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Enzymology of the Acetyl-CoA Pathway of CO_2 Fixation

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ABSTRACT: We know of three routes that organisms have evolved to synthesize complex organic molecules from CO_2: the Calvin cycle, the reverse tricarboxylic acid cycle, and the reductive acetyl-CoA pathway. This review describes the enzymatic steps involved in the acetyl-CoA pathway, also called the Wood pathway, which is the major mechanism of CO_2 fixation under anaerobic conditions. The acetyl-CoA pathway is also able to form acetyl-CoA from carbon monoxide.

There are two parts to the acetyl-CoA pathway: (1) reduction of CO_2 to methyltetrahydrofolate (methyl-H_4folate) and (2) synthesis of acetyl-CoA from methyl-H_4folate, a carboxyl donor such as CO or CO_2, and CoA. This pathway is unique in that the major intermediates are enzyme-bound and are often organometallic complexes. Our current understanding of the pathway is based on radioactive and stable isotope tracer studies, purification of the component enzymes (some extremely oxygen sensitive), and identification of the enzyme-bound intermediates by chromatographic, spectroscopic, and electrochemical techniques. This review describes the remarkable series of enzymatic steps involved in acetyl-CoA formation by this pathway that is a key component of the global carbon cycle.

KEY WORDS: acetyl-CoA, CO_2 fixation, Wood pathway, acetogenic bacteria, acetyl-CoA pathway.

I. INTRODUCTION

Our planet requires a continual source of fixed carbon since heterotrophic beings like ourselves remove organic carbon and produce energy by oxidizing the organic carbon to CO_2. Reconversion of CO_2 to organic carbon occurs by a reductive process called CO_2 fixation. Organisms are defined as autotrophs if they obtain all or virtually all of their cellular carbon from CO_2.[1-3] This implies that the requirement for small quantities of some vitamins or cofactors that are recycled does not disqualify an organism from the autotrophic category. The energy for reductive CO_2 fixation comes from light for photoautotrophs and from chemical energy for chemoautotrophs. Understanding the pathways of CO_2 fixation by autotrophic organisms is important since these processes constitute our energy and carbon sources.

Three mechanisms of CO_2 fixation have been discovered. The Calvin cycle, which is the best known of these, is used by most plants and photosynthetic bacteria. Elucidated by Calvin and co-workers in the 1950s and 1960s, this reaction sequence is included in all biochemical texts. A different autotrophic mechanism has been developed by some phototrophic green sulfur bacteria in which they reverse the tricarboxylic acid cycle so that CO_2 is incorporated into, instead of released from, the cycle.[4-8] Most autotrophic anaerobic bacteria have evolved a third process of CO_2 fixation that is quite distinct from the other two pathways and is called the reductive acetyl-CO_2 pathway. Although organisms using these three pathways all perform reductive CO_2 fixation, there is little or no similarity between them, implying that there is no common evolutionary ancestor. Based on the patterns of isotopic fractionation between ^{12}C and ^{13}C, the sedimentary

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carbon record indicates that autotrophic growth developed soon after the Earth became habitable, approximately 3.8 billion years ago.[9] Anaerobic organisms using the reductive acetyl-CoA path have an isotope fractionation pattern that suggests that they may have been the first autotrophs, using inorganic compounds such as CO and H_2 as an energy source and CO_2 as an electron acceptor approximately 1 billion years before O_2 appeared.[10]

Conceptually, the simplest way to form an organic molecule is to construct it one carbon at a time. Though simple in theory, the condensation of two one-carbon units to form the two-carbon compound, acetate, has intrigued chemists, biochemists, and microbiologists since 1945, when some of the first biochemical experiments using radioactive isotope tracer methods were performed.[11] However, the enzymes and intermediates in this pathway were like a puzzle, recalcitrant of resolution and characterization for a number of years. The major pieces in the puzzle have been discovered only recently. Elucidation of the mechanism of acetyl-CoA synthesis has required a number of different biochemical techniques, including enzyme kinetics, electrochemistry, and spectroscopy. The enzymology of the anaerobic acetyl-CoA pathway of CO and CO_2 fixation is the subject of this review.

The reductive formation of acetyl-CoA from CO_2 has been called the acetyl-CoA pathway, the Wood pathway,[12] and the Ljungdahl-Wood pathway.[13] Anyone reviewing this pathway must pay an enormous debt for the contributions made by Harland G. Wood, who began working on acetate synthesis by acetogenic bacteria in 1952, and Lars G. Lungdahl, who began as a student of Wood in 1958.

Organisms that generate acetic acid as their sole or major end product in the fermentation of organic substrates or respiration on H_2/CO_2 or CO are called acetogenic bacteria. Acetogens that can synthesize acetic acid from CO_2 and H_2 (Equation 1) were first detected in 1932.[15]

$$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O$$
$$\Delta G'_o = -94.9 \text{ kJ/mol}^{14} \quad (1)$$

In 1951, it was suggested that the mechanism of acetate synthesis could constitute a previously unrecognized mechanism of autotrophic CO_2 fixation.[16] The results of labeling suspensions of whole cell of *Clostridium thermoaceticum* suspensions with $^{14}CO_2$ ruled out sugar phosphate intermediates and thus the Calvin cycle as the mechanism of CO_2 fixation.[17] In addition, the extent and pattern of labeling was inconsistent with that expected for the reductive citric acid cycle.[17] Therefore, autotrophic growth by *C. thermoaceticum* apparently was due to a novel pathway. It has since been recognized that acetyl-CoA is the precursor of acetate, so Equation 2 also represents the autotrophic pathway.

$$4H_2 + 2CO_2 + CoASH \rightarrow CH_3\text{–}CO\text{-}SCoA + 3H_2O + H^+ \Delta G'_o = -59.2 \text{ kJ/mol}^{14} \quad (2)$$

$$CH_3\text{–}CO\text{–}SCoA \rightarrow \text{cell carbon} \quad (3)$$

The acetyl-CoA pathway has been most thoroughly dissected in the acetogenic bacterium, *C. thermoaceticum*, as, until recently, there were only a few acetogenic bacteria known. In addition, since this organism is a thermophile, most of its enzymes are more stable to purification at room temperature than those of many of its acetogenic mesophilic counterparts. Over the last approximately 10 years, many acetogenic bacteria have been discovered.[12] It has recently become apparent that other anaerobes, such as methanogenic and sulfate-reducing bacteria, form cell carbon from CO_2 by the acetyl-CoA pathway (via Equations 2 and 3). Also, the enzymes of this pathway mediate acetyl-CoA oxidation by sulfate-reducing bacteria (Equation 4), acetate catabolism by methanogenic bacteria (Equation 5), and CO oxidation to CO_2 by many anaerobes (Equation 6).

$$CH_3CO\text{–}SCoA + SO_4 + H^+ \rightarrow H_2O + 2CO_2 + H_2S + CoASH \quad (4)$$

$$CH_3COOH \rightarrow CH_4 + CO_2$$
$$\Delta G'_o = -36.1 \text{ kJ/mol}^{18} \quad (5)$$

$$H_2O + CO \rightarrow CO_2 + H_2$$
$$\Delta G'_o = -20.1 \text{ kJ/mol}^{14} \quad (6)$$

The CO oxidation activity allows many organisms using the acetyl-CoA pathway to grow on the toxic gas, CO, as sole carbon and electron source (Equation 7).

$$2CO + 2H_2 \rightarrow CH_3COOH$$
$$\Delta G'_o = -135 \text{ kJ/mol} \qquad (7)$$

Growth on either H_2/CO_2 (Equation 1) or CO (Equation 7) is extremely exergonic and allows for the synthesis of ATP via both substrate- level and electron transport-linked ATP synthesis. This pathway occurs in many anaerobic environments, including marine and fresh water sediments; in the soil; and in landfills and waste treatment sites. Acetogenic bacteria are also common in the rumen of cows, horses, and sheep, and in the hindgut of termites and the large intestine of humans.[19-21]

Before discussing how nature manufactures acetic acid, it is interesting to consider one catalytic mechanism used by industrial chemists to synthesize acetate and to compare the similarities between the two catalytic systems. The industrial process is called the "Monsanto process" (Figure 1) in which methanol and CO are the reactants for acetate synthesis.[22,23] Instead of enzymes, a metal complex containing rhodium acts as the catalyst. After conversion of methanol to methyl iodide, the rhodium center is methylated and then carbonylated. Then a methyl migration is proposed to occur, forming an acetyl-rhodium intermediate that is cleaved by iodide to form acetyliodide, regenerating the catalyst. Finally, hydrolysis of acetyliodide yields acetate and hydrogen iodide. Industrial chemists chose iodide; nature selected a thiol compound, coenzyme A, as an acyl group acceptor.

In the Monsanto process, the chemistry occurs at a rhodium center. There is evidence that bacteria assemble and synthesize acetyl-CoA at an iron/nickel-containing metal complex that is a component of a protein, carbon monoxide dehydrogenase (CODH), found in all organisms that can perform anaerobic CO_2 fixation.

II. CHARACTERIZATION OF THE ENZYMES INVOLVED IN ACETYL-COA SYNTHESIS

A. General Aspects of the Acetyl-CoA Pathway

In the acetyl-CoA pathway, which is outlined in Figure 2, 2 mol of either CO or CO_2 and CoA are converted to acetyl-CoA. This pathway has been reviewed previously.[12,14,24-27] Acetyl-CoA serves as a precursor of the cell's macromolecules as well as a source of ATP. Enzymatic cleavage of the high-energy thioester bond to form acetate is coupled to ATP synthesis via the phosphotransacetylase and acetate kinase reactions. This pathway, then, is a mechanism for converting CO_2 or the toxic gas, carbon monoxide, into acetate and into cell material. Overall, this is an irreversible noncyclic pathway that can be divided into three cyclic steps. First, 1 mol of CO_2 or CO is reduced to the oxidation state of a methyl group. This set of reactions involved H_4folate-dependent enzymes and the product of this

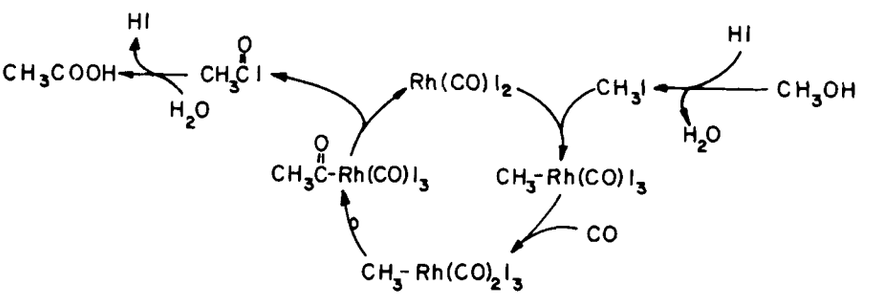


FIGURE 1. The Monsanto process for the industrial synthesis of acetic acid. (Modified from Reference 23.)

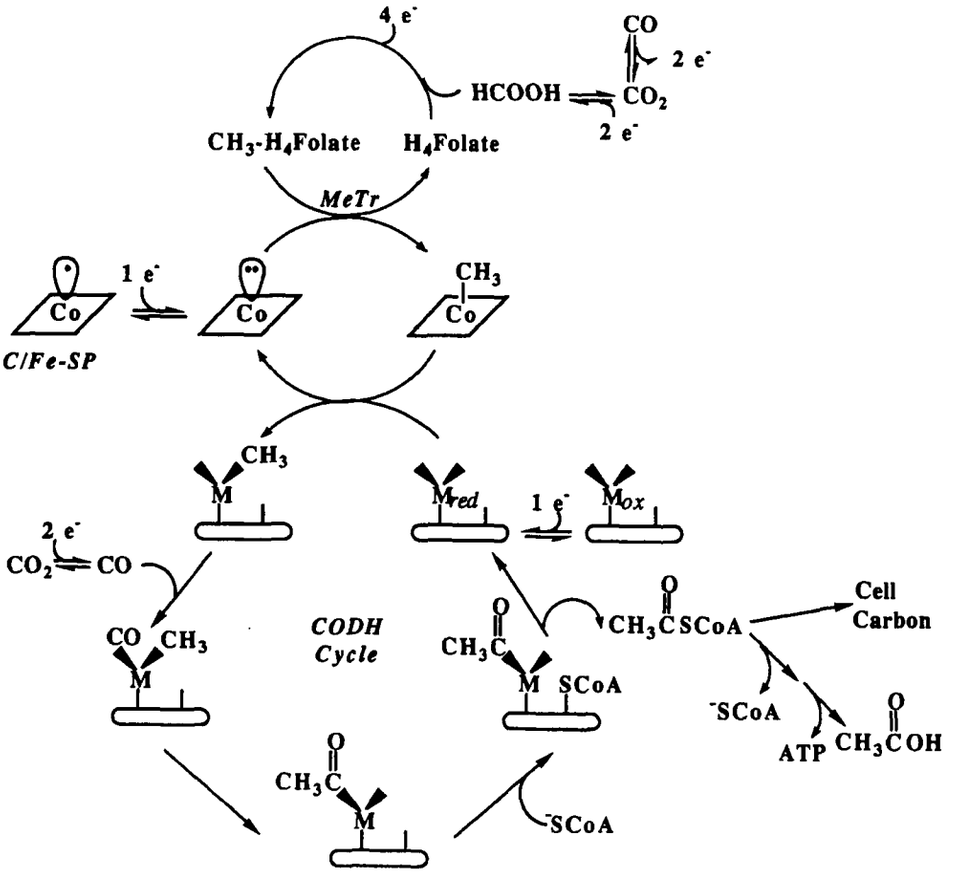


FIGURE 2. Scheme of the pathway of acetyl-CoA synthesis. The square with Co in the center represents the corrinoid ring of the C/Fe-SP; the elongated oval represents CODH; M designates a redox active metal center involved in the assembly of the acetyl group of acetyl-CoA.

action sequence is CH_3- H_4folate (Equation 8). The rest of the pathway involves the synthesis of acetyl-CoA from CH_3- H_4folate, CO, and CoA (Equation 9).

$$H^+ + CO_2 + 3H_2 + H_4\text{folate} + ATP \rightarrow CH_3\text{–}H_4\text{folate} + 2H_2O + ADP + PO_4^{3-} \quad (8)$$

$$CH_3\text{–}H_4\text{folate} + CO_2 + H_2 + CoA \rightarrow H_2O + CH_3\text{–CO-SCoA} + H_4\text{folate} + H^+ \quad (9a)$$

$$CH_3\text{–}H_4\text{folate} + CO + CoA \rightarrow CH_3\text{–CO-SCoA} + H_4\text{folate} \quad (9b)$$

These reactions consist of one- and two-carbon transformations that are unique in that they occur as enzyme-bound intermediates. Conventional methods of following isotope labeling patterns from CO_2 and characterizing the products, therefore, could not be used to identify the intermediates in the acetyl-CoA pathway. In the second step of the pathway, the methyl group of CH_3-H_4folate is transferred to a corrinoid containing protein. In the final steps of the pathway, acetyl-CoA is assembled from the precursor methyl, carbonyl, and CoA moieties. The assembly and the formation of the high-energy thioester bond of acetyl-CoA occurs on CODH.

The design of the acetyl-CoA pathway is quite simple in principle in that it involves the synthesis of a two-carbon compound from one-carbon precursors. The use of acetyl-CoA as the two-carbon

compound appears to be a wise choice since it is a source of ATP and an important metabolic precursor for all cells.

