the latter complex was found to be exchangeable with a variety of thiolates and cyanide. The [Ni-Fe_3-S_4] cluster was also obtained by the insertion of Ni^{2+} into the [Fe_3-S_4] clusters of the *Pyrococcus furiosus* and and *Desulfovibrio gigas* ferredoxins.[126] The spectroscopic properties of the Ni-modified protein were similar to those of Holm's synthetic model. The Ni-containing ferredoxin was shown to bind CN^-, an inhibitor of the CODHs from *R. rubrum* and *C. thermoaceticum*.[126]

b. Functional Models of CODH. The CODH reaction is formally equivalent to a well-known industrial process called the water−gas shift reaction (WGSR) (eq 8) [127]. The WGSR utilizes the reducing power of

$$H_2O + CO \leftrightarrow H_2 + CO_2 \qquad (\Delta H = -42 \text{ kJ/mol}) \tag{8}$$

CO to produce H_2 from water under mild conditions. The hydrogen is used as the reductant for processes such as the Haber−Weiss process for generation of ammonia from N_2. This reaction is also used to remove CO during the Haber process. This reaction is catalyzed by a variety of metal complexes. Current methods of effecting the WGSR involve heterogenous catalysis with Cr_2O_3 at 350 °C or Cu−Zn oxide at 200−300 °C. Homogenous catalysts that have been used for this reaction have been reviewed by Laine and Crawford.[128]

The mechanism of this reaction (Figure 7) is viewed as a nuclophilic attack of hydroxide on metal-bound CO. A β hydride shift is conceived to occur next to eliminate CO_2 and generate a metal hydride complex. The metal−hydride then attacks water to regenerate the hydroxide nucleophile and the active catalyst.

Bimetallic binary complexes for the WGSR show a pronounced synergistic effect. Both mixed Ru-Fe clusters and a mixture of Ru clusters with Fe complexes are much more active than either of the component clusters when used independently. A pyridine solution of $Fe_3(CO)_{12}$ shows no WGSR activity and a solution of $Ru_3(CO)_{12}$ shows a turnover number of 15 mol of H_2 per molecule per day at 100 °C and 0.4 bars of CO. Under the same conditions, the mixed clusters, $FeRu_2(CO)_{12}$ and $Fe_2Ru(CO)_{12}$, have turnover numbers of 220 and 250 per day, respectively.[129]

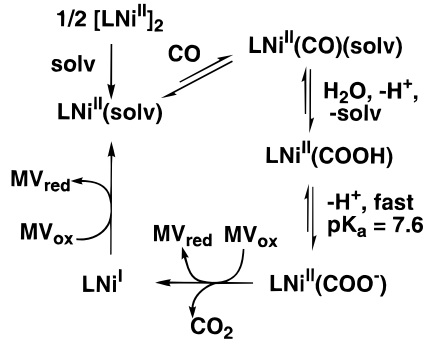


Figure 8. Mechanism of CO oxidation by a model Ni complex. (Modified from ref 131.)

A major difference between the WGSR and CODH is that the former produces H_2 and the latter produces two protons and two electrons. A closer relative to CODH than the WGSR has been developed.[130,131] Dinuclear homologous nickel(II) complexes of $[Ni(tmtss)]_2$ (tmtss is tetramethylsalicyldehyde thiosemicarbazone) containing iminothiolate ligands were shown to catalyze the oxidation of CO to CO_2 and protons in aqueous solution at room temperature with methyl viologen as the electron acceptor. The reaction rate observed was 1.04 h^{-1} under optimal conditions in the presence of sodium acetate as a base; this is 10^7-fold slower than the enzyme. The proposed mechanism (Figure 8) includes (i) binding of CO to the solvated monomeric Ni complex, (ii) nucleophilic attack of water on bound CO to give an intermediate Ni-COOH complex (this step would require loss of a proton from water) and desolvation of the complex, (iii) rapid deprotonation of the Ni-COOH to form Ni-COO^-, (iv) decarboxylation of Ni-COO^- associated with reduction of the first mol of methyl viologen to generate CO_2 and a Ni(I) complex, and (v) completion of the catalytic cycle by reoxidizing Ni(I) to Ni(II) with a second mole of methyl viologen. Step iv is suggested to be rate limiting. The sigmoidal rate−pH profile was fit to a pK_a of 7.6, which is very similar to the value for the enzymatic reaction. In the model reaction, this pK_a was suggested to reflect deprotonation of the Ni-COOH to form Ni-COO^-. The ability of this model complex to reduce MV instead of releasing H_2 was explained by the lack of a nickel−hydride intermediate.

Development of efficient ways to catalyze the reduction of CO_2 has been a major focus of many laboratories for over a century. In 1870, the first successful process described used zinc electrodes with sodium bicarbonate as the electrolyte.[132] Formic acid was the sole electrolysis product. Reduction of CO_2 by 2, 4, 6, or 8 electrons has been accomplished using different reductants and catalysts to give a variety of different one-carbon compounds. The eight-electron reduction of CO_2 (the Sabestian reaction) uses 4 equiv of H_2 to generate methane.[133] The overall reaction is identical to that catalyzed by methanogenic organisms, however, instead of enzymes, heterogenous nickel, ruthenium, and rhodium catalysts are used. Higher alcohols also have been synthesized from $CO_2 + H_2$. (See ref 134 and references therein.) Although the electrochemical reduction on metal cathodes has been the most successful approach, a persistent problem is that a high overpotential is required.[135] Apparently the requirement for such negative reduction potentials (over 1.0 V more negative than the CO_2/CO couple) is that the potential must be negative enough to generate the CO_2 radical anion since the process occurs at potentials near that of the standard potential for the $CO_2/CO_2^{\bullet-}$ radical anion couple.[136] In contrast, the electrochemical reduction of CO_2 to CO by CODH does not require an overpotential.[84] Control of which products are formed is a function of the electrode material, the solvent, and the nature and concentration of the supporting electrolyte. A concept that has emerged from the Aresta laboratory is that if electron transfer occurs faster than proton transfer, then CO will be the product.

Several nickel complexes can catalyze CO_2 reduction. The square-planar Ni(II) complex, $[Ni^{II}Cyclam]^{2+}$ (cyclam = 1,4,8,11-tetraazacyclotetradecane), catalyzes the electrochemical reduction of CO_2 to CO in aqueous solution at a mercury electrode at potentials below −0.9 V.[137−141] The proposed mechanism involves CO_2 binding to electrode-adsorbed $[Ni^{I}Cyclam]^+$ in a η^1 manner followed by sequential proton- and electron-transfer steps. Protons from the cyclam amine appear to play an important role in stabilizing the initial Ni−CO_2 bond by hydrogen bonding. When $[Ni^{I}Cyclam]^+$ was reacted with CO_2 in DMF, formate was produced instead of CO.[142] Electrocatalytic reductions of CO_2 to CO by several other nickel complexes of macrocyclic and polypyridyl ligands have been described.[143−147] Ni-porphyrins have been shown to reduce CO_2 all the way to methanol,[148] whereas, and Ni-pthalocyanines reduce it to CO.[149] Aresta and co-workers have reported that CO_2 bound to a phosphine Ni(0) complex $[Ni(PCy_3)_2(CO_2)]^0$ (PCy = cyclohexylphosphine) can be reduced by thiols to give CO and water. They proposed that the protonation of Ni−CO_2 complex by the thiol (or other Brønsted acids) is the key step in the reaction.[150] Aresta pointed out that this may be relevant to understanding the mechanism of CODH.

Iron complexes also have been described that can catalyze electrochemical CO_2 reduction. Electroreduction of CO_2 to a mixture of CO and formate has been shown to be catalyzed by an iron−sulfur cluster $[Fe_4S_4(SR)_4]^{2-}$ apparently in the tetraanionic state at potentials of ∼−2.0 V vs SCE.[151] The most detailed studies of catalytic CO_2 electrochemical reduction have been performed with iron(0) porphyrins which produce CO as the major product.[136] Conversion of this to a bimetallic system by the addition of Mg^{2+} resulted in a significant increase in catalytic efficiency. This reaction required an ambient potential of approximately −1.8 V vs SCE. It was proposed that the Fe(0)-porphyrin begins the reduction in an inner-sphere process and the Mg^{2+} ion facilitates the reaction by coordinating to CO_2 and providing electrophilic assistance. When a series of weak Brønsted acids such as propanol was added to the CO_2 reduction system, catalysis was so efficient that the rate (for example, 200 s^{-1} at 0.1 M CO_2) appears to be limited by the rate of release of CO bubbles from the mercury electrode.[152] The effect of the acid was postulated to provide electrophilic assistance by

pulling electron density from the metal-bound CO_2 as electrons are pushed into the substrate by the catalyst.