The purification and manipulation of most of the enzymes involved in the acetyl-CoA pathway requires a special environment since most of the enzymes are poisoned by very low levels of oxygen. Anaerobic chambers that are commonly used maintain the oxygen level below 1 ppm. The bacteria are grown and harvested anaerobically and then every step in the purification and manipulation of the enzymes must be performed under strictly anaerobic conditions in order to maintain activity. Methods for preparing anaerobic buffers and description of one style of anaerobic chamber have been described.[28]

The specific activity of acetate synthesis in growing cells of *C. thermoaceticum* is ~115 nmol min^{-1} mg cell $protein^{-1}$.[14] Therefore, the rates of individual steps in the synthesis must occur at least this rapidly. For example, catalysis of a partial reaction in the overall synthesis by a purified protein that is present at a level of 10% of total cell protein should occur with a specific activity of at least ~1.2 μmol min^{-1} mg $protein^{-1}$. A lower specific activity could indicate that the reaction under investigation in kinetically incompetent (an artifact), is being measured under nonoptimal conditions, or that an essential rate-enhancing component is lacking. One criticism of previous studies is that *in vitro* rates of acetyl-CoA synthesis were much lower than *in vivo* acetate synthesis.[14] As described below, we have optimized conditions and now obtain rates using purified enzymes that approximate the *in vivo* rate.

B. The First Step of the Pathway: H_4Folate-Dependent Synthesis of CH_3-H_4Folate

The rate of acetate synthesis *in vivo* is ~115 nmol min^{-1} mg cell protein.$^{-1}$.[14] The lowest activity measured in cell extracts of *C. thermoaceticum* is that of 5,10-methylene-H_4folate reductase, which is 580 nmol min^{-1} mg^{-1}, measured in the reverse direction.[29] Evaluation of whether any of these reactions could constitute rate-limiting steps in the pathway awaits the performance of experiments in the physiological direction. Kinetic data are not available for the forward reactions involving formate dehydrogenase, cyclohydrolase, and the 5,10-methylene-H_4folate reductase.

The first step in the synthesis of acetyl-CoA is the synthesis of CH_3-H_4folate from CO_2 via formate dehydrogenase and a series of H_4folate-dependent enzymes. Since this work has been reviewed previously,[27] this part of the pathway is only covered briefly. A review[30] and a three-volume book[31] on the mechanism of folate-dependent enzymes are also available. In 1966, H_4folate and its derivatives were first postulated to be involved in acetate biosynthesis,[32] based on the demonstration that corrinoids were involved in acetate biosynthesis and corrinoids had been shown to be involved in methionine biosynthesis. In this latter reaction, the methyl group of CH_3-H_4folate is transferred to a corrinoid, forming methylcobamide as an intermediate in the formation of methionine from homocysteine. Subsequently, CH_3-H_4folate was detected as an intermediate in acetate biosynthesis by isolating $^{14}CH_3$-H_4folate from whole cells that had been pulse labeled with $^{14}CO_2$[33] and by demonstrating that the methyl of CH_3-H_4folate is converted to acetate by cell extracts.[34] It was postulated that CO_2 is converted to HCOOH and then to CH_3-H_4folate via a series of H_4folate-dependent reactions.[32,35] The enzymes catalyzing reduction of formate to CH_3-H_4folate had been studied previously in *Clostridium cylindrosporum* and *C. acidi-urici* by Rabinowitz and coworkers.[36]

The biologically active forms of H_4folate and its derivatives have the *6S* configuration.[37,38] H_4folate and H_4folate-dependent enzymes analogous to those involved in the acetyl-CoA pathway play key roles in one-carbon transfers for a number of essential cell functions (synthesis of serine, thymidylate, purines, and methionine) in all cells; however, since these enzymes are involved in a key catabolic pathway in acetogenic bacteria, they are found at levels 1000-fold higher and the purified enzymes have approximately 100-fold higher specific activity than in other organisms.

1. Formate Dehydrogenase

The first reaction in the conversion of CO_2

to CH_3-H_4folate is the two-electron reduction of CO_2 to HCOOH, catalyzed by a tungsten-containing NADP-dependent formate dehydrogenase (FDH) (EC 1.2.1.43) in *C. thermoaceticum* (Equation 10).[12,28]

$$NADPH + CO_2 + H^+ \rightarrow NADP^+ + HCOOH$$

$$\Delta G'_o = +21.5 \text{ kJ/mol}^{14} \quad (10a)$$

$$H_2 + CO_2 \rightarrow HCOOH$$

$$\Delta G'_o = +3.4 \text{ kJ/mol}^{14} \quad (10b)$$

The midpoint reduction potential for the CO_2/HCOOH couple is -420 mV vs. the standard hydrogen electrode (SHE).[39] The rate of this reaction in the physiological direction (CO_2 reduction) has not been determined; however, oxidation of formate by NADP occurs at a rate of 0.5 to 5 and 1050 μmol min^{-1} mg $protein^{-1}$ by the cell extract and purified enzyme, respectively. Rich in metals, the FDH from *C. thermoaceticum* is an $\alpha_2\beta_2$ enzyme (M_r = 340,000) containing, per $\alpha\beta$ dimer, 1 tungsten, 1 selenium, and ~18 iron and ~25 inorganic acid-labile sulfides in the form of iron-sulfur clusters.[12] The tungsten is apparently part of a tungstopterin prosthetic group, like the molybdopterin found in xanthine oxidase, sulfite oxidase, and nitrate reductase[40] and the Mo-containing FDHs of other organisms, including *M. formicicum*.[41,42] The function of the molybdopterin in the methanogenic enzyme or of tungstopterin in the *C. thermoaceticum* enzyme has not been defined, but an involvement in hydride transfer from NADPH to Mo(VI) forming Mo(IV)-H as has been shown for xan- *M. formicicum* enzyme.[41,42] CO_2, rather than bicarbonate, was shown to be the substrate for several FDHs.[43-45] Since CO and HCOOH are at the same oxidation level, it is interesting that FDH is required even when CO is the substrate for growth.[46] Thus, CODH oxidizes CO to CO_2 and FDH reduces CO_2 to HCOOH (Equation 11a, b).

$$CO + H_2O \rightarrow 2e^- + 2H^+ + CO_2 \quad (11a)$$

$$2e^- + 2H^+ + CO_2 \rightarrow HCOOH \quad (11b)$$

2. 10-Formyl-H_4Folate Synthetase

Formate is activated via an ATP-dependent condensation with H_4folate by formyl-H_4folate synthetase (EC 6.3.4.3.), forming 10-formyl-H_4folate (Equation 12).

$$HCOOH + ATP + H_4\text{folate} \rightarrow 10\text{-HCO-}H_4\text{folate} + ADP + P_i$$

$$\Delta G'_o = -8.4 \text{ kJ/mol}^{31} \quad (12)$$

This enzyme has been purified, kinetically characterized, and sequenced from a number of sources, including *C. thermoaceticum* (see Reference 47 and citations within). The *C. thermoaceticum* enzyme is oxygen-stable and has been expressed in active form in *E. coli*.[48] In higher organisms, this enzyme exists as one of the activities of a trifunctional C_1-H_4folate synthase,[49,50] whereas the bacterial enzymes are monofunctional with similar α_4 stoichiometry (monomer M_r = 60,000), turnover rates, Michaelis constants for substrates, kinetic mechanisms, and amino acid compositions. Kinetic and equilibrium exchange studies indicate a random sequential mechanism (see Reference 51 and citations therein). Formation of 10-formyl-H_4folate by the synthetase from *C. thermoaceticum* occurs at a rate of 7 to 18 and 254 μmol min^{-1} mg $protein^{-1}$ for the cell extract and purified enzyme, respectively.[28,52] Monovalent cations activate the enzymes from *C. thermoaceticum* and *C. cylindrosporum*[53] significantly decreasing the K_m for formate.[52] In the presence of K^+, the K_m-values for formate and Mg-ATP are 5 and 0.1 m*M*, respectively. Formyl phosphate appears to be an intermediate, indicating that the synthetase has a "formate kinase" activity, as was shown with the *C. cylindrosporum* enzyme by presteady state kinetics, trapping the intermediate, and utilization of formyl phosphate as the precursor of the formyl group of formyl-H_4folate.[54] A related activity of the synthetase is its ability to synthesize ATP and carbamate from ADP and the formyl phosphate analogue, carbamyl phosphate.[55]

3. 5,10-Methenyl-H_4Folate Cyclohydrolase and 5,10-Methylene-H_4Folate Dehydrogenase

The next two steps in the Ljungdahl-Wood pathway are catalyzed by 5,10-methenyl-H_4folate cyclohydrolase (Equation 13) and 5,10-methylene-H_4folate dehydrogenase (Equation 14).

10-formyl-H_4folate + H^+

$\rightarrow$ 5,10-methenyl-H_4folate + H_2O

$\Delta G'_o = -35.3$ kJ/mol[31] (13)

$2e^- + 2H^+$ + 5,10-methenyl-H_4folate

$\rightleftarrows$ 5,10-methylene-H_4folate

$\Delta G'_o = -4.9$ kJ/mol with NAD(P)H[31],

-23.0 kJ/mol with H_2 (14)

In *C. thermoaceticum* and in most other prokaryotes, these enzymes are part of a bifunctional protein. In eukaryotes, these activities are generally part of a trifunctional C_1-synthase also containing the 10-formyl-H_4folate synthetase. The enzymes from the acetogens, *C. formicoaceticum*[56] and *Acetobacterium woodii*,[57] are monofunctional.

Thermodynamically, the cyclohydrolase (EC 3.5.4.9.) reaction strongly favors cyclization, the equilibrium constant for the conversion of 10-formyl-H_4folate to 5,10-methenyl-H_4folate measured in D_2O at 25°C to be 1.4×10^6 M^{-1}.[58] The reverse reaction, hydrolysis of 5,10-methenyl-H_4folate occurs in *C. formicoaceticum* at a rate of 2.8 and 470 μmol min^{-1} mg $protein^{-1}$ in cell extracts and for the purified enzyme, respectively. The K_m for 5,10-methenyl-H_4folate is 0.19 mM. In the trifunctional C_1-synthase of chicken liver, the cyclohydrolase and dehydrogenase activities appear to share a single folate binding site[59,60] and both NADP and 5,10-methylene-H_4folate inhibit the cyclohydrolase activity.[61] In contrast, for the NAD-dependent bifunctional dehydrogenase/cyclohydrolase of Ehrlich ascites tumor cells, these two sites apparently are kinetically independent.[62] Due to the large favorable equilibrium and the fairly rapid nonenzymatic acid-catalyzed formation of 5,10-methenyl-H_4folate, the requirement for synthesizing this enzyme at high levels is intriguing.

Following the cyclization, 5,10-methylene-H_4folate dehydrogenase (EC 1.5.1.5, NADP; EC 1.5.1.15, NAD) catalyzes the NAD(P)H-dependent reduction to form 5,10-methylene-H_4folate (Equation 14). The E'_o for the methenyl-/methylene-H_4folate redox couple is -295 mV vs. the standard hydrogen electrode (SHE).[31] The dehydrogenase has been isolated from a number of sources and has been observed to be monofunctional only in *C. formicoaceticum*[56] and *A. woodii*.[57] Only the 5,10-methylene-H_4folate dehydrogenases from Ehrlich ascites tumor cells[62] and these two bacterial sources have been found to be dependent on NAD^+ instead of $NADP^+$. The equilibrium constant for this reaction, as written in Equation 14, has been measured to be from 0.13 to 59.[57,63] In most cases, the reaction has been probed only in the direction of formation of 5,10-methenyl-H_4folate, which is the reverse of the physiological reaction. However, the enzyme from *A. woodii* catalyzes both the oxidation of 5,10-methylene-H_4folate and the reduction of 5,10-methenyl-H_4folate with specific activities of ~1700 μmol min^{-1} mg $protein^{-1}$.[57] In cell extracts, the specific activity is ~10 μmol min^{-1} mg $protein^{-1}$.[53] With the *C. thermoaceticum* dehydrogenase, oxidation of 5,10-methylene-H_4folate by $NADP^+$ occurs according to a ternary complex mechanism with a specific activity of 720 μmol min^{-1} mg $protein^{-1}$ at 60°C and K_m values for $NADP^+$ and 5,10-methylene-H_4folate of 30 and 60 μM, respectively.[212] Recently, the NADP-dependent dehydrogenase from the acetogen, *Peptostreptococcus productus*, was shown to use a ternary complex mechanism.[64]

4. 5,10-Methylene-H_4Folate Reductase

The final step in the H_4folate part of the acetyl-CoA pathway is the reduction of 5,10-methylene-H_4folate to 5-CH_3-H_4folate, catalyzed by 5,10-methylene-H_4folate reductase (EC 1.1.99.15) (Equation 15).

$$2e^- + H^+ + \text{5,10-methylene-H}_4\text{folate} \rightleftarrows \text{5-methyl-H}_4\text{folate}$$

$\Delta G'_o = -39.2$ kJ/mol with NAD(P)H[31], -57.3 kJ/mol with H_2 as electron donor (15)

The standard reduction potential for the methylene-H_4 folate/CH_3- H_4folate couple is -130 mV vs. SHE.[65] In acetogenic bacteria, the enzyme has been purified from *C. formicoaceticum*[66] and *C. thermoaceticum*[29] and in both cases was found to be an oxygen-sensitive enzyme containing an iron-sulfur cluster, zinc, and FAD.[66] The gene encoding the reductase from *C. thermoaceticum* has been sequenced recently.[213] In contrast to the eukaryotic enzyme (below), the acetogenic reductase utilizes reduced ferredoxin and $FADH_2$ in the reduction of 5,10-methylene-H_4folate, and pyridine nucleotides were ineffective electron carriers for the reaction in either direction.[66] The activity of this enzyme in the physiological direction has not been reported, but oxidation of CH_3-H_4folate occurs with a specific activity of 337 (35°C) and 290 (55°C) μmol min^{-1} mg $protein^{-1}$ for the *C. formicoaceticum* and *C. thermoaceticum* enzymes, respectively.[29,66] In the cell extract, the *C. thermoaceticum* enzyme has a specific activity of 0.58 μmol min^{-1} mg $protein^{-1}$. The reactions catalyzed by both acetogenic reductases apparently proceed via a ping-pong mechanism.[29,66]

Mechanistic studies of 5,10-methylene-H_4folate reductase have been performed with the FAD-containing enzyme from pig liver, an allosteric enzyme whose activity is decreased 50,000-fold in the presence of *S*-adenosyl-L-methionine. A review of the pig liver reductase is available.[67] The reaction couples the oxidation of NADPH to the reduction of 5,10-methylene-tetrahydrofolate to 5-CH_3-H_4folate via a ping-pong Bi Bi mechanism. The first half reaction, reduction of FAD by NADPH, is rate limiting during steady-state turnover.[68] The *pro-S* hydrogen is stereospecifically removed from NADPH and transferred to FAD, forming $FADH_2$.[69] Then, $NADP^+$ is released before 5,10-methylene-H_4folate binds.[68] In the second half reaction, reduction of 5,10-methylene-H_4folate by bound $FADH_2$ to form CH_3-H_4folate, hydrogen from $FADH_2$ is exchanged with solvent.[70] It is proposed that the imidazolium ring of 5,10-methylene-H_4folate opens to form an iminium cation followed by tautomerization.[67] Intermediacy of the iminium species is deduced from analyses of the mechanism of the nonenzymatic formation of 5,10-methylene-H_4folate from formaldehyde and H_4folate[71] and by sodium borohydride reduction of an intermediate in the reaction.[72] The rate-limiting step in the second half reaction apparently is hydrogen transfer from solvent to 5,10-methylene-H_4folate[70] in which the hydrogen is sterospecifically added to the more sterically accessible face of the pteridine.[73]

C. General Aspects of the Formation of Acetyl-CoA from CH_3-H_4Folate, CO, and CoA

Following the formation of CH_3-H_4folate, the second and third steps involve the formation of acetyl-CoA from CH_3-H_4folate, CO, and CoA. The scheme for acetyl-CoA synthesis shown in Figure 2 has been criticized because the *in vitro* synthesis of acetyl-CoA from CH_3-H_4folate, CoA, and either pyruvate or CO was slower than *in vivo* acetate synthesis. Therefore, critics argued that an essential component in the reaction sequence still needed to be identified. However, we have found that no proteins other than methyltransferase (MeTr), the corrinoid/iron-sulfur protein (C/Fe-SP), CODH, and the electron transfer protein, ferredoxin (Fd), are required to account for the *in vivo* rates of acetate synthesis. This reaction is sensitive to pH, ionic strength, and the relative levels of each of the proteins.[214] When these four homogenous proteins are incubated with CH_3-H_4folate, CO, and CoA under optimal conditions, acetyl-CoA is synthesized at a rate as fast as that at which cells can make acetic acid.[214]

There are two discrepancies in the literature regarding the requirement of additional proteins for the synthesis of acetyl-CoA from CH_3-H_4folate, CO, and CoA. In both cases, the postulated roles of the proteins are to interact with or activate CODH. A CODH disulfide reductase

was proposed to reduce key disulfide bonds in CODH, resulting in stimulation of the exchange reaction between CoA and acetyl-CoA and the synthesis of acetyl-CoA.[74] The second incongruity is a report that CODH contains a third subunit (the disulfide reductase was considered since its molecular weight is equivalent to that seen as the "third subunit"), which under some conditions dissociates during the purification of CODH.[75] This report states that purification of a form of CODH that retains the third subunit yields a more physiologically relevant "three-subunit" enzyme rather than the two-subunit form that had been studied previously.[75] Removal of the disulfide reductase is accomplished routinely by molecular exclusion chromatography.[76] The two-subunit form of CODH lacking the disulfide reductase has been studied in various laboratories since 1983.[46] However, we have demonstrated that *all* reactions known to be catalyzed by CODH are associated with the two-subunit enzyme.[77,78] These reactions included acetyl-CoA synthesis from CO and CoA with methyl-CODH or methylated C/Fe-SP as methyl donor, methylation of CODH, the CoA/acetyl-CoA exchange, exchange reactions between methylated CODH and either acetyl-CoA or the methylated C/Fe-SP, the CO/acetyl-CoA exchange, and CO oxidation.[77,78] Occurring at rapid rates, these reactions proceed at higher specific activities when the disulfide reductase is removed. In addition, the rates of acetyl-CoA synthesis from CH_3-H_4folate, CO, and CoA observed under optimal conditions with homogeneous proteins (including the standard two-subunit form of CODH) are at least as fast as the *in vivo* rate of acetate synthesis.[214] Thus, it seems clear that claims that CODH is a "three-subunit" enzyme are unjustified. If there is a role for the disulfide reductase *in vivo*, it may be to couple the reductive activation of CODH to the oxidation of NADPH. However, even when NADPH, disulfide reductase, and CODH were present, the observed rates of the exchange reaction between CoA and acetyl-CoA were ~14,000-fold slower[74] than the rates determined under optimal conditions in the absence of the disulfide reductase and NADPH.[78] In addition, none of the reactions just described have been observed to require activation by the purified disulfide reductase.

The overall free energy change for the synthesis of CH_3-H_4folate from CO_2, ATP, and H_4folate (Equation 8) can be calculated from the values given above to be -120.6 kJ/mol with H_2 and -66.3 kJ/mol with NAD(P)H as electron donor. Thus, one can also calculate the free energy change for the synthesis of acetyl-CoA from CH_3-H_4folate, CO_2, H_2, and CoA (Equation 9a) to be $+30.1$ kJ/mol.

The remainder of this review concentrates on defining the enzymology of the synthesis of acetyl-CoA from CH_3-H_4folate, CO, and CoA (Equation 9b), a reaction sequence involving enzyme-bound organometallic intermediates. Using the $\Delta G'_o$ values given above, one can calculate that this reaction is mildly endergonic with a free energy change of $+9.3$ kJ/mol.

D. The Second Step: Methylation of C/Fe-SP

MeTr catalyzes the second step in acetyl-CoA synthesis, which is the transfer of the methyl group of CH_3-H_4folate to the cobalt center of a separate protein, the C/Fe-SP (Equation 16).

$$\text{Cob(I)amide} + \text{CH}_3\text{–H}_4\text{folate} \rightleftarrows \text{CH}_3\text{–Cob(III)amide} + \text{H}_4\text{folate} \quad (16)$$

This is the first step in the series of enzyme-bound intermediates in the pathway. This reaction is similar to the first step in the reaction mechanism of cobalamin-dependent methionine synthase.

1. History of the Discovery of the Role of Corrinoids in Acetyl-CoA Synthesis

The first hint of the role of corrinoids in acetyl-CoA synthesis came in 1964 when Poston et al.[79] showed that intrinsic factor, an inhibitor of vitamin B_{12}-dependent reactions, inhibits acetate synthesis and that the methyl group of methyl-B_{12} could be incorporated into C-2 of acetate. Figure 3 depicts the structure of the corrinoid found in the corrinoid protein involved in acetyl-CoA synthesis. Corrinoids contain a tetrapyrrolic

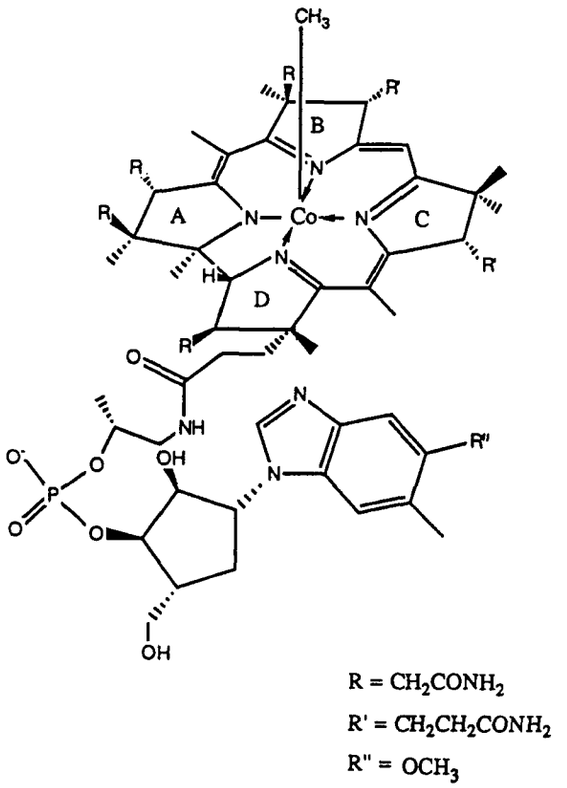


FIGURE 3. Structure of a base-off corrinoid in the methyl-Co^{3+} form.

corrin ring with a cobalt nucleus, giving a nearly planar Co-N_4 ligand environment. In biochemical reactions, cobalt can exist in the 1+, 2+, and 3+ oxidation states. Chemistry of the cobalt ion is profoundly influenced by the axial ligands. A nitrogen atom from benzimidazole with electron-releasing properties can ligate to the lower axial position of cobalt. The upper axial ligand is a methyl group in the case of methylcobalamin, adenosine for coenzyme B_{12}, and cyanide for cyanocobalamin or vitamin B_{12}. A two-volume text on corrinoid biochemistry and chemistry has been published.[80]

Once it was known that corrinoids were important in acetyl-CoA synthesis, an exploration of their role began. Vitamin B_{12} derivatives had been shown to be directly involved in the synthesis of methionine[81] as a prosthetic group in methionine synthase[82] and the methyl group of CH_3-H_4folate had been found to serve as the precursor of the S-methyl group of methionine.[83] Methyl-B_{12} had been detected as an intermediate in methionine biosynthesis.[84] *C. thermoaceticum* is a rich source of corrinoids, containing over 20 different types that total 300 to 700 nmol/g of frozen cells.[32] The levels of corrinoids and their structural diversity in anaerobic bacteria have been reviewed recently.[85] When cells of *C. thermoaceticum* were pulse-labeled with $^{14}CO_2$, $^{14}CH_3$-labeled corrinoids were isolated.[86] When the methylated corrinoids were added to cell extracts, pyruvate, and CoA, ^{14}C-labeled acetate was formed.[86] Although these experiments utilized free cobamide cofactors, it was likely that the *in vivo* catalyst was a corrinoid containing protein; therefore, efforts concentrated on isolating such an enzyme. A corrinoid protein (M_r = 27 kDa) was isolated from *C. thermoaceticum*, which accounted for 50% of the 5-methoxybenzimidazolylcobamide.[87] Based on the evidence that this was a major form of cobamide that was radiolabeled by $^{14}CO_2$ pulse labeling,[86] this protein was postulated to be involved in acetate synthesis.[87] However, it was not until 1984 that a corrinoid protein that was active in the synthesis of acetate was isolated. Hu *et al.*[88] partially purified an 88-kDa corrinoid protein, showed that it has an $\alpha\beta$ structure consisting of 55 and 33 kDa subunits, and demonstrated that the protein accepts the methyl group of CH_3-H_4folate, forming a methylcorrinoid species.[88] When the methylated corrinoid protein was incubated with CO, CoA, and a protein fraction containing CODH activity, the methyl group was incorporated into acetyl-CoA.[88] This 88-kDa protein was then purified to homogeneity and, when it was shown to be the first example of a corrinoid protein to contain an iron-sulfur cluster, was named the corrinoid/iron-sulfur protein (C/Fe-SP).[89]

2. Recent Studies of Methylation of the C/Fe-SP

We have used electron paramagnetic resonance (EPR) spectroscopy and electrochemical methods to probe the mechanism of catalysis of the C/Fe-SP, since much of the biochemistry in the Wood pathway occurs at metal centers. Definition of the oxidation state of a paramagnetic metal center and identification of the ligands that bind the metal center can often be accomplished

by EPR spectroscopy. Several general texts on EPR spectroscopy and reviews on the uses of EPR in biochemistry (e.g., References 90 to 92) and a review that focuses on EPR spectroscopy of corrinoids[93] are available.

a. Reductive Activation of the C/Fe-SP

As shown in Figure 2, transfer of the methyl group of CH_3-H_4folate to the cobamide site of the C/Fe-SP involves a nucleophilic attack of the reduced (Co^{1+}) cobamide on the electrophilic methyl group, and the 2+ and 3+ oxidation states of cobalt are inactive.[89,94] However, by quantitating the concentration of Co^{2+} by EPR, the only EPR active from of cobalt, we showed that all the cobamide in the isolated protein from *C. thermoaceticum* is in the inactive Co^{2+} form.[94] Thus, the C/Fe-SP requires a reductive of Co^{2+} to Co^{1+}. Reduction of Co^{2+} in vitamin B_{12} and in other cobamides requires a very strong reductant at a reduction potential of about −610 mV.[95-97] This potential is thought to be much lower than the ambient potential inside anaerobic cells and is near the potential at which water is electrolyzed to H_2 and O_2; thus, generation of electrons at this low a potential would be unlikely. An important question is how can *C. thermoaceticum* accomplish this strongly endergonic reaction? It appears that *C. thermoaceticum* has taken advantage of two mechanisms to circumvent the problem of a very difficult reduction (see Reference 98 for review).

The first mechanism was to evolve a way to increase the reduction potential of the Co center into a physiologically relevant range. This was shown by an EPR spectroelectrochemical study in which the C/Fe-SP was reduced electrochemically and the extent of reduction was monitored by EPR spectroscopy.[94] Since Co^{2+} is the only EPR active form of cobalt, reduction of Co^{2+} to Co^{1+} was quantitated by measuring the intensity of the EPR signal. A quantitative treatment of the EPR spectroelectrochemical data by the Nernst equation (Equation 17)

$$E = E_o' + (59/n)\{\log([ox]/[red])\} \quad (17)$$

yields a midpoint redox potential of −504 mV vs. SHE and a slope of 60 mV, indicating a one-electron process.[94] Comparison of this value with that of other corrinoids reveals that the Co^{2+} center of the C/Fe-SP is approximately 60-fold easier to reduce than in other corrinoids. This is important since redox potentials in the range of −500 mV are considered to be in the physiological range for anaerobic bacteria. There are a number of electron donors and carriers with midpoint potentials in this range. In addition, we have shown that CODH can reduce the Co^{2+} center of the C/Fe-SP with CO in a reaction that is stimulated by ferredoxin.[89]

How was the redox potential of the Co center increased? We have found the apparent answer to this question by characterizing the coordination state of cobalt. The upper EPR spectrum (Figure 4) is of the C/Fe-SP and reveals a major structural difference between the cobamide in the C/Fe-SP and other corrinoids and corrinoid-containing proteins that had been observed in the past. The lower EPR spectrum is that of the 27 kDa corrinoid protein from *C. thermoaceticum* and is typical of other cobalamins and cobamides. The reason for the unusual spectrum of the C/Fe-SP relates to the cobalt coordination state. When the nitrogen of the benzimidazole base is ligated to the lower axial position of the cobalt center (see Figure 3), there is an interaction between the nuclear spin of nitrogen (I = 1) and the electron spin of Co^{2+}, causing a three-line splitting of each peak in the EPR spectrum. At low pH values, the benzimidazole nitrogen is protonated (pK_a = 2.9)[96] and a single line replaces the triplet in the high field region of the EPR spectrum. The equilibrium between these two states is depicted in Figure 5. In fact, the EPR spectrum of the C/Fe-SP is typical of corrinoids at pH 2 to 3 and demonstrates that the benzimidazole base, although clearly present in the structure of the cobamide,[89] is not coordinated to cobalt in this protein. The importance of this unusual coordination state is appreciated by considering studies of model cobalt complexes. The $Co^{2+/1+}$ couple for "base-off" cobamide was predicted to be −500 mV,[96] close to the value determined for the Co center in the C/Fe-SP. Therefore, control of the coordination chemistry around the axial position can alone account for the 60-fold greater ease of reduction of the Co

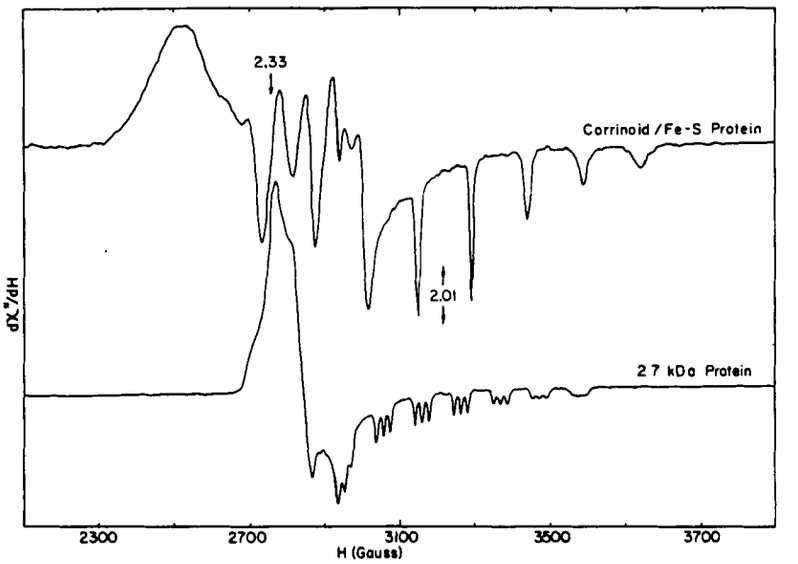
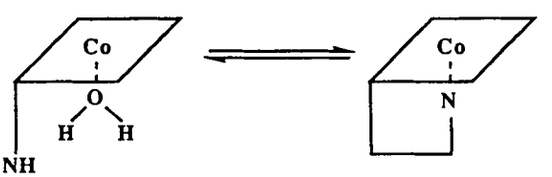


FIGURE 4. EPR spectra of the as-isolated corrinoid proteins. Both the C/Fe-SP and 27 kDa corrinoid protein were in 50 m*M* Tris/HCl, pH 7.6. EPR conditions: temperature, 100 K; field set, 3100 G; gain, 4 × 10^3; power, 10 mW; scan rate, 100 G/min; and frequency, 9.038 GHz. (From Ragsdale, S. W. et al., *J. Biol. Chem.*, 262, 14289, 1987. With permission.)