2. Comparison between the Models and CODH

There are several major differences between the models and CODH. CODH oxidizes CO to CO_2 $\sim 10^7$-fold faster than the Ni model complex described above. It also is more efficient ($\sim$1.0 V overpotential is required for the models and none for the enzyme[84]) and more specific (CODH does not generate formic acid or products more reduced than CO) in the CO_2 reduction reaction. There is another enzyme, formate dehydrogenase, that is specific for producing formate from CO_2.[153] These two aspects of CODH are not overly surprising since enzymes are known to be highly specific and to accomplish huge rate accelerations for many reactions. How does CODH accomplish such high specificity and provide such a low activation energy barrier? It is likely that one part of the answer involves the use of a bimetallic mechanism for catalysis.[92] By bridging an electrophilic Ni ion to the iron−sulfur cluster in cluster C, one accomplishes the electrophilic assistance discussed above when weak Brønsted acids were added to the Fe(0)−CO_2 catalyst or in the case of the Ru−Fe catalyst for the WGSR. In addition, nonenzymatic CO oxidation requires nucleophilic attack of water from solution and CO_2 reduction requires the elimination of hydroxide from bound CO_2. We have proposed that the Ni component of cluster C binds and deprotonates water to provide a nucleophilic hydroxide species to attack the Fe−CO complex.[92] Thus, Ni is proposed to serve a role similar to that of zinc in carbonic anhydrase. Another important aspect of the enzymatic catalysis is that when the metal-bound carbohydroxy intermediate ($^-$COOH) is formed, electron density can be efficiently delocalized into a heterometallic cluster with soft sulfur ligands, aiding in the release of CO_2 and protons. In one of the model systems,[131] decomposition of the carbohydroxy intermediate appears to be the rate-limiting step. In the WGSR, the unstable carbohydroxy complex rapidly decarboxylates to give metal−hydride and CO_2 by β-hydride elimination.[154] A further advantage enjoyed by CODH is that it contains two other redox clusters (clusters A and B) that can serve as intramolecular electron acceptors before the electrons finally are transferred to other proteins (ferredoxin, flavodoxin, etc.) or mediators (viologens, etc.).

IV. Mechanism of Acetyl-CoA Synthesis

The acetyl-CoA pathway is described in Figure 9. The metabolism of CO, CO_2, and organic carbon by the acetyl-CoA pathway has interested scientists for at least 50 years. When ^{14}C first became available, H. A. Barker, Martin Kamen, Harland Wood, and others began to investigate this pathway. An early experiment showed that $^{14}CO_2$ was converted to both the methyl and carboxyl groups of acetic acid.[155] Further attempts to characterize the steps in the pathway using radioisotope labeling methods were met with frustration. On the other hand, Calvin, Benson, and others were able to completely elucidate the reductive pentose phosphate pathway by using radioisotope labels to isolate and characterize the key

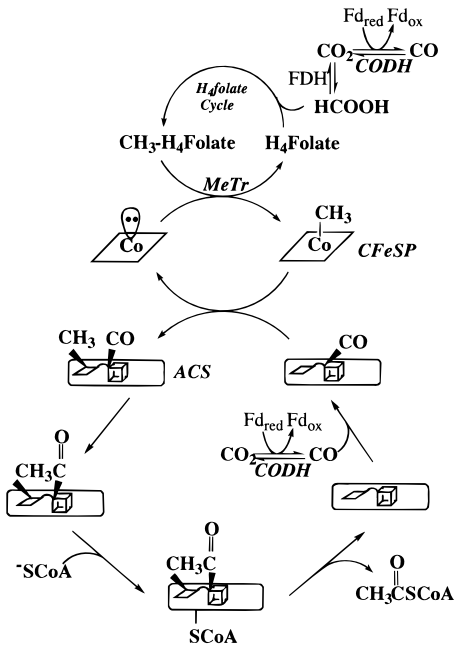


Figure 9. The reductive acetyl-CoA pathway.

intermediates. As should become clear in this review, elucidation of this pathway was a major challenge to biochemists because the standard methods to elucidate organic compounds could not possibly identify enzyme-bound intermediates like those in the acetyl-CoA pathway. The unique steps in the acetyl-CoA pathway were only detected and characterized by studying highly concentrated solutions of purified enzymes by sensitive spectroscopic methods that focus directly on the active-site metal centers. These unique reactions are summarized by eq 9. The $\Delta G°$ for eq 9 was calculated to be −65.0 kJ/mol yielding an equilibrium constant of 9×10^{10} M^{-1} at 37 °C.[37]

$$CO + CH_3\text{-}H_4\text{folate} + CoASH \rightarrow CH_3\text{-}CO\text{-}SCoA + H_4\text{folate} + H_2O \quad (9)$$

An enzyme that catalyzed CO oxidation was discovered in the late 1950s. CODH was then discovered in a variety of organisms and the role appeared simply to allow organisms the luxury of growing on CO as a sole carbon source. There was growing evidence in the early 1980s that CODH played some role in the acetyl-CoA pathway. Various roles for CODH in acetate synthesis were suggested before 1985, including reduction of an electron carrier or enzyme prosthetic group involved in CO_2 reduction to acetate[72] and formation of an enzyme-bound [HCOOH] group from pyruvate[156,157] or CO.[158] The finding that the CODH from acetogenic bacteria also had acetyl-CoA synthase activity was completely unexpected.[23] The history of this discovery has been described.[159] At the time, acetyl-CoA or acetate were thought to be synthesized by a corrinoid or a corrinoid-containing protein. A series of experiments were performed that demonstrated that CODH was the only protein required for an isotope exchange reaction, discovered by Harold Drake several years earlier,[158] between CO and acetyl-CoA labeled in the

carbonyl group (eq 10). These experiments showed

$$CO + CH_3\text{-}^{14}CO\text{-}SCoA \rightarrow CO + CH_3\text{-}CO\text{-}SCoA \quad (10)$$

that CODH must be able to bind the methyl, carbonyl, and SCoA groups of acetyl-CoA, equilibrate the carbonyl group with CO in solution, and then condense these three groups to resynthesize acetyl-CoA. Thus, it was proposed that the synthesis and assembly of acetyl-CoA occur on CODH, that the role of the CFeSP was to donate the methyl group to CODH, and that a more appropriate name for the acetogenic CODH is *acetyl-CoA synthase*.[23]

The proposal that CODH was the acetyl-CoA synthase in the pathway was not universally accepted[160] because the methyl-CODH, CODH-CO, and CODH-SCoA intermediates required in the mechanism had not been detected. Now that the key intermediates have been identified and characterized (Figure 10), it is clear that the acetogenic CODH does indeed function as an acetyl-CoA synthase allowing acetogens to fix CO and CO_2 into cell carbon. Evidence that the methanogenic CODH is involved in carbon assimilation has been reviewed.[2,7] The central role of CODH/ACS and the acetyl-CoA pathway in autotrophic growth of methanogens was strikingly demonstrated by the isolation and characterization of a CODH mutant of *Methanococcus maripaludis* that required acetate and could not grow on H_2/CO_2.[161] Evidence that certain sulfate-reducing bacteria also grow autotrophically by the acetyl-CoA pathway has been reviewed.[160]

The detection and characterization of the enzyme-bound intermediates in the ACS reaction has uncovered unusual metal clusters in which nickel is bridged to an [Fe_4-S_4] unit; the first example of a biological organometallic reaction sequence; new enzymatic mechanisms of C−C and C−S bond formation; and novel roles for metals in biology including the first demonstrated biological example of an alkylnickel species, the first unambiguous and definitive assignment of a role for nickel in any enzyme, and a new role for iron−sulfur clusters in the chemical steps of catalysis. In the following few sections we will describe what is known about the mechanism of ACS and discuss chemical models that are relevant to ACS. All CODHs are not bifunctional; many like the *R. rubrum* and carboxydobacterial enzymes lack ACS activity. Therefore, the following discussion is pertinent to the bifunctional CODH/ACS enzymes of acetogens, methanogens, and sulfate reducers.