Base-off Cobalamin **Base-on Cobalamin**

FIGURE 5. Base-off/base-on equlibria of the cobamide center.

center of the C/Fe-SP than expected for a corrinoid. It is felt that when the electron-releasing benzimidazole nitrogen is coordinated to Co^{2+}, addition of another electron to form Co^{1+} is less favorable than when a weaker and less electron-releasing ligand is present. Facilitation of the reduction of the cobalt center of a corrinoid protein involved in methanogenesis is also apparently accomplished, altering the coordination state of the cobalt center.[215] An extreme modification of the nucleotide moiety was observed with the corrinoid from the acetogen, *Sporomusa ovata*, in which *p*-cresolylcobamide and *p*-phenolylcobamide replace the benzimidazole base.[99,100] These aromatic bases cannot coordinate to cobalt; however, it was found that in a corrinoid protein from *S. ovata*, a histidine ligand from the protein ligates to Co^{2+}, giving EPR spectroscopic properties identical to those of the benzimidazolylcobamides.[101] Purification and characterization of a corrinoid protein containing a noncoordinating phenolic or cresolic base are expected to yield further information on the chemistry and physiological role of the nucleotide base.

b. Methylation of the C/Fe-SP by CH_3-H_4Folate

Once Co^{1+} is formed, it reacts with CH_3-H_4folate, forming H_4folate and methyl-cobamide. Methylation of the C/Fe-SP requires another protein, MeTr, which has been purified to homogeneity[102] and its gene has been cloned[103] and sequenced.[216] The intermediacy of Co^{1+} in the reaction of the C/Fe-SP with CH_3-H_4folate has been demonstrated by experiments in which methyl-Co^{3+} was shown to be the product of the reaction of Co^{1+} with CH_3-H_4folate.[89] More re-

cently, we have established the kinetic competence of Co^{1+} in a stopped-flow investigation that demonstrates that methyl-Co^{3+} is formed at a rapid rate of $\sim 200\ s^{-1}$ by reaction of Co^{1+} with CH_3-H_4folate and that the kinetic profile of Co^{1+} decay is paralleled by the appearance of CH_3-Co^{3+}.[217] Methylation of the cobalt center of the C/Fe-SP is similar to the first step of the reaction catalyzed by methionine synthase in which rapid reaction kinetic experiments have established the kinetic competence of Co^{1+}.[104]

Co^{1+} is a powerful nucleophile;[105,106] however, the methyl group of *N*-5-CH_3-H_4folate adjacent to a tertiary amine, is not electrophilic. Displacement of this methyl group, therefore, would be expected to require an activation step through a quaternization or oxidation of the amine (see Reference 98 for a discussion). Presumably, one of the functions of MeTr is to facilitate this activation.

Methylation of the C/Fe-SP occurs at redox potentials even more positive than -505 mV, the value measured by EPR spectroelectrochemistry of the $Co^{2+/1+}$ couple in the absence of substrates, because the reduction is coupled to the exergonic reaction of Co^{1+} with CH_3-H_4folate. When methyl iodide is the methyl donor, the apparent midpoint potential for the $Co^{2+/1+}$ couple of the C/Fe-SP is much more positive than the actual E_o.[77] In a related study, it was found that the apparent E_o' of the $Co^{2+/1+}$ couple of methionine synthase was increased by 80 mV by the addition of CH_3-H_4folate, indicating that it was $\sim$20-fold easier to reduce Co^{2+} in the presence of CH_3-H_4folate than in its absence.[107] In the presence of *S*-adenosyl-methionine, which contains an electrophilic methyl group bound to a sulfonium ion, the coupled reduction-methylation reaction becomes irreversible, favoring the reduction by a factor of at least 3×10^7.[107] Interpretation of reactions involving an electron transfer step followed by a chemical or binding step(s) is not straightforward unless the thermodynamics and kinetics of both the redox and the subsequent steps are well characterized.[108] Application of this theory to CODH has been considered in detail.[78] If the reduced form of a redox center has higher affinity for a ligand or is more catalytically active relative to the oxidized species, the apparent midpoint potential of the redox reaction is shifted toward a positive value since the effect of the coupled reaction is to lower the concentration of the reduced species relative to the inactive oxidized species. The opposite effect will be observed if the oxidized form of the enzyme binds the ligand. Examples of this shift in midpoint potential have been well studied.[109,110] The magnitude of the shift depends on how exergonic the coupled reaction is and how rapidly the coupled reaction occurs relative to the electron transfer reaction.

In summary, *C. thermoaceticum* has two mechanisms of favoring the methylation of the C/Fe-SP. First, control of the cobalt coordination chemistry decreases the thermodynamic barrier for reduction of Co^{2+} to form active Co^{1+} by approximately 60-fold. This is likely an evolutionary response that permits Co^{1+} to exist at equilibrium in the cellular milieu. Second, the coupled methylation of Co^{1+} by CH_3-H_4folate decreases the apparent thermodynamic barrier by approximately 20-fold.

Unexpectedly, the methylcobamide product of the methyl transfer reaction is in the "base-off" conformation,[89] unusual at pH values above 3. Analysis of the reactivity of model compounds indicates that the base-off form is more susceptible to nucleophilic attack by $\sim$4.2 kcal/mol,[111,112] presumably because approach of a nucleophile is less hindered in the *base-off* conformation.[113] In addition, in the "base-on" conformation, the electron donating character of benzimidazole would decrease the electrophilicity of the coordinated methyl group. Base-off methylcobamides have been found to be protected against homolytic fission.[114-116] The quantum yield for cleavage of the Co-C bond of base-off coenzyme B_{12} was recently shown to be five-fold lower than that of base-on coenzyme-B_{12}.[117] We have proposed that the next step in the pathway of acetyl-CoA synthesis occurs by a nucleophilic attack on methylcobamide by CODH.[77,94] Thus, the methyl group of base-off methylcobamide, protected against radical attack

and activated for nucleophilic displacement, appears to be poised for the next reaction in the pathway, methylation of CODH.

c. Structure and Function of the Iron-Sulfur Cluster

The $[4Fe\text{-}4S]^{2+/1+}$ cluster of the C/Fe-SP appears to be important in the methyl transfer reactions.[89] The redox potential of this cluster is -523 mV,[94] very similar to that of the $Co^{2+/1+}$ couple (see below). Removal of the cluster cripples methyl transfer reactions; however, it has no effect on the redox potential[94] or the EPR spectra of the cobamide.[89] Similarly, removal of the cobamide does not affect the redox potential of the cluster.[94] Thus, it is likely that the cluster is not located extremely close to the cobalt center. The amino acid sequences of the two subunits of the C/Fe-SP have been determined based on the DNA sequence[218] of the cloned genes.[103] The locations of the cysteinyl ligands to the iron sites in the cluster have been tentatively assigned to the N terminal region of the 55 kDa subunit of the C/Fe-SP based on comparison of the sequence to that of other [4Fe-4S] containing proteins.[218] The location of the cobamide binding site has not been deduced.

E. The Third Step: Assembly of Acetyl-CoA by CODH

Step three of the Wood pathway consists of a series of reactions involving the remarkable nickel/iron-sulfur protein, CODH. The enzyme from *C. thermoaceticum* has an $(\alpha\beta)_3$ structure with subunits of 77 and 71 kDa,[46] whose amino acid sequences have been deduced from the sequences of the cloned genes.[118]

1. History of the Discovery of the Role of CODH in Acetyl-CoA Synthesis

One role for CODH, as indicated by its name, is to oxidize CO to CO_2. CO is used as a carbon and electron source for a number of bacteria,[3,119-121] including acetogenic bacteria such as *C. thermoaceticum*.[122,123] When it was discovered that *C. thermoaceticum* contains CODH and that alkyl halides inhibit both the oxidation of CO and the reduction of CO_2 to acetate, a role for CODH in acetate synthesis was suggested.[124] The role was speculated to be enzymatic reduction of an electron carrier or enzyme prosthetic group involved in CO_2 reduction to acetate.[124] Then a protein fraction containing CODH was discovered to be one of the components involved in the synthesis of acetate from CH_3-H_4folate and either CO or pyruvate.[102,125] This protein fraction was later shown to include the C/Fe-SP and hydrogenase. Demonstration that the CODH-containing protein fraction catalyzed an intriguing exchange reaction between CO and the carbonyl group of acetyl-CoA indicated that CODH binds a formate oxidation-level C_1 intermediate ([HCOOH]) that could serve as the precursor of the carbonyl of acetyl-CoA.[125] It was suggested that the same [HCOOH] intermediate was formed during the synthesis of acetyl-CoA from CH_3-H_4folate, pyruvate, and CoA via coupling of CODH with pyruvate ferredoxin oxidoreductase.[102] Further evidence for a CODH-bound C_1 intermediate was provided by isolation of a $^{14}C_1$-CODH complex from [1-^{14}C]pyruvate.[126]

If CODH-[HCOOH] was the precursor of the carbonyl group of acetyl-CoA, how was the acetyl group of acetyl-CoA formed? It had been assumed since corrinoids had been first discovered to play a role in acetate synthesis that the formation of acetate occurred at the cobalt center. *C. thermoaceticum* extracts convert carboxymethylcobalamin to acetate, and pulse-labeling experiments with $^{14}CO_2$ generated a labeled corrinoid that, when photolyzed, yielded the photolysis products of carboxymethylcobalamin.[86] In addition, alkylcobamides have been termed biological Grignard reagents[127] and a mechanism involving attack of the methyl carbanion on a carboxy group forming an acetoxycorrinoid had been considered.[128] The latter mechanism was supported by an experiment using the deuterated substrates, CH_3-H_4folate and $[CD_3]$-methylcobalamin in which over 50% of the acetate formed had a trideuteromethyl group.[128] Formation of an acetyl-cobalamin product via a radical mechanism was suggested when acetyl-cobalamin was

formed by photolysis of methylcobalamin at elevated levels (31 atm) of CO.[129] It was found that when the methylated C/Fe-SP was incubated with CO (i.e., in the absence of CoA), a low level of acetate formed, indicating that there was an intermediate acetyl group in the pathway that could be hydrolyzed to acetate, but in the presence of CoA was converted to acetyl-CoA.[88] Thus, it was thought that the assembly of acetate occurred on a corrinoid protein and it was controversial whether the synthesis occurred from an acetylcobalt, acetoxycobalt, or a carboxymethylcobalt intermediate.

Before 1985, it was thought that the only activities of CODH were (1) CO oxidation and (2) formation of a C_1 intermediate that became the carboxyl of acetate. When Wood and Ragsdale found that the purified CODH from *C. thermoaceticum* per se catalyzes an exchange reaction between CO and the carbonyl group of acetyl-CoA,[76] they postulated that the assembly of acetyl-CoA occurs not on the C/Fe-SP but on CODH. Table 1 shows that elution of ^{63}Ni, CO oxidation, and CO/acetyl-CoA exchange activities all occurred simultaneously during the purification of CODH to homogeneity.[76] During this exchange, the methyl-carbonyl and carbonyl-SCoA bonds must be broken and since there are no acceptors in the reaction mixture other than CODH, the results indicated that CODH must bind the methyl, carbonyl, and SCoA groups in the resynthesis of acetyl-CoA. Thus, it was proposed that the synthesis and assembly of acetyl-CoA occur on CODH, that the role of the C/Fe-SP was to donate the methyl group to CODH, and that a more appropriate name for CODH is *acetyl-CoA synthase*.[76] An indication of the general view at the time this postulate was offered can be gained by considering a concurrent study of cyanide inhibition of acetate synthesis by whole cells of *C. thermoaceticum*, in which the only role for CODH in acetyl-CoA synthesis was proposed to be CO oxidation/CO_2 reduction.[130] I have found only one reference in the literature to the possible role of CODH in assembly of acetyl-CoA prior to 1985. This is in the form of a question raised by Hu et al.[88] in 1984, "Is the C_2 intermediate formed on the nickel of the CO dehydrogenase . . . or on the corrinoid protein itself or elsewhere?". The discovery that CODH was the acetyl-CoA synthase in the pathway[76] was revolutionary; however, it was still subject to criticism because the methyl-CODH, CODH-CO, and CODH-SCoA intermediates had not been detected and are relatively unusual species (see, for example, Reference 14). Indeed, we have launched a major effort to detect, isolate, and characterize intermediates involved in the pathway. In summary, it now appears that the assembly of acetyl-CoA does indeed occur on CODH and that much of

TABLE 1
Copurification of the CO Oxidation, CO/Acetyl-CoA Exchange, and ^{63}Ni Activities[a]

	CO → CO_2 (U[b]/mg)	Exchange (mU[c]/mg)	^{63}Ni (cpm/mg × 10^{-3})
Cell extract	6.6	nd	3.5
DEAE-Sephacel	55	25	17
Phenyl-Sepharose	130	nd	43
Bio-Gel HTP hydroxylapatite	290	115	95
Bio-Gel A-0.5 m gel filtration	330	160	100
Agarose-CoA	390	170	118

[a] Modified from Reference 76.
[b] 1 unit = 2 μmol MV reduced min^{-1}.
[c] 1 mU = 1 nmol CO exchanged with 1 nmol [1-^{14}C]acetyl-CoA.

the assembly process occurs on metal centers and involves organometallic bonds, as shown in Figure 2.

2. Properties of the Metal Centers in CODH

Since metal centers play a key role in the assembly of acetyl-CoA, it is important to characterize the metal sites in CODH. The role of nickel in CODH has been reviewed.[131] Stimulation of the CODH activity in *C. pasteurianum*, *C. thermoaceticum*, and *C. formicoaceticum* by addition of nickel to the medium first suggested that CODH was a nickel-containing enzyme.[132,133] Stronger evidence was provided when CODH activity and radioactivity were shown to comigate in polyacrylamide gels of cell extracts of *C. thermoaceticum* grown in the presence of ^{63}Ni.[134] Then, the *C. thermoaceticum* enzyme was purified to homogeneity and shown to contain ~2 nickels per αβ dimer.[118] Simultaneously, CODH from *C. thermoaceticum* was purified to near homogeneity by Diekert and Ritter[135] and shown to contain nickel. Involvement of nickel in formation of an organometallic bond to CO was shown by EPR spectroscopy (discussed below).