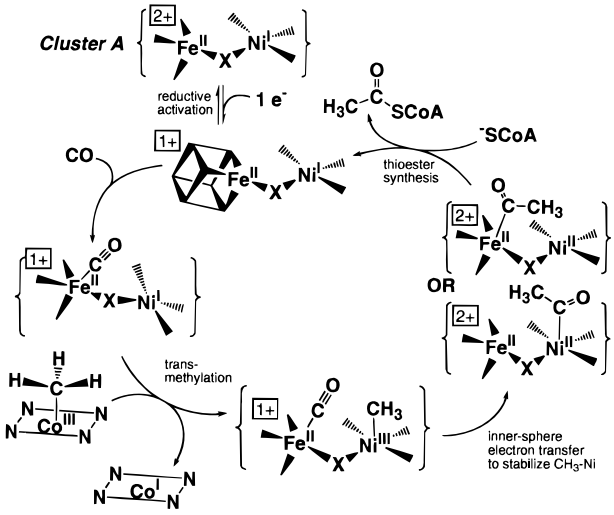


Figure 10. ACS-bound organometallic intermediates in the acetyl-CoA pathway.

A. Discovery of the ACS Active Site and Its Structure

Cluster A, the site of acetyl-CoA synthesis, appears to have a structure similar to that of cluster C in which Ni is bridged to a [Fe_4-S_4] cluster. The first hint of an unusual structure for cluster A was based on EPR studies of the CO-reacted enzyme. When CODH/ACS from *C. thermoaceticum* was reacted with CO for over 10 s, a slow relaxing EPR signal is observed at temperatures as high as 130 K with *g* values at 2.08, 2.07, and 2.03.[83] These *g* values and relaxation properties are unlike those of [Fe_2-S_2], [Fe_3-S_4], or [Fe_4-S_4] clusters or of Ni(III) or Ni(I). That this EPR signal does not come from an isolated Ni ion or Fe−S cluster was demonstrated by isotope substitution methods (Figure 11). Reaction of the ^{61}Ni- ($I = 3/2$) and ^{57}Fe- ($I = 1/2$) substituted enzymes with CO gave significant spectral broadening demonstrating that cluster A is an unusual cluster containing both Ni and Fe.[162,163] When CODH was reacted with ^{13}CO in place of ^{12}CO, the resulting EPR

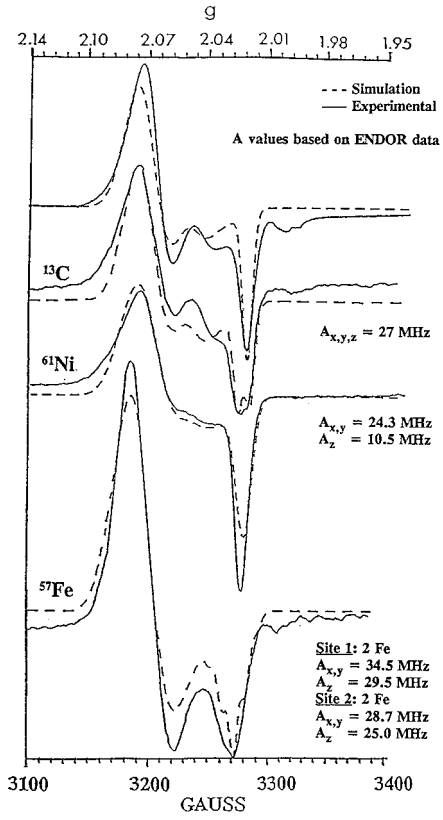


Figure 11. EPR studies of isotopically enriched ACS after reaction with CO. Experimental (—) and simulated spectra (- - -) using *A* values derived from the ENDOR experiments of CODH enriched with ^{13}CO, ^{61}Ni, and ^{57}Fe. For simulation of the ^{57}Fe spectra, four irons in two different types of iron were assumed with the *A* values shown. (Modified from ref 165.)

spectrum split into a doublet. This demonstrated that CO binds to a novel Ni−Fe cluster on CODH. This adduct became known as the NiFeC species and the EPR signal was called the NiFeC signal. An almost identical EPR spectrum was observed when the methanogenic ACS was reacted with CO, indicating that cluster A exists in these organisms.[39,164] The conditions for formation of this signal in *Ms. barkeri* were recently studied. The NiFeC signal was formed at a CO concentration of ~25 μM. Generation of the NiFeC species was shown to require a one-electron reduction with a midpoint reduction potential of −541 mV.[165] Apparently, formation of the adduct involves binding of CO to the reduced enzyme.[166]

Electron−nuclear double resonance (ENDOR) spectroscopy was used to better characterize cluster A in the first reported Ni ENDOR study of an enzyme.[162] ENDOR can directly measure the strength of interaction, *A*, between the electronic and nuclear spins. In addition, each magnetic nucleus located in a different environment gives a distinct ENDOR signal. Two ENDOR signals were observed in the ^{57}Fe-containing enzyme suggesting that two types of iron were part of cluster A. Simulation (dotted lines in Figure 11) of the EPR spectrum with the ENDOR-derived coupling constants suggested that the cluster contained at least three iron atoms. ENDOR signals also were observed from the ^{61}Ni-containing and ^{13}C-treated enzymes. Simulation of the EPR spectra suggested a single Ni and one ^{13}CO were present in the complex. On the basis of the combined ENDOR and EPR results, the CODH-CO complex was proposed to contain CO, Ni, and ~3−4 iron atoms.[162] The ^{57}Fe coupling constants were similar to those obtained earlier by Mössbauer spectroscopy.[86] The Mössbauer parameters (hyperfine coupling constants, isomer shift, and quadrupole splitting parameters) of cluster A had been found to be similar to those of $[Fe_4\text{-}S_4]^{2+}$ clusters and led to the proposal that cluster A contained a Ni ion bridged to a $[Fe_4\text{-}S_4]^{2+/1+}$ cluster.[86] The ENDOR results were thus consistent with this hypothesis. The coupling constant for ^{13}CO was similar to that obtained for the ^{13}CO adduct with the *C. pasteurianum* hydrogenase (this is a Fe-only hydrogenase) which has a unique Fe−S cluster at the H_2 activation site.[167]

On the basis of combined EPR, ENDOR, and Mössbauer spectroscopic evidence, it appeared that cluster A contained one nickel ion and three to four irons with properties resembling a $[Fe_4S_4]^{2+/1+}$ cluster and that it could bind a single molecule of CO. The possibilities included (i) a Fe_4S_4 cluster linked to Ni through a ligand bridge with CO binding either to Ni or Fe, (ii) the same arrangement of metals but with CO acting as the ligand bridge, or (iii) [Ni−Fe_3S_4] cluster. Of these possible structures, only the Ni−Fe_3-S_4 complex had a purely synthetic precedent.[96,123,124] A Ni−Fe_3-S_4 center also was assembled by incorporating Ni into the unoccupied edge of a [Fe_3-S_4] center in a ferredoxin.[168] EXAFS experiments were inconsistent with a [Ni−Fe_3-S_4] cluster[169,170] although this structure could not be unambiguously ruled out.

By Fourier-transform infrared (FTIR) spectroscopy, the CO stretching band was located at 1995 cm^{-1}, which ruled out the possibility of a bridging CO and provided strong support for a terminally bound CO, *i.e.*, metal−C≡O.[171] Isotope-chase experiments using ^{13}CO in the presence of [^{12}C]acetyl-CoA demonstrated that the carbonyl group that is marked by the 1995 cm^{-1} band is the precursor of the carbonyl group of acetyl-CoA. On the basis of similar isotope-chase experiments that had been done by EPR,[165] this vibrational band was accepted as the IR signature for the NiFeC species. On the basis of combined magnetic resonance and IR results, a working model of the structure of the cluster A−CO adduct was written, [Ni−X−Fe_4-S_4]−C≡O, where X is an unknown bridge between the Ni ion and the Fe_4-S_4 cluster.[171]

The above structural model is consistent with recent spectroscopic studies of the dissociated α subunit and an $\alpha\beta_2$ form.[20] The isolated α subunit contains one Ni and four Fe and has spectroscopic properties[21] similar to those of cluster A of the native enzyme. The Ni is coordinated to two S donors at 2.19 Å and two N or O donors at 1.89 Å in a distorted square plane and the irons are in an $S = {}^3/_2$ [Fe_4-S_4]$^{2+/1+}$ cluster.[21]

B. Enzymatic Mechanism of Acetyl-CoA Synthesis

1. CO Binding to Cluster A

A variety of spectroscopic experiments were described above that helped to understand the structure of cluster A and define the adduct between ACS and CO. These studies indicated that CO is a terminal ligand to either the Ni or Fe site in the [Ni−X−Fe_4-S_4]. A Ni−CO complex was expected since the cluster was presumed to facilitate electron transfer reactions during acetyl-CoA synthesis. Most scientists had focused on Ni as the active site of acetyl-CoA synthesis because this metal is relatively exotic in biology. There were many descriptions of cluster A as "the Ni center in CODH" and many chemists considered that modeling the Ni site was sufficient in understanding the reactivity of the enzyme.