One of the four known nickel-containing proteins thus far discovered in nature, CODH, contains 1.7 ± 0.2 nickels, 11 ± 2 irons, 1 ± 1 zinc, and 14 acid-labile inorganic sulfides per αβ dimer.[46] The role of zinc has not been investigated. Inquiry into the structure and possible roles of the iron and nickel complexes in catalysis has revealed that at least one nickel and three irons are within a single cluster that appears to be involved in the assembly of acetyl-CoA. This is fully discussed below. Since this review focuses on the enzymology of acetyl-CoA synthesis and no role has yet been assigned to the metal centers other than the Ni-Fe-CO complex, characterization of the remaining metal clusters by spectroscopic and electrochemical methods is only briefly treated. We have observed at least seven EPR signals from CODH, all of which exhibit hyperfine broadening when ^{57}Fe replaces normal iron, indicating that there are seven distinguishable iron-containing metal complexes in the protein.[136] However, some of these signals certainly result from different conformations of the metal center(s) and/or the protein. The Ni-Fe-C species gives rise to two interconvertible signals with g-values at 2.08, 2.02 and at 2.05, 2.02.[137] Two EPR signals also appear to result from the reduced form of a $[4Fe\text{-}4S]^{2+/1+}$ center, both with g-values at 2.04, 1.94, and 1.90, differing only in the linewidths of the two signals.[136] An iron-containing center that also appears to exist in two conformations is thought to elicit EPR signals at 2.01, 1.81, and 1.65 and 1.97, 1.87, and 1.75.[136] The structure of this center is unknown. In addition to these signals resulting from $S = 1/2$ systems, there are EPR signals that account for significant spin concentration in the regions between $g = 4$ and 6.[136] These are most likely due to high spin-state forms of the same metal centers just described. Therefore, it appears that the metals in CODH are organized into three distinct clusters that can each occur in different forms and appear to be indistinguishable by chromatographic and electrophoretic analyses, yet spectroscopically distinct.[136,138] At present, we can only speculate that the roles of the $[4Fe\text{-}4S]^{2+/1+}$ cluster and the center giving rise to the 2.01, 1.81, 1.65 and 1.97, 1.87, 1.75 EPR signals involve electron transfer processes.

We have found that other than its ability to assemble acetyl-CoA and to oxidize CO/reduce CO_2, CODH can perform oxidation reduction reactions with CO and CO_2 analogs. In the presence of a suitable reductant, it can convert nitrous oxide to N_2 at a rate approaching those of the nitrous oxide reductases from denitrifying bacteria.[78] The E_o' of the N_2O/N_2 redox couple is +1175 mV.[139] Interestingly, the nitrous oxide reductase from *Pseudomonas stutzeri* is a copper enzyme that oxidizes CO to CO_2 and CO binding was proposed to occur at the metal site.[140] Carbonyl sulfide (COS) is a structural analog of CO_2, in which a sulfur atom replaces one of the oxygens. COS can act both as an inhibitor as well as an alternate substrate of CODH. COS inhibits the oxidation of CO to CO_2 competitively (K_i = 0.4 m*M*) with respect to CO.[219] In the presence of reduced methyl viologen, CODH acts as a COS reductase.[219] Furthermore, under reducing conditions, COS was found to bind directly to the Ni-Fe-containing center, presumably forming

the same complex that is formed when CO_2 or CO are reacted with CODH.[219] Thus, COS apparently acts both as a structural and kinetic analog of CO_2 for the CODH from *C. thermoaceticum*. CODH from *Rhodospirillum rubrum* also is competitively inhibited by COS with respect to CO with a binding constant of 2.2 μM.[141] It was found to not reduce the iron-sulfur centers of CODH and thus was presumed to not act as an alternate substrate;[141] however, this conclusion does not seem well founded since, if COS is a substrate, its properties would only allow it to act as an oxidant instead of as a reductant.

3. Methylation of CODH

That CODH per se catalyzes an exchange reaction between CO and acetyl-CoA[76] implies that there is a methyl binding site of CODH. Methylation of CODH is the first of a series of reactions in the assembly of acetyl-CoA by CODH. This reaction is a protein-to-protein one-carbon transfer of a methyl group from methylated C/Fe-SP to CODH. This methyl group is the precursor of the methyl group of acetyl-CoA.

For simplicity, Figure 2 depicts CODH first binding the methyl group of the methylated C/Fe-SP and then CO in an ordered mechanism. In fact, it appears that CODH can bind the methyl and CO groups in a random order. This is based on the observations that (1) methylated CODH can react with CO[77] and carbonylated CODH can react with the methylated C/Fe-SP[220] to form acetate, (2) methylation of CODH occurs in the absence of CO,[77] and (3) carbonylation of CODH occurs in the absence of the methylated C/Fe-SP.[137,142]

Before considering the methylation of CODH, it is germane to discuss the stereochemistry of acetyl-CoA synthesis from CH_3-H_4folate, CO, and CoA. Lebertz et al.[143] found that the conversion of the chiral methyl group of CHTD-H_4folate to the methyl of acetate occurs with overall retention of configuration. Intermolecular methyl transfer reactions result in inversion of configuration; thus, it is expected that transfer of the methyl group from CH_3-H_4folate to the C/Fe-SP, yielding methylcobamide, results in a single inversion of configuration. Conversely, the migration of a methyl group to a metal-carbonyl (also described as a carbonyl insertion) yields retention of configuration at the acetyl group (Reference 143 and references therein). Since formation of acetyl-CoA and then acetate from any acetylated intermediate (acetyl-X) would not change the configuration at the methyl group, an odd number of inversions (most likely, one) must occur in the conversion of methylcobamide to the acetyl-CODH intermediate. This is expected to be the intermolecular transfer of the methyl of methylcobamide to methyl-CODH. Another stereochemical investigation showed that the CO/acetyl-CoA exchange reaction occurs with retention of stereochemistry at the methyl group of acetyl-CoA.[144] However, this study provided little mechanistic information since it was already known that acetyl-CoA synthesis from CH_3-H_4folate does not scramble the sterochemistry,[143] and the principle of microscopic reversibility demands that any exchange reaction yield no net change in the original stereochemical configuration.

In the first experiments on the methylation of CODH, the enzyme was methylated with $^{14}CH_3I$ or $^{14}CH_3$-C/Fe-SP and a stable $^{14}CH_3$-CODH complex was isolated, hydrolyzed, and identified as *S*-methyl-cysteine.[145] In addition, the $^{14}CH_3$-CODH complex was stable to sodium dodecyl sulfate gel electrophoresis locating the radioactivity in the β (71 kDa) subunit.[145] When $^{14}CH_3$-CODH was treated with CO and CoA, the methyl-cysteine peak was absent from the profile of the radioactive amino acids after hydrolysis.[145] These combined results indicated that the mechanism of methyl transfer involves attack of a cysteinyl residue of CODH on the methyl group of the methylated C/Fe-SP, forming an intermediate methyl-cysteine product, and it was further postulated that *S*-acetylcysteine was a likely intermediate in the final steps of the synthesis.[145] Formation of acetyl-cysteine was invoked to satisfy the known stereochemical constraints on acetyl-CoA synthesis from CH_3-H_4folate, since migration of the methyl from cysteine to a metal center would result in a prohibited additional methyl transfer.[145] However, formation of acetyl-cysteine from methylcysteine would involve unprecedented chemistry in which there would be

a carbonyl insertion into a very stable methyl thioether bond.

We have recently obtained strong evidence that the site on CODH for binding the methyl group is a low potential metal center and that formation of methyl-CODH requires a prior reduction of this metal center. Much of this evidence relies on investigation of the rate of methyl transfer as a function of reduction potential by a method called controlled potential enzymology.[77,146] Controlled potential enzymology is defined as the measurement of enzymatic reactions at defined oxidation reduction potentials and is especially important in examining reactions involving redox-active sites. Performing reactions at defined redox potentials is sometimes as important as performing reactions at defined pH values (see below). As pointed out by Hungate,[147] when a solution is in equilibrium with the oxygen in our standard atmosphere there is ~0.245 m*M* oxygen in solution giving a redox potential of +0.8 V vs. SHE, and this value only decreases by 15 mV with every tenfold decrease in the oxygen concentration in the solution. Thus, an adequate system to remove oxygen and a low potential reducing system are required to lower the potential.[147] The importance of maintaining a low redox potential is underscored by recent estimates that the potential in aerobically growing *E. coli* is probably between −280 and −420 mV:[148] over 1 V from the potential in aerobic reaction mixtures! The intracellular redox potential of anaerobic bacteria is expected to be even lower; for example, methanogenic bacteria only grow in media with a redox potential below −330 mV.[149,150]

Figure 6 shows an electrochemical cell we have developed to poise the redox potential of a reaction mixture.[78,146] The potential is applied between a gold working electrode and a silver/AgCl electrode. The cell is so airtight that the redox potential can be maintained as low as −650 mV vs. SHE. Once a poised potential is stable, a reaction is initiated via addition of a substrate or enzyme through a small septum-stoppered hole in the top half or a small open hole under positive gas flow. Samples are removed and the reactions are stopped at different times, and the amount of substrate depleted or product formed is measured by conventional means such as HPLC. The electrochemical cell can be used for spectroscopy by modification of the lower half, i.e., fusing an EPR tube for analysis of the solution by EPR spectroscopy or fusing a quartz cuvette for UV-visible spectroscopy.

Table 2 summarizes the properties of the methyl binding site on CODH. Methylation of CODH with methyl iodide or with the methylated C/Fe-SP is dependent upon the reduction potential.[77] With methyl iodide, the C/Fe-SP is methylated first, followed by the transfer of its methyl group to CODH (Equations 18 and 19).

$$CH_3I + [Co^{1+}]\text{–C/Fe-SP} \rightarrow CH^3\text{–}[Co^{3+}]\text{–C/Fe-SP} + I^- \quad (18)$$

$$CH_3\text{–}[Co^{3+}]\text{–C/Fe-SP} + CODH \rightleftarrows [Co^{1+}]\text{–C/Fe-SP} + CH_3\text{–CODH} \quad (19)$$

Since methyl iodide is a highly reactive methyl group group donor and there is excess methyl iodide, the reaction is irreversible. The half turnover potential for methylation of CODH with methyl iodide is ~−400 mV and the rate at 0 mV was approximately 20-fold slower than the rate at −550 mV.[77] When the methylated C/Fe-SP was used directly, the rate was stimulated by approximately 50-fold by poising the potential at ~−300 mV relative to the rate at ~0 mV.[77] This appears to be a reversible reaction since it exhibits a bell-shaped curve and reveals that the equilibrium favors formation of methyl-C/Fe-SP at potentials lower than −350 mV (Equation 19). These two experiments clearly show that a low potential center on CODH is involved in the methylation reaction. A third reaction, exchange between the methyl group of methyl-CODH and the C-2 of acetyl-CoA (Equation 20) was examined, since in this case the only redox-active groups involved are on CODH.

$$CH_3\text{–CODH} + {}^{14}CH_3\text{–CO–SCoA} \rightleftarrows {}^{14}CH_3\text{–CODH} + CH_3\text{–CO-SCoA} \quad (20)$$

This exchange reaction occurred at least 130-fold faster at −500 mV relative to the rate at ~0 mV.[77] These combined results provide compel-

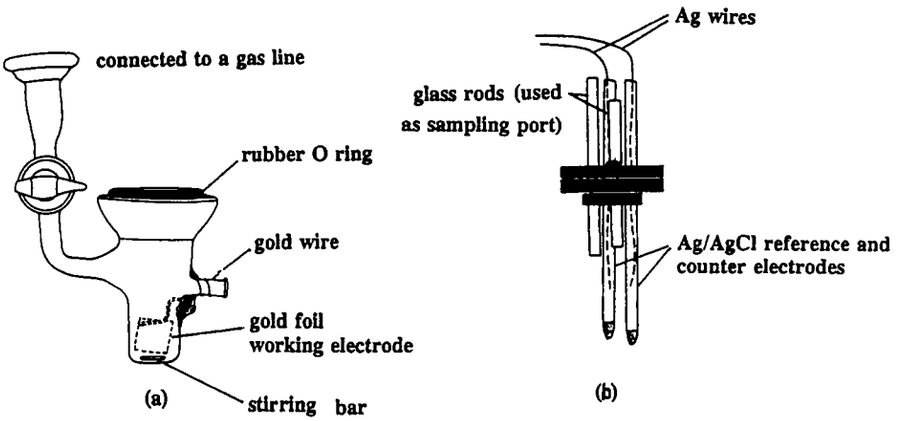


FIGURE 6. General design of the electrochemical cell used for controlled potential enzymology and spectroscopy. For spectroscopy, the bottom half is modified by fusing either a quartz cuvette for UV-visible or an EPR tube for EPR studies. Side views of the top (a) and bottom (b) halves. (From Reference 131, which was modified from Reference 78.)

TABLE 2
Properties of the Methyl Binding Site on CODH

Redox potential < -450 mV[77]
Methyl cysteine has been identified;[145] however, methylation of CODH and formation of acetyl-CoA from methyl-CODH are not inhibited by SH inhibitors[77]
Methylated CODH can perform several reactions:[77]

Acetyl-CoA synthesis:	CH_3-CODH + CO + CoA → CH_3-CO-SCoA + CODH
Acetate synthesis:	CH_3-CODH + CO + H_2O → CH_3-COOH + CODH
Methylation of C/Fe-SP	CH_3-CODH + C/Fe-SP → CH_3-C/Fe-SP + CODH
Exchange:	CH_3-CODH + *CH_3C/Fe-SP ⇄ *CH_3-CODH + CH_3-C/Fe-SP
Exchange:	CH_3-CODH + *CH_3-CO-SCoA ⇄ *CH_3-CODH + CH_3-CO-SCoA

It appears that the active methyl acceptor site is a low potential metal center on CODH[77]

ling evidence that a metal center rather than a cysteinyl thiol is the initial nucleophile on CODH that acts as the methyl acceptor.[77] First, methylation of CODH occurs over a wide pH range, from 5.5 to 7.5.[145] This pH profile is not consistent with the normal profile for cysteine ionization and could reflect changes in coordination state around a metal center, although there is precedent for cysteine residues in proteins with low pK_a values. Second, the dependence on redox potential of the methylation of CODH is unlike that which would be expected for reduction of a dithiol to an active thiolate.[77] Most disulfide/dithiol redox reactions occur in the range of -200 to -300 mV, yet, as discussed above, the methylated redox center has a redox potential of < -400 mV. Third, methylation of CODH at low potentials is not inhibited by thiol reagents.[77] Fourth, based on model chemistry, formation of an acetyl intermediate would be expected to involve a methyl migration (CO insertion), a reaction that has been well documented for organometallic chemistry (References 151 and 152, for example), but we are not aware of such reactions occurring at a thioether.

Methylated CODH can donate its methyl group in several reactions, including acetyl-CoA synthesis, in the presence of CO and CoA,[77]

indicating that methyl-CODH is a competent intermediate in the pathway.

One possible explanation for the identification of methyl-cysteine from the amino acid hydrolysate[145] is that the methyl group may migrate from a metal center to a cysteinyl residue. Formation of the methyl-thioether is expected to be an irreversible reaction. Support for the postulate of a methyl migration is offered by the observation that dithiothreitol can slowly demethylate CODH.[77]

In summary, as indicated in Figure 2, methylation of CODH is proposed to occur by a reductively activated methyl displacement reaction. This reaction would then most likely involve cleavage of the methylmetal bond on the C/Fe-SP to form a methyl-metal bond on CODH. A likely candidate for the methyl acceptor site is the Ni-Fe-C center (discussed below) since it appears to be the binding site for the CO that will become the carbonyl of acetyl-CoA and it has a low midpoint reduction potential. However, the possibility that another of the low potential metal centers in CODH could be involved in methylation should not be rejected and further inquiries will be required to fully characterize the methyl binding site. For example, it is important to spectroscopically identify the methyl-CODH species.

4. Carbonylation of CODH

When CODH binds CO, an intermediate is formed that is the precursor of the carbonyl group of acetyl-CoA. This intermediate has been probed by spectroscopic and electrochemical methods that indicate that the site of CO binding is the same "M" shown in Figure 2 to be involved in binding the methyl and acetyl groups by CODH (discussed below).

CODH is isolated in a EPR-silent state. Upon reaction with CO, which is both a ligand and a reductant, CODH exhibits seven different EPR signals when spectroscopy is performed at liquid helium temperatures (<4 K).[136,153] Most of these EPR signals also are seen upon dithionite or electrochemical reduction and these spectra and their interpretation were briefly considered above. Here, the formation of an EPR detectible metal-carbonyl complex, which is the precursor of the carbonyl of acetyl-CoA, is discussed.

a. Properties of the Ni-Fe-CO Center of CODH

Binding of CO to CODH elicits an EPR signal that can be observed at the relatively high temperatures of liquid N_2 (~77 K) where most Fe-S centers relax too quickly to be detected (Figure 7). Though this is a fairly simple axial EPR signal arising from a system with a single unpaired electron, its origin has been traced to an interesting metal complex. Since the g-values and lineshape are significantly different from those of simple free radicals, it was likely that at least a metal was associated with the center. The clas-

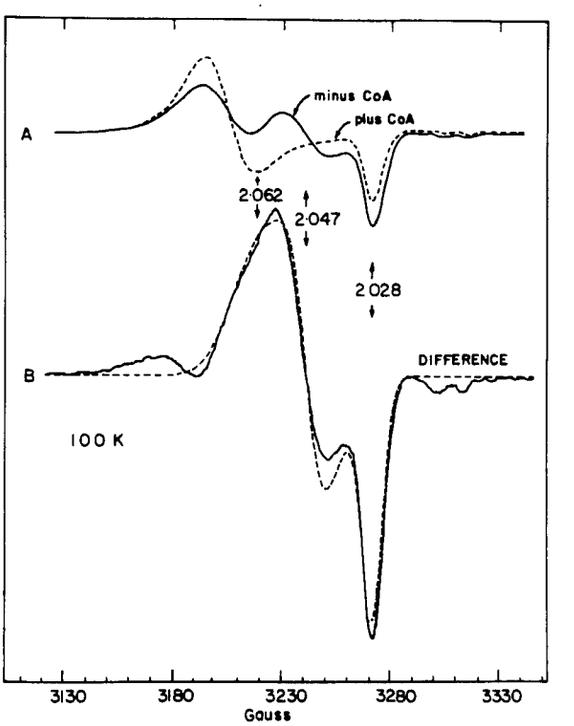


FIGURE 7. EPR spectrum of the Ni-Fe-CO center showing the effect of CoA binding on the spectrum. EPR conditions: temperature, 100 K, field set, 3190 G; gain, 3.2×10^3; power, 10 mW; scan rate, 100 G/min; and frequency, 9.286 GHz. (From *Bioinorganic Chemistry of Nickel*, Lancaster, J. R., Ed., VCH Publishers, Weinheim, 1988, 311. With permission.)

sic way to infer which metals are involved in a spin system is to perform substitution by an isotope that has a nuclear spin (I). The nuclear magnet attracts the unpaired electron spin (S) that alters the properties and the EPR spectrum relative to the system lacking the nuclear spin. The strength of this interaction is described by a tensor $\hat{\mathbf{A}}$, the magnetic hyperfine coupling tensor, which relates the nuclear and electron spin, $\mathbf{S}\cdot\hat{\mathbf{A}}\cdot\mathbf{I}$. For example, if a nucleus with a nuclear spin of 1/2 (e.g., ^{1}H) is a component of a system that exhibits an EPR signal, two lines (2 I + 1) will be observed at each resonance position. Often, instead of distinct lines, only a broadening of the spectrum will be observed. CODH contains nickel, iron, and zinc and, fortunately, natural abundance nickel and iron (both predominantly I = 0) can be substituted by isotopes with a nuclear spin. In addition, CO is available as ^{13}CO.

We performed a series of isotope substitutions (Figure 8).[142,154] When CODH is reacted with ^{13}CO in place of ^{12}CO, the resulting EPR spectrum exhibits a doublet centered at the g-value of the high field resonance of the spectrum of the ^{12}CO-reacted enzyme. This experiment demonstrates that CO is part of the spin system, most likely as a ligand to a metal(s). In order to perform an isotopic substitution with nickel, it was necessary to culture *C. thermoaceticum* in a medium containing ^{61}Ni (I = 3/2) in place of natural abundance nickel that is mostly composed of ^{58}Ni (68%) and ^{60}Ni (26%), both with I = 0. After isolation of CODH from these cells and reacting with CO, a significant broadening of the spectrum is observed relative to the enzyme containing natural abundance nickel. This experiment demonstrates that Ni is part of this complex that binds CO. Finally, CODH was isolated from cells grown in the presence of ^{57}Fe (I = 1/2) in the place of natural abundance iron,[137,154] which is 92% ^{56}Fe (I = 0). When ^{57}Fe-containing CODH is reacted with CO and analyzed by EPR spectroscopy, the spectrum was significantly broadened relative to that of the ^{56}Fe-containing enzyme, revealing that iron also is a component of this EPR active center. Therefore, the quest to identify the metal complex in CODH involved in binding CO revealed the presence of a novel metal center containing both nickel and iron.

Using electron-nuclear double resonance (ENDOR) spectroscopy,[154] we confirmed the EPR evidence for a Ni-X-Fe mixed metal center that binds CO. ENDOR is a method that can directly reveal the strength of interaction, $\hat{\mathbf{A}}$, 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-contain-

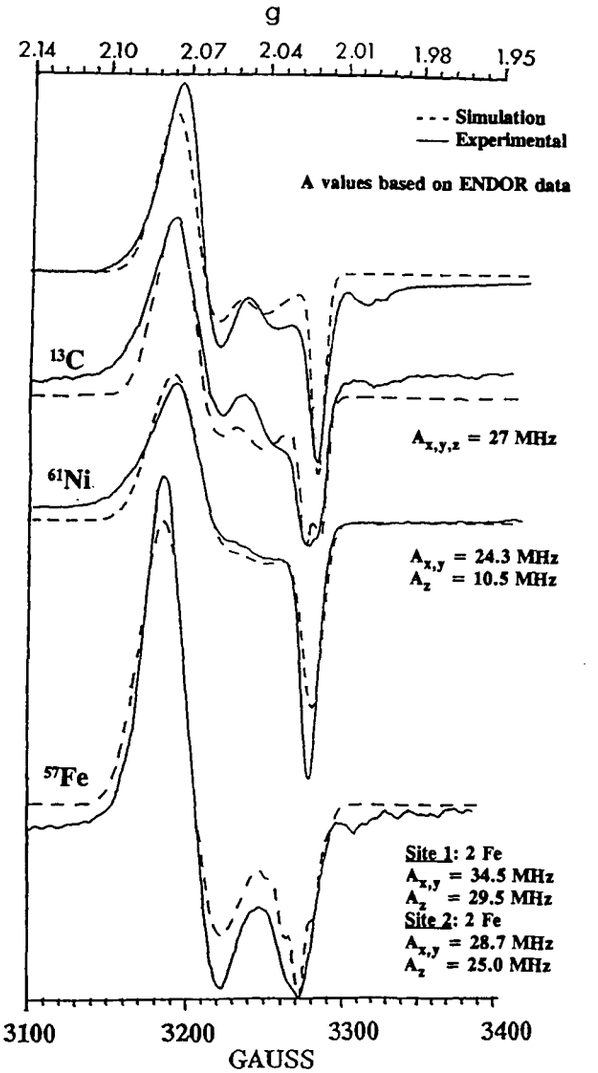


FIGURE 8. Isotope studies of isotopically enriched CODH after reaction with CO. Experimental spectra (——) and simulated spectra (– – –) using A values derived from the ENDOR experiments of CODH enriched with ^{13}CO, ^{61}Ni, and ^{57}Fe. For simulation of the ^{57}Fe spectra, four irons in two different types of iron were assumed with the A values shown. (Modified from Reference 154.)

ing enzyme, suggesting that at least two irons were part of the complex. A reasonable simulation (dotted lines in Figure 8) of the EPR spectrum with the ENDOR-derived coupling constants required the inclusion of at least three iron atoms. ^{61}Ni-containing and ^{13}CO-treated enzymes also were analyzed by ENDOR, and the use of a single Ni and CO with the ENDOR-derived coupling constants (Å values) was sufficient to accurately simulate the EPR spectra. This[154] was the first reported Ni ENDOR study of an enzyme. Therefore, the CODH-CO complex contains CO, Ni, and ≥3 iron atoms.[154] The coupling constants observed for the ^{57}Fe containing enzyme are similar to those obtained for Fe-S clusters and those for ^{13}CO are similar to those obtained for the ^{13}CO-reacted hydrogenase from *C. pasteurianum* in which an iron-sulfur cluster, implicated as the site of H_2 activation, binds CO[155] to inhibit the enzyme.

The environment of the iron sites in CODH has been investigated by Mössbauer spectroscopy.[138] This method requires the substitution of ^{57}Fe and has been important in the characterization of the metal environment in a number of iron-containing proteins. In the Mössbauer spectrum of the Ni-Fe-CO complex of CODH (Figure 9), the solid line is the simulated spectrum using the parameters stated in the legend and the hatched lines represent the experimental data. The same parameters were successful in simulation of the Mössbauer spectrum obtained at higher temperatures. In addition, the hyperfine coupling values used in the simulation are nearly identical to those observed in the ENDOR experiment (above). The hyperfine coupling values, isomer shift, and quadrupole splitting parameters used to fit the Mössbauer spectra are very similar to those of $[4Fe\text{-}4S]^{2+}$ centers.[138]

The environment of the Ni-Fe_4-S_4-CO center is markedly influenced when a tryptophan residue involved in CoA binding is modified, suggesting that the metal-CO intermediate is near both a tryptophan residue and the site of CoA binding.[156] Other evidence for proximity of the Ni-Fe_4-S_4-CO center and the CoA binding site is provided by the demonstration that CoA has a direct effect on the EPR spectrum of the metal-carbonyl intermediate (see Figure 7).[137]

b. Possible Structures of the Ni-Fe-C Center

Based on spectroscopic evidence, several working models for the "M-CO" precursor of the carbonyl of acetyl-CoA can be forwarded (Figure 10). These models have in common at least three irons, one nickel, and a single CO. The M-CO could be a [4Fe-4S] cluster linked to Ni through a ligand bridge and CO could bind either to Ni or Fe. One possibility is that CO could serve as the bridging ligand. Incorporation of Ni into the corner of a cubane Fe_3-S_4 cluster also is possible, in which case, CO could bind either to the Ni or Fe components of the complex. Of these possible structures, thus far only the Ni-Fe_3-S_4 complex has a purely synthetic precedent.[157] In addition, a Ni-Fe_3-S_4 center has been assembled by incorporation of Ni into the unoccupied edge of a [3Fe-4S] center in a ferredoxin.[158] A nickel EXAFS analysis of the *C. thermoaceticum* CODH indicates that the Ni site is sulfur rich,[159,160] consistent with all the above models. The bridged Ni-X-Fe_4S_4 structure is supported by EXAFS studies in which inclusion of a Ni-Fe bond of ~3.3 Å resulted in a better fit to the data.[160] Further studies are required to choose among these or perhaps other models of the M-CO site in CODH.

c. Ni-Fe Complexes Analogous to the Ni-Fe-CO Center of Acetogens

A protein complex containing CODH from *Methanosarcina thermophila* was shown to contain a metal center that, when the enzyme is reacted with CO, exhibits EPR signals very similar to those of the Ni-Fe-CO center in the CODH from *C. thermoaceticum*[161] These signals can be observed at liquid nitrogen temperatures and have g-values of 2.089, 2.078, 2.03 and 2.057, 2.049, 2.027.[161] Interconversion between these EPR signals is influenced by the presence of acetyl-CoA.[161] Based on isotopic substitution with ^{61}Ni, ^{57}Fe and ^{13}CO, these EPR signals were shown to result from a Ni-Fe-CO complex.[161] The *M. thermophila* enzyme complex contains five subunits with Ni, Fe, Zn, and Co in a corrinoid center.[162]

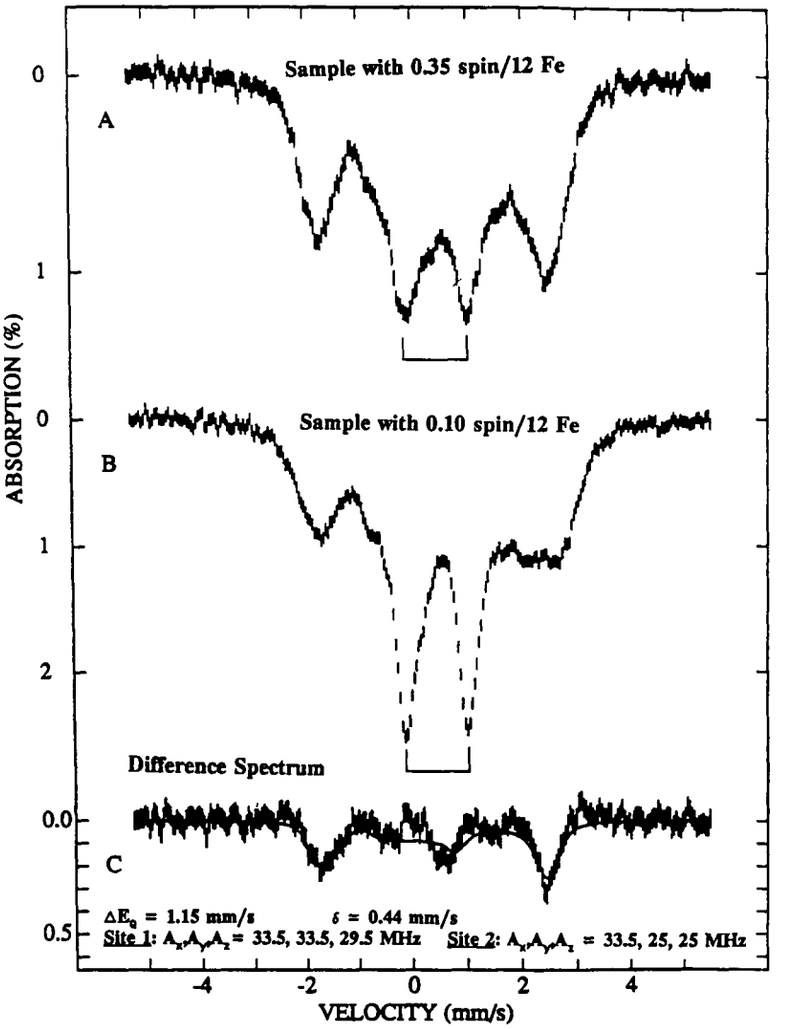


FIGURE 9. 4.2 K Mössbauer spectrum of CO-reduced CODH. Spectra were recorded in a 60-mT applied parallel field. Spectrum C is a difference spectrum obtained by subtracting the spectrum in B from that in A and removing the central quadrupole doublet. The solid line in C is a spectral simulation including two sites, both with ΔE_Q = 1.15 mm/s and isomer shift = 0.44 mm/s. (From Reference 138, which the reader can see for details.)

Nickel and iron are associated with the CODH component and the corrinoid and iron are within the corrinoid/iron-sulfur component.[163] As in the acetogenic CODHs, the *M. thermophila* enzyme complex can perform exchange reactions between CO and acetyl-CoA,[164] CoA and acetyl-CoA,[164] and acetyl-CoA synthesis from methyl iodide, CO, and CoA.[163]

A metal center containing nickel and iron also has been described in the CODH from *Rhodospirillum rubrum*,[165] a monomeric protein containing 7 irons, 6 sulfur, 1 nickel, and 0.4 zinc.[166,167] Two EPR signals are observed from the oxidized *R. rubrum* CODH, one with g-values at 2.04, 1.90, and 1.71 and another at 1.95, 1.88, and 1.79. When the *R. rubrum* enzyme is treated with CO, these EPR signals disappear.[165,166] The lineshapes and g-values of these EPR signals are similar to two EPR signals from a center of undefined structure in the CODH from *C. thermoaceticum* with g-values at 2.01, 1.81, and 1.65 (E_o' = −220 mV) and 1.97, 1.87, and

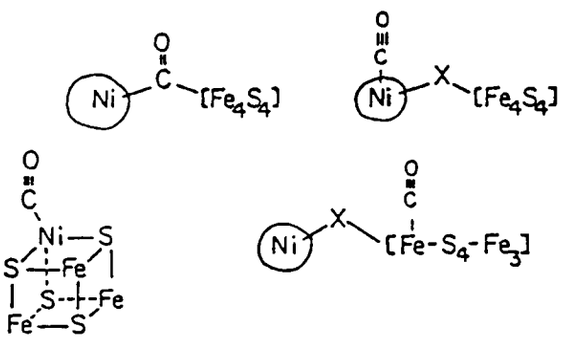


FIGURE 10. Minimal working models of the Ni-Fe-CO center.