We felt that it was important to determine whether CO binds to Ni or Fe at the active site of cluster A. NMR and RR spectroscopy were considered as methods to distinguish between a Ni−CO and an Fe−CO intermediate. We recognized that both would be difficult experiments for various reasons. For example, RR would be difficult since cluster A was probably fully reduced in its adduct with CO and reduced Fe-S clusters are notoriously poor Raman scatterers. Tom Spiro and his student, Di Qiu, made a difficult experiment work. The RR experiment worked beautifully because, although most of the Fe−S bands were suppressed, the metal−CO vibration was enhanced (Figure 12). It was a startling discovery that the cluster A−CO bond is between carbon and Fe, not Ni.[91,171,172] The Fe−CO vibration (ν_{Fe-C} = 360 cm^{-1}) was sensitive to ^{13}C and ^{54}Fe isotope substitutions and insensitive to ^{64}Ni substitution.[172]

As with any putative reaction intermediate, it was crucial to determine if the Fe−CO band and the NiFeC EPR signal represent the same species and if

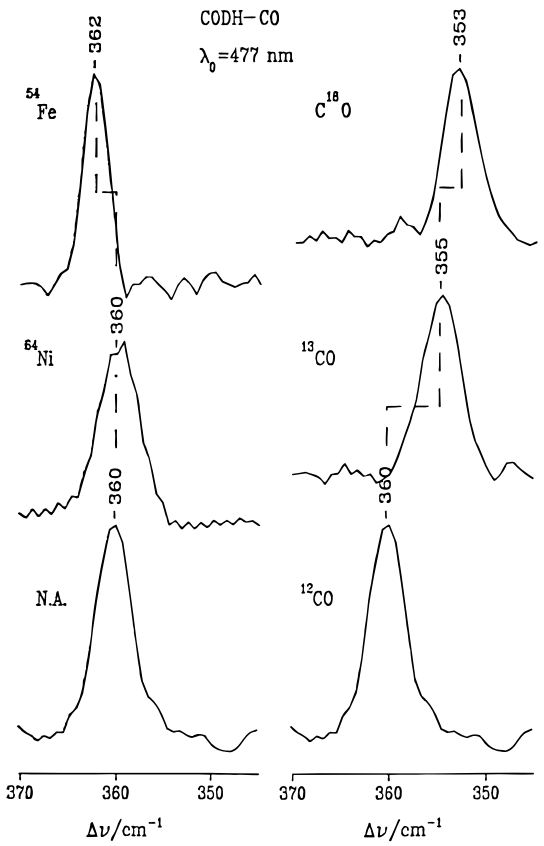


Figure 12. RR spectroscopic studies of isotopically substituted ACS. (From ref 172.)

they derive from catalytically important species or are just interesting artifacts. Kinetic studies have shown that the RR band and the NiFeC species form at similar rates, that they are formed fast enough to support acetyl-CoA synthesis,[80,91,165] and that the "carbon" in both species undergoes exchange with the carbonyl group of acetyl-CoA.[165,171,172] These experiments strongly indicate that the Fe−CO band is the RR signature of the adduct between CO and cluster A (the NiFeC species). The NiFeC EPR signal has been observed when CODH/ACS is reacted with acetyl-CoA (through a reversal of the pathway),[165] when the enzyme was reacted with CO_2 under reducing conditions,[84] and when CODH/ACS is reacted with pyruvate in the presence of pyruvate: ferredoxin oxidoreductase (Menon and Ragsdale, manuscript submitted). Additionally, the NiFeC EPR signal was modified by substrates (CoA and acetyl-CoA)[173] and inhibitors (*N*-bromosuccinimide)[174] of ACS. Besides appearing at kinetically competent rates, the NiFeC species was shown to disappear when the CO-reacted methanogenic CODH/ACS was treated with the methyl donor, methyltetrahydrometanopterin.[175] The combined results provide unambiguous evidence that the adduct between CO and cluster A involves an Fe−CO, not a Ni−CO, bond and that the carbonyl group serves as the precursor of the carbonyl moiety of acetyl-CoA.

$$\text{CODH-(Ni-Fe)-CO} + \text{CH}_3\text{-}^{13}\text{CO-SCoA} \rightarrow \text{CODH-(Ni-Fe)-}^{13}\text{CO} + \text{CH}_3\text{-CO-SCoA} \quad (11)$$

2. Methyl Group Transfer from the Methylated CFeSP to ACS

Since CO binds to iron, then what is the role of nickel? It seemed unlikely that Ni was a bystander in the catalytic mechanism. Ni had earlier been shown to be required for acetyl-CoA synthesis.[13,14,166] We proposed a bimetallic mechanism for acetyl-CoA synthesis that included the iron−carbonyl intermediate and postulated a methylnickel intermediate as the precursor of the methyl group of acetyl-CoA.[172]

Identification of the methyl-ACS intermediate was accomplished by a combination of stopped-flow kinetics and RR spectroscopy.[176] These experiments were technically difficult because they required high concentrations of the methylated CFeSP as the methyl group donor. First, the rate of the transmethylation reaction was measured by rapidly mixing the methylated CFeSP with CODH/ACS in the stopped-flow instrument and following the spectra of the reaction mixture (Figure 13). Since Co^{1+}, Co^{2+}, and methyl-Co^{3+} have distinct UV−visible spectra, the product of the reaction (Co^{1+}) could be unambiguously assigned. These studies demonstrated that the methyl transfer to Ni occurs by a heterolytic cleavage of the methyl−cobalt bond (discussed in more detail below). Reaction of the CO-treated methanogenic CODH/ACS with CH_3-H_4MSPT (methyltetrahydrosarcinapterin) resulted in disappearance of the NiFeC EPR signal consistent with formation of the acetyl−enzyme adduct.[175] This reaction was not observed to affect the EPR signals of the other metal centers in the protein. After pretreating the *Ms. barkeri* enzyme with CO and CH_3-H_4MSPT, acetyl-CoA synthesis activity increased by 44-fold.[175] Disappearance of this EPR signal also was observed when the methyl group was provided by methyl iodide in the presence of the CFeSP.[165] Under the defined conditions, methyl iodide generates CH_3-CFeSP, which serves as the direct methyl donor to CODH/ACS.[177]

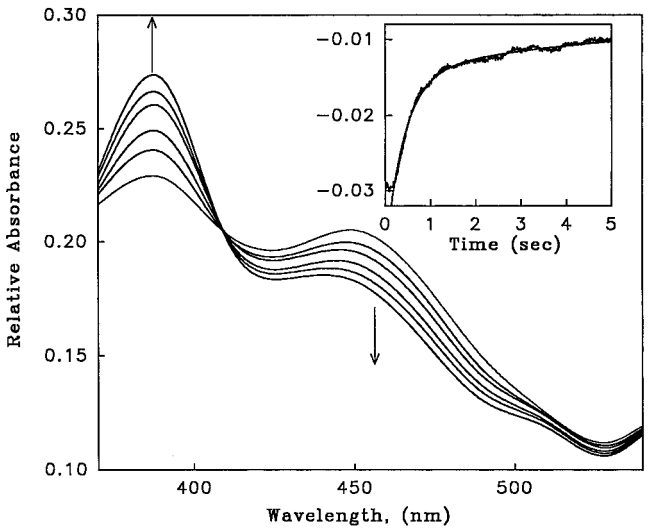


Figure 13. Reaction of reduced ACS with methylated corrinoid/Fe-SP. CODH/ACS was incubated with CO for 15 min and rapidly mixed with the methylated corrinoid/Fe-S protein. Spectra were obtained by collating individual kinetic traces. (Inset) Single-wavelength-monitored kinetics of the reaction of 10 μM ACS with 10 μM methylated corrinoid/Fe-S protein followed at 390 nm. (From ref 176.)