1.75 (E_o' = −530 mV).[137] In the *R. rubrum* CODH, this is the center that is proposed to be a Ni-Fe complex based on hyperfine broadening of the EPR spectrum of the ^{57}Fe- and ^{61}Ni-containing enzyme and on the absence of this EPR signal in samples of the enzyme lacking nickel.[165] In addition, cyanide, which is a competitive inhibitor with respect to CO,[168] alters the EPR signals described above.[165] These EPR signals are also similar to that of one of the centers of the CODH from *Methanosarcina barkeri* with a redox potential of −35 mV, g-values of 2.005, 1.91, and 1.76, and the g = 1.76 resonance shifts to g = 1.73 upon incubation with CO.[169] This EPR spectral shift also occurs when whole cells of *M. barkeri* are performing methane synthesis from acetate.[170] A similar EPR signal is seen in the CODH from *Methanothrix soehngenii*.[170a]

5. Methyl Migration for Form Acetyl-CODH

After carbonylation, there appears to be a methyl migration to form acetyl-CODH. Much evidence for this exists. First, acetate is the exclusive product of the reaction of CH_3-CODH with CO in the absence of CoA.[78] Second, acetate has been identified as a product of the reaction of CH_3-C/Fe-SP with CO and either purified CODH (Ragsdale's unpublished results described in Reference 74) or a protein fraction containing CODH.[88] Third, acetate has been detected as a minor product of the synthesis of acetyl-CoA from CH_3-H_4folate, CO, and CoA.[125] Finally, the rate of the CoA/acetyl-CoA exchange reaction is ~200-fold faster than that of the CO/acetyl-CoA exchange reaction (discussed below), indicating that cleavage of the C-S bond of acetyl-CoA, forming the acetyl intermediate, occurs faster than formation/breakage of the C-C (acetyl) bond of acetyl-CoA.[75,78] So far, the acetyl-metal intermediate has not been spectroscopically detected.

6. Binding of CoA to CODH

Quantitation of the strength of interaction between CoA and CODH has yielded quite disparate values. Kinetic evidence for binding of CoA to CODH was provided by the demonstration that CODH per se catalyzes an exchange reaction between CO and acetyl-CoA and that CoA is a potent inhibitor of this reaction[76] with K_i of 7 μM.[171] The K_m for acetyl-CoA in the CO/acetyl-CoA exchange reaction is 600 μM.[171] In the exchange reaction between labeled CoA and acetyl-CoA, the K_m for CoA is 50 μM.[75] Equilibrium dialysis experiments indicate that there are two binding sites for CoA with approximately equal occupancy at a low affinity (K_d = 50 μM) and a high affinity (K_d = 2.6 mM) site.[78] In the synthesis of acetyl-CoA from CoA, CO, and either methyl iodide[77] or CH_3-H_4folate,[172] K_m values for CoA of ~4.7 mM have been measured.

Based on EPR spectroscopy, CoA appears to bind to CODH near the Ni-Fe-CO complex, as described above. An arginine residue was proposed to be involved in CoA binding possibly via a charge-charge interaction with the pyrophosphate bridge of CoA since the exchange reaction between CO and acetyl-CoA is inhibited by glyoxals.[76] Quenching of the fluorescence of a tryptophan residue on CODH upon addition of CoA indicates that a tryptophan residue is near the CoA binding site.[156,173] Further evidence for a CoA-tryptophan interaction has been obtained by a chemical modification approach. When exposed tryptophan residues are oxidized by *N*-bromosuccinimide (NBS), the CO/acetyl-CoA exchange reaction is significantly inhibited and CoA protects against this inhibition.[173] In order to determine that tryptophan is at the CoA binding site, CODH was labeled with 2,4-dinitrophenylsulfenyl chloride (DNPS-Cl), a reagent specific

for tryptophan residues, treated with trypsin, and the labeled tryptic peptides were sequenced.[118] One peptide containing a *trp*-his-thr-gly-gln-*arg* sequence was protected by CoA against DNPS-Cl modification. This peptide was located in the 78 kDa subunit of CODH based on location of the peptide sequence within the deduced amino acid sequence.[118] The presence of an arginine residue close to the protected tryptophan suggests that it is the residue that is modified by glyoxal.[76]

Further experiments are required to explain the apparent existence of two CoA binding sites per $\alpha\beta$ form of CODH and to resolve the discrepancies between the binding constants described above. One possibility is that there are two forms of CODH, a high activity form that binds CoA tightly and a form that binds CoA weakly. Alternately, a single form of CODH could have a high affinity catalytic CoA binding site and an additional site that could play a structural or regulatory role. It also is possible that CODH exhibits half-of-the-sites reactivity. EPR spectroscopic studies (discussed below) also provide evidence for different conformations of CODH.[136,137]

7. Condensation of the Bound Acetyl and Thiol Groups to Form Acetyl-CoA

If one considers the final steps in the pathway, synthesis of the thioester bond can be probed by following an isotope exchange reaction between CoA and the CoA moiety of acetyl-CoA.[74,75,78] Pezacka and Wood[74] demonstrated that CODH catalyzes a slow exchange reaction between [^{3}H]CoA and acetyl-CoA in the presence of a reducing system consisting of either reduced ferrodoxin or NADH and disulfide reductase. A considerably higher rate of CoA/acetyl-CoA exchange in the presence of CO was reported by Ramer et al.,[75] with rates approximately sevenfold faster than the CO/acetyl-CoA exchange. An even faster rate was measured when the reaction was performed at low redox potentials.[78] 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}), ~14,000-fold higher than the values measured earlier,[74] presumably due to the stimulatory effect of the low redox potential. The rate at -575 mV is 2000-fold faster than that at -80 mV.[78] Treatment of the kinetic data by a Nernst analysis revealed that a group on CODH with a midpoint reduction potential of ≤ -486 mV must be activated by a one-electron reduction in order to catalyze this exchange reaction.[78] The CoA/acetyl-CoA exchange reaction is inhibited by CO, N_2O, CO_2, CN^-, and CoA analogs.[78]

As shown in Figure 11, the redox-sensitive CoA/acetyl-CoA exchange reaction is proposed to involve (1) interaction of acetyl-CoA with CODH, (2) cleavage of the C-S bond forming an acetyl-CODH-CoA intermediate, (3) release and rebinding of CoA, and (4) resynthesis of acetyl-CoA.[78] The redox step apparently involves a one-electron reductive activation of a metal center on CODH with a midpoint potential < -486 mV. Interaction of acetyl-CoA with CODH occurs through arginine and tryptophan residues (described above). We showed that binding of CoA to CODH is unaffected by the redox potential and presume that acetyl-CoA binding also is redox insensitive. Therefore, if steps 1 and 3 are not affected by the redox potential, cleavage of the C-S bond of acetyl-CoA, formation of the acetyl-CODH intermediate, or resynthesis of acetyl-CoA is assumed to be the redox sensitive step. Our data would be consistent with reaction of the reduced metal center with acetyl-CoA to cleave the C-S bond and form an acetyl-metal intermediate.[78]

8. Evidence for the Assembly of Acetyl-CoA at a Single Metal Center on CODH

Based on combined results obtained from electrochemical, spectroscopic, and kinetic studies, a single metal center on CODH appears to be reductively activated to form "M", which is the site of assembly of the methyl, carbonyl, and acetyl moieties of acetyl-CoA. Interpretation of the results obtained from study of reactions involving a reduction step followed by a chemical or binding step(s) may be aided by reference to Figure 12. The S-shaped curve on the left represents the dependence of the ratio of the concentrations of oxidized and reduced metal centers (M_{ox}/M_{red}) on the measured potential for the

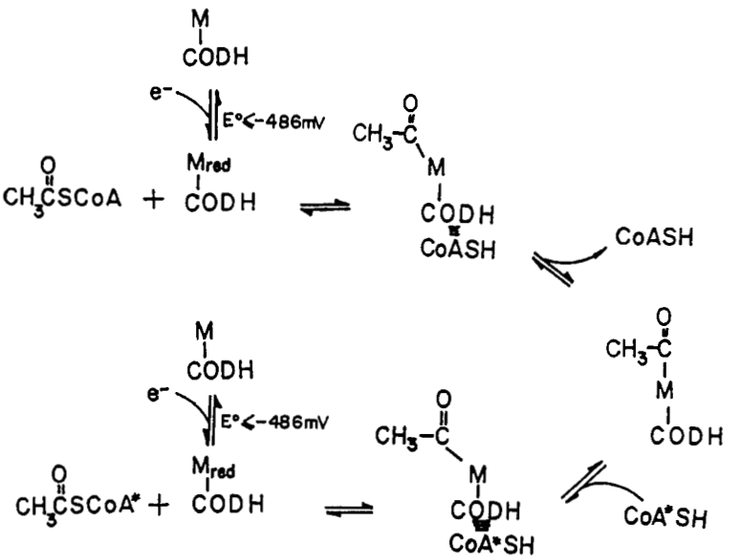


FIGURE 11. Scheme of the CoA/acetyl-CoA exchange reaction. This diagram explains the redox dependence of this reaction by coupling the reaction of acetyl-CoA to the reduced form of a metal center on CODH, followed by an exchange reaction between radioactive CoA and unlabled acetyl-CoA. (From Lu, W. P. and Ragsdale, S. W., *J. Biol. Chem.*, 266, 3554, 1991. With permission.)

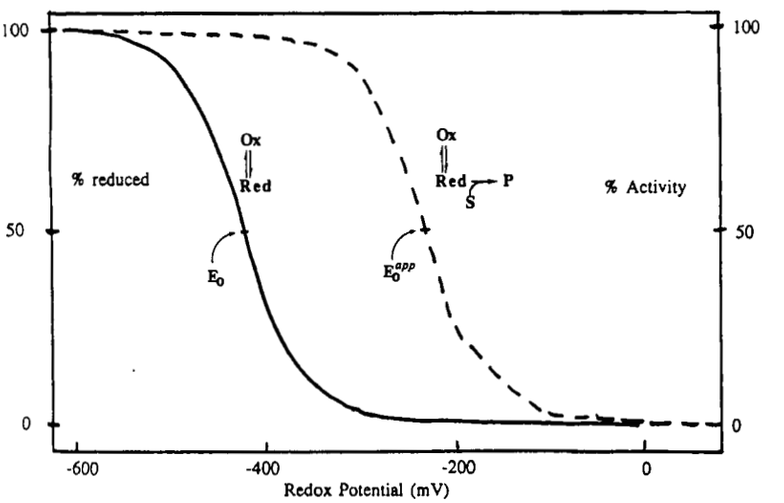


FIGURE 12. Diagram explaining the shift in apparent midpoint potential by coupling a chemical reaction to an oxidation-reduction reduction.

isolated electron transfer reaction. The curve on the right (shifted to more positive potentials) represents the same M_{ox}/M_{red} ratio as a function of potential when M_{red} is coupled to a reaction. More detailed theory of these types of reactions is presented in Reference 108 and has been applied to CODH[78] and methylation of the C/Fe-SP (above). If M_{red} has higher affinity for a ligand or is catalytically more active than M_{ox}, the apparent midpoint potential of the redox reaction shifts in a

positive direction since the coupled reaction lowers the M_{red}/M_{ox} ratio. The magnitude of the shift depends on how exergonic the coupled reaction is and how rapidly the coupled reaction occurs relative to the electron transfer reaction. Similarly, if one measures the dependence of the rate of a reductively activated reaction on the redox potential, the potential at which half-maximal activity occurs will be more positive than the actual midpoint potential for that active site redox center in the absence of the coupled reaction. Therefore, even if one cannot strictly determine the redox potential for the reductively activated center that is involved in catalysis and binding, it can be stated that the midpoint reduction potential for the reductively activated center must be less than or equal to the midpoint turnover potential.

Based on the above considerations, a metal center on CODH with a reduction potential of ≤ -486 mV is reductively activated by one electron to a state in which it can form acetyl-CODH. Methylation of CODH required reductive activation of a center with an E_o' of ≤ -450 mV. The rate of reduction of CO_2 to CO and formation of the EPR signal of the Ni-Fe-C complex from CO_2 exhibit apparent midpoint potentials of ~ -430 mV,[136] demonstrating that the midpoint reduction potential(s) for the center(s) involved in these reactions is ≤ -430 mV. Formation of the Ni-Fe-CO EPR signal from acetyl-CoA occurs according to a one-electron reduction with a midpoint potential of -541 mV, implying that the Ni-Fe-CO species has a midpoint potential of ≤ -541 mV vs. SHE.[219] By definition, isotope exchange reactions at equilibrium do not yield a net free energy change; therefore, we predict that -541 mV may be near the true E_o' of the Ni-Fe-$C^{ox/red}$ couple. Based on the combined results, it is likely that the same low-potential metal site on CODH is being reductively activated in each of these reactions, suggesting that the same metal center is the site of methylation, carbonylation, and acetylation. Since formation of the Ni-Fe-CO EPR signal associated with CO binding correlates well with the redox potential dependence of the above reactions, the reductively activated metal center shown as M in Figure 2 has been postulated to be the Ni-Fe_{3-4}-S_4 center.[78]

F. Summary of the Reductive Acetyl-CoA Pathway

In summary, in the acetyl-CoA pathway, 1 mol of CO_2 or CO undergoes a net six- or four-electron reduction, respectively, to the level of a methyl group as CH_3-H_4folate. Once the C/Fe-SP is reductively activated to the Co^{1+} state, MeTr catalyzes the transfer of the methyl group of CH_3-H_4folate to the C/Fe-SP forming an enzyme-bound methylcobalt species. The prejudiced view of this author describes the steps that occur on CODH as follows. A one-electron reductive activation of CODH precedes a series of reactions involved in the assembly of acetyl-CoA. Formation of a methyl-metal species and a carbonylation step precede the methyl migration to form an acetyl-metal intermediate. After CoA binds, thiolysis of the acetyl intermediate by CoA yields acetyl-CoA. Insight into the chemistry involved in acetyl-CoA formation would benefit from the study of biomimetic models including defined nickel and iron containing complexes that are analogous to the proposed intermediates. Stable and structurally defined methyl-Ni^{2+}, Ni^{1+}-CO, and acetyl-Ni complexes have been synthesized that can undergo reaction with a thiol to form an acetylthioester.[174] Further study of these complexes is expected to enhance our understanding of the way that nature makes acetyl-CoA.