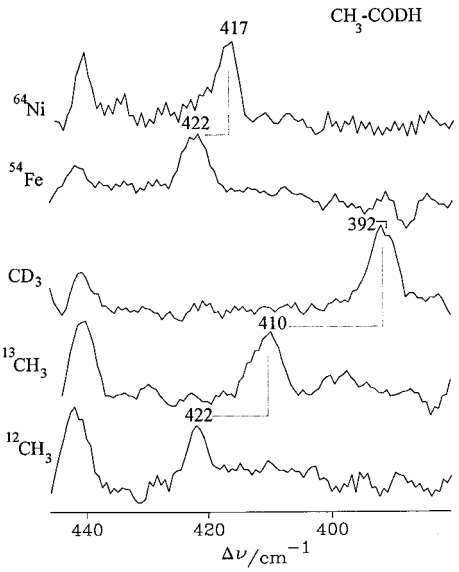


Figure 14. Identification of the methyl−Ni intermediate on ACS. RR spectra were collected of the indicated isotopomers of reduced ACS after reaction with methylated CFeSP (the CH_3-CFeSP spectrum was subtracted digitally).

With the rate of the methyl-transfer reaction established, we began to look for the methyl−metal bond on ACS by RR spectroscopy. After the RR spectrum of the Co−CH_3 bond of the methylcobamide substrate was assigned (ν_{Co-C} = 429 cm^{-1}), the methylated CFeSP (containing CH_3−, CD_3−, or $^{13}CH_3$−Co) was reacted with ACS (containing natural abundance metals, ^{54}Fe, ^{58}Fe, or ^{64}Ni). The reaction mixture was quenched at the appropriate time by freezing in liquid nitrogen, and RR spectra were measured (Figure 14). These studies identified a methyl−nickel adduct of ACS (ν_{Ni-C} = 422 cm^{-1}).[176] This was the first example of a nickel−carbon bond in nature. This work confirmed the previous proposal of a bimetallic mechanism for the final steps in acetyl-CoA synthesis, provided direct evidence for a new biological role of nickel, and established a unique organometallic mechanism of biological carbon−carbon bond formation. It also resolved a controversy over whether the methyl group bound to a cysteine residue[178] or to a metal center[177] on ACS.

3. Methyl Migration To Form Acetyl-CODH

After carbonylation and transmethylation, there is either a methyl migration or carbonyl insertion to form an acetyl-ACS intermediate. That there is an acetylated enzyme was first demonstrated by isolation of acetate as the exclusive product of the reaction of CH_3-ACS with CO in the absence of CoA[103] and as a product of the reaction of CH_3-CFeSP with CO and either purified CODH (Ragsdale, S. W., unpublished results described in ref 179) or a protein fraction containing CODH.[180] Kinetic studies also predict an acetyl-ACS intermediate because the rate of the CoA/acetyl-CoA exchange reaction is ~200-fold faster than that of the CO/acetyl-CoA exchange reaction, indicating that cleavage of the C−S bond of acetyl-CoA (that would form the acetyl intermediate) is faster than breakage of the C−C (acetyl) bond.[103,181] So far, the acetyl−metal intermediate has not been spectroscopically detected.

4. CoA Binding to ACS

Characterization of the mode of CoA binding to ACS (K_d ~ 7 μM) was reviewed recently.[1] EPR spectroscopic results indicated that CoA binds near or at cluster A and that arginine and tryptophan helps to position CoA in the active site.[23,174,182] Consistent with the role of the large subunit in acetyl-CoA synthesis, a peptide containing the active site tryptophan residue was located in this subunit.[183] Tryptophan and arginine residues directly upstream of a cysteine-rich region of the *M. thermophila* β subunit are conserved.[27] It has been suggested that CoA may serve as a ligand to cluster A.[184]

5. Carbon–Sulfur Bond Formation To Produce Acetyl-CoA

The final step in the mechanism involves the synthesis of the "high-energy" thioester bond of acetyl-CoA. Remarkably, this is accomplished by ACS without utilizing ATP or any other conventional phosphotransfer chemistry. The organisms then harnass the high energy of the thioester bond and synthesize ATP and acetate by the combined actions of phosphotransacetylase and acetate kinase. The reverse reaction is the first step in acetyl-CoA utilization by methanogens. In methanogens, this reaction has been clearly shown to require the β subunit of the enzyme complex.[40] The kinetics of thioester formation have been studied by following an exchange reaction between [^{3}H]CoA and acetyl-CoA.[103,179,181] One of these studies[103] demonstrated that optimal conditions include performing the reaction at low redox potentials. The specific activity of the exchange between CoA and the CoA moiety of acetyl-CoA at −520 mV and pH 7.0 at 55 °C is 200 μmol min^{-1} mg^{-1} (500 s^{-1}). Treatment of the kinetic data by a Nernst analysis revealed that the active site for this reaction has a midpoint potential of ≤−486 mV and requires a one-electron reductive activation.[103] After reductive activation of cluster A, there are two possible mechanisms of C−S bond formation. Possibly the CoA thiol could in one step attack and displace the bound acyl group in a standard S_N2 type reaction. The other possibility[184] is a two-step mechanism in which CoA first binds to cluster A and then performs reductive elimination of the acetylmetal species. In either case, after acetyl-CoA forms and dissociates from the enzyme, the active reduced form of ACS could reenter the catalytic cycle.

6. Inhibitors of Acetyl-CoA Synthesis

Several inhibitors of CO oxidation have been described; only one reversible inhibitor that appears to be specific for the ACS active site has been found, CS_2.[60] EPR spectroscopic and steady-state kinetic studies indicate that CS_2 interacts with cluster A of the *C. thermoaceticum* enzyme. In the isotope exchange reaction between acetyl-CoA and CO, CS_2 was found to be a competitive inhibitor with respect to CO (K_i = 0.47 mM) and a mixed inhibitor with respect to acetyl-CoA (K_{i1} = 0.30 and K_{i2} = 1.1 mM). The reaction of CODH with CS_2 resulted in an EPR

spectrum with *g* values of 2.200, 2.087, and 2.017, which is similar to that seen for the adduct between anions and cluster C (described above). EPR spectroelectrochemical titrations demonstrated that the CODH-CS_2 complex has three redox states and that the intermediate state is paramagnetic. A maximum of 0.3−0.4 spins/mol of CODH could be obtained. Fitting this data to the Nernst equation indicated that integral spin intensities could not be obtained because the reduction potentials for the two redox couples were isopotential (∼−455 mV). We suggested that similar redox chemistry may limit the spin intensity of the adduct between cluster A and CO. Although CS_2 did not bind to cluster C, it inhibited reactions that occur at cluster C. CS_2 was a noncompetitive inhibitor vs CO_2 in CO_2 reduction and vs CO in CO oxidation. Interestingly, CS_2 appears to have a different mode of reactivity with the CODH from *R. rubrum* and is a potent competitive (with respect to CO_2) inhibitor of CO_2 reduction exhibiting a K_i of 43 μM.[106]

Several irreversible inhibitors of ACS have been described. Mersalyl acid, which disrupts Fe-S clusters, strongly inhibits acetyl-CoA synthesis.[23] The tryptophan-specific modification reagent, *N*-bromosuccinimide, inhibits acetyl-CoA synthesis and also modifies the cluster A EPR signal.[174] Phenyl glyoxal, which modifies arginine residues, is another reagent that inhibits ACS, probably by disrupting the CoA binding site.[23]

C. Structural or Functional Models of the ACS Reaction

Organometallic chemistry is the foundation of ACS enzymology. Each reaction step is a separate chapter in a stimulating inorganic chemistry book with a table of contents that includes: metal−carbonyl, alkyl−metal, and acyl−metal bond formation, methyl group transfer between metals, metal-promoted carbonyl insertions or methyl migrations, metal−sulfur bond formation, and metal-catalyzed thioester formation. Nature wrote a separate book on the synthesis of catalytically active heterometallic clusters. Comprehending the catalytic, electronic, magnetic, and electrochemical properties of these clusters will require understanding these properties of heterometallic structural models and of the component metal ions in thiolate-rich coordination environments.