Higher growth yields than can be generally accounted for by substrate-level phosphorylation are observed when acetogens, such as *C. thermoaceticum*, grow heterotrophically.[175] In addition, when acetogens grow autotrophically on H_2/CO_2 and other one-carbon compounds (reviewed in References 12 and 14), there apparently is no net ATP gain by substrate-level phosphorylation, since the one ATP generated from acetyl-CoA conversion to acetate is used for synthesis of formyl-H_4folate. The need to explain these results leads one to expect that acetogenic bacteria couple the synthesis of acetate with generation of ATP via oxidative phosphorylation. Discussion of possible mechanisms for energy generation via electron transport is outside the scope of this review and has been covered recently.[27,176]

G. Heterotrophic Growth of Acetogens

Historically, the acetyl-CoA pathway was recognized first as a heterotrophic pathway. Early studies were carried out with *C. thermoaceticum*, first isolated by Fontaine et al.[177] and found to ferment glucose to approximately 3 mol of acetate. Acetate synthesis occurs by oxidation of glucose to pyruvate by the Embden-Meyerhof pathway and three acetates are then formed from the two pyruvates. One pyruvate reacts with pyruvate ferredoxin oxidoreductase (PFOR, denoted E-TPP), ferredoxin, and CoA to yield CO_2, acetyl-CoA, and reduced ferredoxin (Equation 21). CO_2 is reduced to CH_3-H_4folate via the Hy-folate-dependant enzymes described in Equation 22. Reaction of M with the second mol of pyruvate and CoA generates two additional mol of acetyl-CoA (Equation 23).

$$CH_3\text{–CO-COOH} + Fd_{ox} + HSCoA \rightarrow CH_3\text{–CO-SCoA} + Fd_{red} + CO_2 + 2H^+ \quad (21)$$

$$7H^+ + CO_2 + H_4\text{folate} + 6e^- \rightarrow CH_3\text{–}H_4\text{folate} + 2H_2O \quad (22)$$

$$CH_3\text{–CO-COO}^- + CH_3\text{–}H_4\text{folate} + 2HSCoA \rightarrow 2CH_3\text{–CO-SCoA} + H_4\text{folate} + H_2O \quad (23)$$

Drake et al.[102] isolated PFOR from *C. thermoaceticum* and studied the formation of acetyl-CoA from CH_3-H_4folate, pyruvate, and CoA. Five protein fractions were isolated which were sufficient to catalyze the reaction shown in Equation 23, of which MeTr, PFOR, and Fd were purified to homogeneity.[102] Other components have been later shown to include CODH and the C/Fe-SP. The carboxyl of pyruvate does not equilibrate with free CO_2 during its conversion to the carbonyl of acetyl-CoA in Equation 23;[178] however, in the absence of CH_3-H_4folate, PFOR can catalyze an exchange reaction between CO_2 and the carboxyl of pyruvate[102] (Equation 24).

$$*CO_2 + CH_3\text{–CO-COO}^- \rightleftarrows CO_2 + CH_3\text{–CO-}*\text{COO}^- \quad (24)$$

Surprisingly, CoA is necessary for this exchange reaction, even though the reaction apparently does not directly involve CoA.[102] It is thought that an enzyme-bound C_1, likely on CODH at the formate oxidation level, denoted CODH-[HCOOH], is formed via a direct transfer from PFOR to CODH. Pezacka and Wood[126] demonstrated the formation of a $^{14}C_1$ complex on CODH upon reaction of CODH with PFOR and [1-^{14}C]pyruvate, and, though it was formed in very low yield, this CODH-C_1 formed the C-1 of acetate. The existence of this CODH-C_1 complex apparently obviates the need to postulate a bound C_1 on PFOR. Thus, synthesis of acetyl-CoA from CH_3-H_4folate, pyruvate, and CoA involves the combined actions of CODH, MeTr, the C/Fe-SP, Fd, and PFOR (E-TPP). How PFOR is able to direct the carboxyls of the two pyruvates in different directions is not understood. It is likely that CODH is involved in this partitioning.

H. The Reductive Acetyl-CoA Pathway in Organisms Other Than Acetogenic Bacteria

Though the acetyl-CoA pathway has been most thoroughly studied in the acetogenic bacteria, other anaerobes form cell carbon from CO_2 by this pathway. Methanogens are of the archaebacterial kingdom[179] and utilize several cofactors that are different from those of eubacteria;[180,181] for example, tetrahydromethanopterin (H_4MPT) replaces H_4folate. An excellent general review of methanogenesis is available.[182] Experiments leading to a discovery that methanogens use the acetyl-CoA pathway have been reviewed,[14,26] thus, this information is greatly abridged here. Surprisingly, some methanogens are able to produce low levels of acetic acid.[183] In the methanogens, CO_2 is converted to CH_3-H_4MPT via a series of H_4MPT-dependent enzymes analogous to the H_4folate-dependent enzymes of

acetogens (Equation 25).[180] Then CH_3-H_4MPT, CO, and CoA are thought to be converted to acetyl-CoA via a methyltransferase, a corrinoid protein, and CODH (Equation 26).

$$CO_2 + 7H^+ + 6e^- + H_4MPT \rightarrow\rightarrow\rightarrow CH_3\text{–}H_4MPT + 2H_2O \quad (25)$$

$$CH_3\text{–}H_4MPT + CO + CoAS^- \rightarrow H_4MPT + CH_3\text{–CO-SCoA} \quad (26)$$

The most convincing evidence that the acetyl-CoA pathway is involved in autotrophic growth of methanogens was provided by a genetic approach. Loss of the ability of *Methanococcus maripaludis* to grow autotrophically was associated with mutation of CODH and restoration of autotrophic capacity was provided by recovery of CODH activity.[13]

Enzymes of the acetyl-CoA pathway apparently mediate acetyl-CoA oxidation by some sulfate-reducing and methanogenic bacteria (reviewed in Reference 184). In the catabolism of acetate by methanogens, the acetate is first converted to acetyl-CoA, which binds and is disassembled by CODH into an acetyl-CODH-SCoA intermediate. Cleavage of the C-C bond of the acetyl intermediate would generate CH_3-CODH-CO. The CODH complex from acetate-grown *M. thermophila*[164] and purified CODH from *M. soehngenii*[170a] have been shown to be competent in C-S and C-C bond cleavage, and thus can bind the methyl, CO, and SCoA fragments of acetyl-CoA.[164] CO of the CH_3-CODH-CO complex is thought to be oxidized and CO_2 released, yielding the CH_3-CODH intermediate. The two electrons liberated in CO oxidation are thought to be coupled to the formation of CH_4 in the final steps of methanogenesis. Reaction of CH_3-CODH with the reduced corrinoid protein would generate a methyl-C/Fe-SP intermediate. Evidence exists for a corrinoid protein in acetate catabolism.[162,185] Formation of enzyme-bound methyl-cobamide intermediates upon incubation of acetyl-CoA with cell extracts of *Methanosarcina barkeri* has been demonstrated.[186] More recently, two corrinoid proteins have been identified that become methylated with the methyl group of acetate, one of which is methylated during methanogenesis and demethylated when methane formation ceases.[187] In addition, a complex containing CODH and a corrinoid protein has been isolated from *M. thermophila* and the two components resolved.[188] The complex catalyzes the synthesis of acetyl-CoA from methyl iodide, CO, and CoA,[163] implying the formation of a methylcobamide intermediate, transfer of the methyl group to CODH, and assembly of acetyl-CoA by CODH as in the acetogenic system.[77,78] Thus, a methylcobamide intermediate is apparently involved in the early steps of formation of methane from the methyl group of acetyl-CoA. It is likely that the next step involves transfer of the methyl group from the methylated cobamide intermediate to H_4MPT by a methyltransferase, forming methyl-SCoM. Both CH_3-H_4MPT and CH_3-SCoM[189-192] have been shown to be intermediates in methane formation from acetate. There is evidence for both a corrinoid and a noncorrinoid protein in the transfer of the methyl group from methyl-H_4MPT to SCoM.[193-195] The final step in methane formation is the two-electron reduction of CH_3-SCoM which is catalyzed by CH_3-SCoM reductase a protein containing a nickel porphyrin (see References 196 and 197, and references therein).

J. Perspectives and Future Studies

This review has focused on the enzymology of the acetyl-CoA pathway in acetogenic bacteria. The advances that have been made in the understanding of the pathway raise many new questions.

The enzymes have all been purified to homogeneity and, with these enzymes under optimal conditions, acetyl-CoA synthesis occurs at a rate as fast as the *in vivo* rate. What reaction(s) limit and regulate the rate of acetyl-CoA formation? To answer this question, each of the individual steps in the pathway must be studied in the physiological direction and their rates compared with the *in vivo* rate of acetate synthesis. Several of the H_4folate-dependent enzymes and formate dehydrogenase have been studied only in the reverse direction and, though thermodynamic barriers sometimes make the physiological reaction difficult to study, this information is exceedingly relevant.

By what mechanism does MeTr transfer the methyl group from CH_3-H_4folate to the C/Fe-SP to form enzyme-bound methylcobamide? Is the methyl group of CH_3-H_4folate activated via quaternization of the N-5 of the folate derivative or by some other mechanism? Stabilization of the base-off forms of Co^{2+} and methylcobamide in the C/Fe-SP is an important ingredient of the methyl transfer reaction. Is the base-off conformation established by sterically restricting approach of the benzimidazole base to the Co center or by another mechanism? Does the [4Fe-4S] cluster of the C/Fe-SP, required for the methyl transfer,[89] play an electron transfer role or could it also have some catalytic function? Many of these questions are expected to be answered by spectroscopic and kinetic studies. Determination of the three-dimensional structure of MeTr and the C/Fe-SP would be a great boon to our understanding of the mechanism of the methyl transfer.

Assembly of acetyl-CoA on CODH is an extremely interesting reaction sequence in which putative intermediates have been detected and/or characterized. Are the proposed intermediates kinetically competent? It behooves us to measure their rates of formation and decay and to establish that these rates are consistent with the overall turnover number for the relevant half reactions and for *in vivo* acetate synthesis. If not, it should be considered that the proposed intermediate could be an artifact or that a rate-enhancing factor is absent. What is the structure of the metal center(s) involved in assembly of the acetyl group? A working model for a Ni-Fe_{3-4}-S_4 center has been proposed (discussed above). Comparison of spectroscopic data on CODH with similar analyses of model complexes[157,158] are anticipated to help rule out incorrect structures and better understand the role of this unique metal center in the synthesis of acetyl-CoA. Generation of biomimetic models of the active site centers of CODH[174] and description of their mechanism of performing one-carbon transformations are important in defining the enzymatic mechanism.

Elucidation of the roles of the other ~8 iron atoms and the zinc in CODH is important. Are the other iron-sulfur clusters in CODH involved in electron transfer or catalytic processes? One puzzling result from the spectroscopic and binding studies is that CODH apparently can exist in two distinct forms (discussed above). Do the two conformers differ in activity? The importance of protein-protein interaction in the pathway should be considered since it often leads to alteration of kinetic profiles. It is anticipated that many of the structural and mechanistic questions will be answered via determination of the three-dimensional structure of crystals of CODH: However, this protein has yet to be crystallized.

Can we extrapolate the mechanistic details accumulated by the study of acetyl-CoA synthesis in *C. thermaceticum* to other acetogens, and, even further, to other anaerobes, such as sulfate-reducers and methanogens? It appears that those acetogens that use the acetyl-CoA pathway contain enzymes that differ in their kinetic, spectroscopic, and structural properties in only minor ways (see References 12, 27, 156, and 198 for comparative biochemistry). Extrapolation of the mechanistic details to the methanogens is riskier. Evidence for the existence of the acetyl-CoA pathway in the methanogens was considered above with the conclusion that the basic pathways are similar. However, the properties of the methanogenic CODHs, corrinoid proteins, and methyltransferases (see References 161, 162, 169, 188, 194, 199 to 201, for example) and coenzymes[181] are quite different from their acetogenic counterparts. Whereas acetogens catalyze an exchange reaction between CO and the carbonyl group of acetyl-CoA, methanogens require CO_2 and require hydrogen or a low-potential reductant (Reference 202 and references therein). The Ni-Fe-C EPR signal that apparently represents the precursor of the carbonyl of acetyl-CoA in acetogens (discussed above) is not observed in the purified CODHs of *M. barkeri*[169] or *Methanococcus vannielii*,[199] yet it is present in the enzyme complex of *M. thermophila*.[161] These examples should temper any eagerness to presume that the details will be identical in the two systems and emphasize the importance of studying the mechanistic details of acetyl-CoA synthesis and catabolism in methanogens.

How do acetogenic bacteria grow autotrophically when there is no net ATP synthesis via substrate-level phosphorylation? One ATP is used in the 10-formyl-H_4folate synthetase reaction and one ATP is generated in the coupling of acetyl-

CoA hydrolysis to ATP synthesis. Thus, additional ATP must be generated via a mechanism of oxidative phosphorylation linked to electron transport. ATPases, membrane-bound electron carriers, and membrane-associated enzymes that catalyze oxidation of electron donors have been isolated (see References 12 and 14 for review). It is important to perform further studies in order to explain the energetics of autotrophic growth and the higher than expected growth yield on organic substrates.

How ecologically important is the acetyl-CoA pathway and how common is the pathway among diverse organisms and habitats? Since the unique enzymes are CODH, the C/Fe-SP, and MeTr, the genes encoding these proteins could be used to screen diverse populations and habitats for the occurrence of the pathway. A related question concerns the interaction and competition between acetogens and methanogens. Methanogens tend to dominate most anoxic habitats that are low in sulfate and nitrate, because they tenaciously scavenge the hydrogen that is produced during anaerobic biodegradative processes, maintaining a low steady-state level of H_2 between 10^{-5} and 10^{-3} atm.[21,203] However, in the guts of various invertebrates and vertebrates, acetogenesis outcompetes methanogenesis.[21] Understanding the mechanisms that control the relative levels of methanogenesis vs. acetogenesis is important since acetate serves as a nutrient, whereas the formation of methane could be looked upon as a loss of energy.

Besides acetogenesis and CO detoxification, acetogens play a role in biodegradation of aromatics, utilizing lignin-derived compounds such as methoxylated aromatics as substrates with 4 mol of methoxyl group fermented to 3 mol of acetate.[204-206] Acetogens also can catalyze the reduction of the double bond of several phenylacrylate compounds (Equation 27)[204] and integrate the carboxyl group of benzoic acid into acetic acid via an aromatic acid-dependent decarboxylase.[207] What are the properties of these interesting enzymes? Few details are available concerning aromatic bioremediation process. Anaerobes are able to dehalogenate and thus detoxify a number of compounds, including Aroclor, a complex mixture of polychlorinated biphenyls (PCBs),[208] the chlorinated pesticide, Kepone,[221] and chlorinated C_1 hydrocarbons.[209,210] In the latter two cases, corrinoids and corrinoid proteins are involved. Free corrinoids have been shown to catalyze reductive dehalogenation with suitable electron donors, such as titanium (III) chloride or dithiothreitol.[210] CCl_4 was converted to $CHCl_3$, CH_2Cl_2, CH_3Cl, and CH_4 with organocorrinoids detected as intermediates.[210] The mechanisms of the dehalogenation reactions are poorly understood.

$$\text{Ph–CH} = \text{CH–COOH} + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{Ph–CH}_2\text{–CH}_2\text{–COOH} \quad (27)$$

Development of a genetic system in the acetogens is an important research objective. A recent genetic study clearly showed the requirement for the acetyl-CoA pathway in autotrophic growth of a methanogen on H_2/CO_2.[13] The inability to express the genes encoding the C/Fe-SP and CODH in active form in *E. coli*[103] provides one rationale for undertaking this task. The uninvestigated assembly of the metal centers could constitute the limitation and may also involve a complex series of reactions. For example, formation of the active site Mo-$Fe_{6\text{-}8}S_{6\text{-}9}$ center of nitrogenase requires the products of at least six genes in *Klebsiella pneumoniae* in addition to the structural genes for the two subunits of the protein (see Reference 211 and references therein). Development of the ability to perform site-directed mutagenesis on these proteins requires a suitable host and vector system. In addition, creation of a genetic system would allow one to investigate the effects of alteration of specific genes systematically. Acetogenic bacteria have been shown to maintain the ability to grow heterotrophically, yet lose the capacity for autotrophic growth on CO or H_2/CO_2 without apparent loss of CODH and hydrogenase activities.[122] Characterization of these and other biochemical defects will lead to a better understanding of the enzymology and regulation of the acetyl-CoA pathway.

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