1. Monsanto Process

There are striking similarities between the mechanisms of biological acetyl-CoA synthesis and the Monsanto modification of the Reppe process (Figure 15) which synthesizes acetic acid from methanol and CO using a homogenous Rh catalyst $[RhI_2(CO)_2]^-$ and HI.[185,186] This industrial process generates approximately 10^6 tons of acetic acid per year world-wide. The reaction (eq 12) is performed at 180 °C and 30 bar and provides 99% yield of acetic acid.

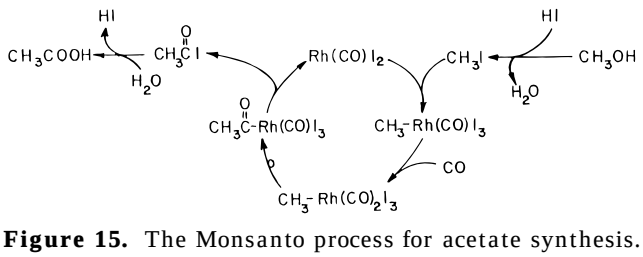


Figure 15. The Monsanto process for acetate synthesis.

$$CH_3OH + CO \rightarrow CH_3COOH \qquad (12)$$

Both the biological and industrial processes apparently involve metal−carbonyl, methyl−metal, and acyl−metal intermediates. The Monsanto process includes (i) conversion of methanol to methyl iodide, (ii) methylation of the active Rh complex by methyl iodide, (iii) CO insertion to form an acyl−Rh complex, (iv) cleavage of the acyl−Rh bond by iodide forming acetyl iodide, and (v) hydrolysis to form acetic acid. Iodide, which serves as an acyl acceptor from the acyl−Rh complex, is analogous to CoA in the enzymatic mechanism.

2. Fe and Ni Carbonyls

One of the first steps in both the CODH and ACS mechanisms is the binding of CO to an iron site in the active site metal cluster. A variety of iron−carbonyl complexes are known. Ferrous heme−CO adducts have been well studied; the CO complex with cytochrome oxidase is responsible for the extreme toxicity of CO. Many of the mononuclear Fe carbonyl complexes that have been studied contain nonbiological ligands like phosphine and cyclopentadienyl ligands $[Fe(CO)(P(OPh)_3)(n\text{-}C_5H_5)]$, $[Fe(CO)(PPh_3)(Cp)]$.[187] Iron−sulfur complexes containing CO and CH_3 moieties are also known.[188] An Fe(II) cysteine complex has been observed to take up CO to give $Fe(cys)_2(CO)_2$, which may be isolated in the acid form or as a sodium salt.[189] Addition of ^{13}CO to $[Fe_4S_4(SPh)_4]^{3-}$ was reported to generate a ^{13}C-sensitive EPR spectrum, accounting for 5−10% of the Fe.[190] Solutions prepared in this way contain a complex mixture of species.[191] Other iron thiolate complexes with CO include $[Fe(cp)(CO)_2(SR)_2]^+$, $[Fe(Cp)(CO)(SEt_2)(CH_3)]$, [(1,3-dithiane)$Fe(CO)_4$], and $[Fe(CO)_4(S(CH_2)_4)]$.[188] Reaction of 1-(methylthio)benzene-2-thiol in alkaline medium with Fe(II) and CO produced $[cis\text{-}Fe(CO)_2(CH_3S\text{-}C_6H_4\text{-}S)_2]$, which binds CO reversibly.[192] Sulfur-bridged Fe(II) complexes containing the tetradentate N_2S_2 donor ligand (N is *N*,*N*′-dimethyl-*N*,*N*′-bis(2-mercaptoethyl)ethylenediamine) react with CO reversibly to give $Fe(N_2S_2)(CO)_2$.[193] There is an example of intramolecular carbonyl insertion into a sulfur donor in an Fe complex that leads to the formation of derivitized ligand.[194]

There are many reports of nickel(I) and nickel(II) complexes that can bind CO.[195] When CO reacts with nickel complexes, pentacoordinate Ni^I−CO complexes are commonly formed that exhibit IR stretching frequencies over a wide range between 2026 and 1940 cm^{-1}. These values compare well with the 1995 cm^{-1} band for the Fe−CO adduct at cluster A in ACS.[171] It has been shown that binding of CO to the Ni^{II}−thiolate complexes [Ni(terpy)$(SC_6H_5)_2(CH_3CN)$] and [Ni(terpy)(*S*-2,4,6-*i*-$Pr_3C_6H_2$)] require a reducing agent that presumably generates Ni(I).[196−198] A $[Ni(tpttd)(CO)]^-$ (tpttd = 2,2,11,11-tetraphenyl-1,5,8,12-tetrathiadodecane) complex was found to be paramagnetic

and gave a CO stretching vibration at 1940 cm^{-1}. The Ni complex $[Ni(NS_3tBu)]^+$ reacts with CO to give $[Ni(NS_3tBu)CO]^+$, which was characterized by IR (the C−O stretch was at 2026 cm^{-1}) and EPR (*g* values were 2.119 and 2.008). There are also two reports of sulfur-rich mononuclear nickel complexes (NiS_4 and $NiS_2N(O)_2$ containing thiolate and thioether ligands) which bind to CO to give Ni−CO.[199,200]

3. Alkyl and Acyl Complexes with Ni and Fe

There are examples also of stable methyl−nickel complexes.[201] In one of the most exciting studies, Ram and Riordan have modeled the methyl transfer from the methylated CFeSP to ACS and provided the first example of alkyl group transfer from methyl−Co^{III} to Ni(I).[202] They reacted CH_3-Co(dmgBF_2)py with 2 equiv of $[Ni(tmc)]^+$ (tmc = 1,4,8,11-tetramethyl-1,4,8,1-tetraazacyclotetradecane and dmgBF_2 = (difluoroboryl)dimethylglyoximato) to produce 1 equiv of the methyl−nickel complex, $[Ni(tmc)Me]^+$, and 1 equiv of $[Ni(tmc)]^{2+}$. The proposed mechanism involves the following steps (eqs 13−15). One of the Ni^{1+} complexes undergoes sacrificial oxidation and the other one reacts with the methyl group.

$$Ni^{1+} + \text{methyl}-Co^{3+} \rightleftharpoons \text{methyl}-Co^{2+} + Ni^{2+} \quad \text{(electron transfer initiation)} \quad (13)$$

$$\text{methyl}-Co^{2+} \rightleftharpoons Co^{1+} + CH_3^{\bullet} \quad \text{(homolysis)} \quad (14)$$

$$Ni^{1+} + CH_3^{\bullet} \rightleftharpoons \text{methyl}-Ni^{2+} \quad \text{(radical capture)} \quad (15)$$

Recent studies using radical traps support the homolytic mechanism of methyl group transfer (Riordan, personal communication). Such studies suggest that the enzyme could use a similar mechanism since the methyl−Co^{2+} bond is weak; homolytic fission of methyl−Co^{2+} occurs at a rate of ~4400 s^{-1}.[203,204] As discussed below in section E, studies with the enzyme strongly indicate that the methyl group is removed by a heterolytic mechanism (eq 16):

$$Ni^{1+} + \text{methyl}-Co^{3+} \rightleftharpoons \text{methyl}-Ni^{3+} + Co^{1+} \quad (S_N2 \text{ displacement of the methyl group}) \quad (16)$$

Many Ni−CH_3 complexes have been shown to undergo migratory insertion reactions when a carbonyl group is also present.[195] A series of studies has been performed in Holm's laboratory that mimic the ACS reaction mechanism with a mononuclear nickel complex. A nickel complex containing the tripodal ligand NS_3R [$NS_3R = N(CH_2CH_2SR)_3$ (R= *i*-Pr, *t*-Bu)], was reacted with MeMgCl to form a methyl−nickel intermediate.[205] In contrast to the enzyme's mechanism, the methyl group is added as an anion. This CH_3−Ni intermediate reacts with CO to form an acyl−Ni(NS_3) complex. Reaction of Ni−acyl(NS_3-tbu)$^+$ complex in THF with thiol forms the thioester R′SCOMe (R = Et, CH_2Ph, Ph) in high yields. Although the system is not catalytic, chemical reactivities of these species provide credibility to the metal-centered reaction cycle of the enzyme. This reaction could have occurred either through an intermolecular nucleophilic attack of the thiol group at the nickel−acyl complex or through an intramolecular process involving a Ni−thiolate intermediate. With a second recent set of X-ray crystallographically characterized, planar nickel complexes, [Ni(bpy)-(CH_3)(SR)] (SR = *p*-$C_6H_4CH_3$, 2,6-$C_6H_3(CH_3)_2$, mesityl, 2,4,6,-C_6H_2(*i*-Pr)$_3$, and 2,6-$C_6H_3Cl_2$), it was shown clearly that thioester synthesis occurred by a nickel-mediated intramolecular process.[184] Ni(bpy)(CO)(CH_3)-(*S*-2,6,$C_6H_3Cl_2$) was generated with reacting an equivalent amount of CO. Further reaction of the acyl−Ni complex with CO leads to the quantitative formation of thioesters, establishing the Ni^{II}−acyl−thiolate complex as an intermediate in thioester formation. The reaction can be summarized by eq 17.

$$[Ni(bpy)Me(SR)] + 3CO \rightarrow RSCOMe + [N(bpy)(CO)_2] \quad (17)$$

There are other examples of methyl−nickel formation and the carbonylation of such complexes to form acyl−nickel. The acyl−nickel complexes are generally unstable and are labile in acidic solutions.[201] Reaction of $[Ni(MeS_2)]$ (MeS_2 = *o*-(methylthio)-thiophenolate^{1-}) with methyl−lithium yields a methyl−nickel complex $[NMe_4][NiCH_3(MeS_2)]$ that reacts with CO to form a labile Ni−acyl complex (ν_{CO} = 1602 cm^{-1}).[130] A Ni(I)−methyl adduct $[Ni(terpy)(SAr)_2(CH_3)]^{2+}$ (R = Me_2-C_6H_3, 2,4,6, I-Pr_3-C_6H_2) has been reported by Mascharak's group.[198] Another square-planar $[Ni(PS)_2]^{2+}$ (PS = $Ph_2PC_2H_4SEt$, $^1/_2Ph_2PC_2H_4SC_3H_6SC_2H_4PPh_2$) can be reduced by Na/Hg amalgam producing $[Ni^0(PS_2)]$, which reacts with methyl iodide forming $[Ni(PS_2)CH_3]^+$.[206] These Ni−methyl complexes react with CO at −60 °C to produce labile acyl complexes that lose CO at −30 °C and revert to the methyl complex. An octahedral Ni(III)−methyl species is generated by reaction of the Ni(I) complex, $[Ni(OEcBC)]^-$ (OEcBC = 2,3,7,8,12,13,17,18-octaethylisobacterochlorin), with methyl iodide.[207,208] [Ni(acac)(PMe_3)(COMe)X] (acac = acetylacetonate^{1-}) and $[Ni(PMe_3)_2X(COMe)]$ (X = halide) were prepared by the reaction of the corresponding methylated species with CO.[209,210]

In reactions reminiscent of the CO/acetyl-CoA exchange reaction catalyzed by ACS, the square-planar Ni^{II}−acyl dithiocarbamate complexes, Ni^{II}-(COR)($S_2CNR'_2$)(PMe_3) (R = Me_3SiCH_2, Me_ePhCCH_2; R′ = Me, Et, I-Pr) participate in CO exchange reactions with $[NiRCl(PMe_3)_2]$ complexes to give $[NiR(S_2CNR'_2)(PMe_3)]$ and $Ni(COR)Cl(PMe_3)_2]$.[211,212] Mononuclear nickel(bipyridyl)thiometallacyclopropane reacts with CO to give a cyclic thioester, i.e., γ-thiobutyrolactone.[213] In this reaction, the insertion of CO into the Ni−S or Ni−C bond generates thiolactone (thioester) by reductive elimination. We consider it a strong possibility that generation of acetyl−CoA occurs by a reductive elimination mechanism.

Sellmann et al. recently synthesized $[Ni(S_4C_3Me_2)]$ complexes ($S_4C_3Me_2$ = 1,3-bis[2-(mercaptophenyl)-thio]-2,2-dimethyl-2-propane^{2-}) (complex 1) that can be reduced by Na/Hg to yield a trinuclear complex.[214] Reaction of the trinuclear complex with ligands like pyridine and trimethylphosphine yielded a mono-

nuclear complex [Ni($S_2C_3Me_2$)(L)] (L = Py or PMe_3) ($S_2C_3Me_2$ = 1-[2-(mercaptophenyl)thio]-2,2-dimethylpropyl^{2-}) that reacts with CO to yield a cyclic thioester $S_2C_3Me_2CO$ (2,3-benzo-6,6-dimethyl-8-oxo-1,4 dithiacyclooctane) and $Ni(CO)_4$. When L was PMe_3, they could isolate and characterize the intermediate acyl−Ni(II) thiolato complex [Ni(S_2C_3-Me_2-CO)(PMe_3)]. The nickel center in this complex apparently functions to mediate the two-electron transfer reactions and to facilitate acyl group formation.[201]

$$\text{complex 1} \rightarrow \text{Ni}-\text{alkyl} \rightarrow \text{Ni}-\text{acyl} \rightarrow \text{complex 1} + \text{thioester} \quad (18)$$

net reaction:

$$S_4C_3Me_2H_2 + CO \rightarrow S_2C_3Me_2CO + S_2H_2 \quad (19)$$

There are also examples of methyl−iron complexes. Alkyliron(III) porphyrin complexes have been synthesized and characterized using NMR spectroscopy.[215] In the iron phosphine complex containing both a methyl and a carbonyl group, [(η^5-Cp)Fe($CO)_2$-Me], a migratory insertion reaction to form a Fe−acyl complex does not occur.[188]

4. Bimetallic Models of the ACS Active Site

Clusters A and C are both bimetallic clusters containing nickel and iron. There are a few relevant Ni/Fe−S heterobimetallic models. One example is a tetranuclear complex containing a square-planar [$Ni^{II}(N_2S_2)$] flanked by thiolate bridged square pyramidal Fe^{2+} ions, {[Ni(BME-DACO)$FeCl]_2(\mu Cl_2)$} (BME-DACO = *N*,*N*′-bis(2-mercaptoethyl)-1,5-diazacyclononane).[216] A similar example is a five-coordinate Fe(II) ion with two double and one single thiolate ion bridged to a square-planar Ni(II) center, [Ni(dmpn)$]_3Fe]^{2+}$ (dmpnH_2 = *N*,*N*′-dimethyl-*N*,*N*′-bis(2-mercaptoethyl)-1,3-diaminopropane).[217] No spectroscopic studies have been reported on these complexes for comparison with biological centers.

There are some relevant functional bimetallic models containing metals other than Ni and Fe. A heterobimetallic complex containing methyl−Zr and Mo−CO undergoes further carbonylation to form an acetyl complex, [$Cp_2Zr(C(O)Me(\mu$-$OC)_2Mo(CO)_2Cp$].[218] Reaction of (methyl)$Mn(CO)_5$ with a Fe−CO complex yields a heterobimetallic bridging complex, [Cp(CO)-Fe(μ-C(O)CH_3)(μ-PPh_2)$Mn(CO)_4$].[219] In this complex, the acetyl group bridges the Fe and Mn centers with the acetyl carbon attached to Fe and the oxygen bound to Mn. The synthesis, structure, and mechanism of formation of [Cp(CO)Fe(μ-C(O)-*p*-tolyl)(μ-CO)Mo(NO)Cp], containing a rare π-bound μ-acyl complex has been reported.[220]

5. Is a Bimetallic System Better in a Chemical Biomimetic?

The idea of using binuclear complexes for ligand activation has been recognized.[221,222] Understanding the properties of heterobimetallic catalysts would help explain how Ni and Fe cooperate in the various steps of acetyl-CoA synthesis. Both hetero- and homonuclear bimetallic complexes without metal−metal bonds are being studied.[223−225] In these complexes, two or more metals are bridged by a common ligand. The joint action of two metal centers could enhance the activation of an organic substrate by several mechanisms. Bimetallic systems can provide enhanced reaction rates, better selectivity, and higher turnover numbers and can offer enhanced stability and integrity during a reaction. The improved properties of the bimetallic systems are primarily due to the synergistic effect of the two metal centers, based on their electronic distribution and site specificity. A simple view is that a bimetallic system should be able to do at least twice as many things as a mononuclear one. This view is supported by studies of ligand substitution reactions. In addition, bimetallic systems exhibit "heterosite reactivity", e.g., the discrete properties of each metal site in a bridged complex can exhibit different reaction types or modes. For example, different phosphine ligands substitute CO on different metal atoms in $H_2FeRu_3(CO)_{13}$ depending on their size and basicity.[226] In the heterometallic $RuCo_2(CO)_{13}$ complex, Ru reacts with H_2 and Co reacts with acetylene.[227]

An example of catalytic enhancement by Lewis acid activation that may be relevant to CODH and ACS is provided by studies of the octahedral metal complex [$MeFeW(CO)_9^{2-}$].[228] A pronounced effect on a methyl/CO migratory insertion reaction was offered by the heterobimetallic $MeFeW(CO)_9^-$ relative to $MeFe(CO)_4^-$. The heterobimetallic system apparently furnishes an internal, readily available transition metal-based Lewis acid that accelerates the migratory insertion process by interacting at the acyl oxygen site. In the same study, although $MeFe(CO)_4^-$ did not react with CO_2, the bimetallic assembly readily complexed CO_2 to yield $MeC(O)OW(CO)_5^-$. Similarly, when the bimetallic complex was reacted with CS_2, a heterobimetallic μ-Me-$CS_2FeW(CO)_8^-$ was produced. The rate of hydroformylation catalyzed by a Zr−Rh heterobimetallic catalyst was enhanced apparently by Lewis acid activation of CO by Zr.[229] A series of complexes, $Cp_2M(\mu$-$PPh_2)_2M'$-(H)$(CO)PPh_3$, containing Zr and Rh have been shown to catalyze the hydroformylation reaction of 1-hexene at rates that are slower than the monometallic Rh complex but with higher product selectivity.[230] That the catalyst was quantitatively recovered after the reaction indicates that the heterobimetallic complex offers an integral catalytic system.

D. Why are Ni and Fe-S Used in the ACS Active Site?

Among transition metals of the first-row, nickel has a preferred ability to form organometallic complexes containing CO and alkyl groups.[231] This could be an important property since one role of nickel in cluster A is to accept and bind a methyl group from the CFeSP. A nickel site is better suited for acylation because of its likely planar geometry, possible coordinative unsaturativity, and its relatively stronger nucleophilic nature to accept CO by migratory insertion. Although an Fe site at Center A is the CO acceptor, it is possible that during the CO migratory insertion, the acyl group is formed at Ni and could include a transitory complex in which CO and the methyl group bind to the same metal.

It is clear that iron plays an important role in catalysis by ACS since a ferrous component of cluster

A binds CO. Another possible role for the Fe-S component of cluster A is to provide a rapid inner-sphere electron transport pathway to facilitate some of the reactions that could presumably occur at a mononuclear nickel complex. For example, the Holm model complexes are not catalytic because, at the end of the reaction sequence, Ni(0) forms, precipitates and is catalytically inactive. If a second redox-active metal had been present to act as an electron acceptor, possibly Ni(I) could have been formed to reenter the reaction cycle.

E. Comparison of the Models with the Enzyme

One major difference between the present models and ACS is the mechanism of methyl group transfer from methyl−Co to Ni. In the relevant model chemistry, the methyl group appears to undergo homolytic scission to leave as a methyl radical; in the enzyme, the methyl group appears to undergo nucleophilic displacement. There are several experiments that favor the heterolytic mechanism for the enzyme. The first argument is that chiral CHTD-H_4folate is converted to acetyl-CoA with retention of configuration.[232] Chiral acetyl-CoA also retains stereochemical integrity (retention) during the exchange reaction between CO and acetyl-CoA.[233] These experiments are consistent with one inversion of configuration associated with conversion of CH_3-H_4folate to methyl-CFeSP and another inversion occurring in the transfer of the methyl group to CODH. Clean inversion of configuration in a reaction is often used as an argument for a S_N2 displacement (heterolytic) mechanism since most radical reactions occur with scrambling of the stereochemistry. A second argument is that methyl−Co bound to the CFeSP appears to be protected from homolytic fission since a solution of methylated CFeSP (CH_3-Co^{3+}) does not undergo homolysis or conversion to CH_3-Co^{2+} even after extensive (over 4 h) incubation at −620 mV potential in an electrochemical cell.[234] Finke argued that homolysis of methyl−Co is avoided in enzyme reactions because the $CH_3-Co^{3+}/CH_3-Co^{2+}$ couple is too negative to be reached by biochemically relevant electron donors (<1.0 V vs NHE).[204] The third argument is based on stopped-flow studies of the methyl group transfer.[176] When CODH was rapidly reacted with the methylated CFeSP, Co^{1+} was generated at a rate that mirrored the rate of methyl−Co^{3+} decay. A mechanism involving homolysis of methyl−Co^{3+} followed by one electron reduction to Co^{1+} was clearly ruled out because the rate of Co^{2+} reduction was found to be ∼20-fold slower than the rate of Co^{1+} formation in the methyl transfer reaction.

Reactions of functional models of the ACS reaction, like the enzyme, involve metal−CO, metal−methyl, and metal−acyl intermediates that can undergo either intra- or intermolecular thioester formation. However, all the chemistry is shown to occur on the single nickel metal site, whereas, in the enzyme, a bimetallic mechanism has been established.

Study of the models has provided important insight into the ACS mechanism. In a typical organometallic acylation reaction, which is also shown by nickel model systems, the following features are observed: (a) groups undergoing insertion or migration are adjacent (*cis*) to each other in the coordination sphere of the metal, (b) a vacant coordination site is created for the forward reaction and is important to drive the reaction by the next incoming substrate or ligand, (c) the incoming ligand occupies a coordination site *cis* to the newly formed acyl group, (d) although alkyl migration and CO insertion have both observed, the former is more common. Regarding the mechanism of acyl formation, in a classic study, stereochemical analysis at the metal site could distinguish between methyl migration that will lead to inversion, vs CO insertion, resulting in retention.[235] Mononuclear nickel complexes can undergo intramolecular insertion to form acyl complexes and even thioesters. However, the reaction is not catalytic due to disintegration of the Ni complex itself.

F. Future Studies

Future mechanistic studies of CODH and ACS are expected to play a major role in understanding how organometallic catalysis takes place in biology. A major effort is underway to characterize the metal−C bond in the acyl-ACS complex. This will help clarify whether the carbonyl group inserts into a metal-Ni bond or if the methyl group migrates to an iron-carbonyl site. It is not understood how the extraordinary reaction between the methylated CFeSP and ACS occur. In this reaction, the enzyme-bound cobamide and the Ni−X−FeS cluster must interact to accomplish the methyl transfer reaction. It would be important to identify whether or not a metal−SCoA bond is an intermediate in acetyl-CoA synthesis. This experiment should establish whether thioester formation occurs by a reductive elimination mechanism.

CODH appears to be involved in generation of an electrochemical potential across the bacterial membrane to allow for CO-dependent ATP formation. How this occurs is not clear; for example, is CODH also a proton pump? It is clear that CODH releases protons and electrons. It will be important to determine at what stages in the catalytic cycle they are released and if they are coupled. Another bioenergetic principle that needs further elucidation is how binding energy is translated in to synthesis of the acetyl-CoA thioester bond.

A number of mechanistic questions could be answered by obtaining a three-dimensional structure of CODH and ACS. These studies are underway in several laboratories. A variety of questions involving the structural and functional importance of certain amino acids and metal clusters could then be answered and more focused questions could be posed. Future studies on the structure and function of CODH may rely on the availability of a genetic system to express site-directed mutants of CODH/ACS. At present, there is not a genetic system available for the acetogens although important strides are paving the way for the development of such a system.[236]

It is important to establish structural and functional models of the CODH and ACS reactions. The synthesis and study of heterobimetallic bridged complexes containing Ni and Fe-S clusters[237] will greatly enhance our understanding of this fascinating one-carbon, ancient chemistry.

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