

Enzymology of the Wood–Ljungdahl Pathway of Acetogenesis

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The biochemistry of acetogenesis is reviewed. The microbes that catalyze the reactions that are central to acetogenesis are described and the focus is on the enzymology of the process. These microbes play a key role in the global carbon cycle, producing over 10 trillion kilograms of acetic acid annually. Acetogens have the ability to anaerobically convert carbon dioxide and CO into acetyl-CoA by the Wood–Ljungdahl pathway, which is linked to energy conservation. They also can convert the six carbons of glucose stoichiometrically into 3 mol of acetate using this pathway. Acetogens and other anaerobic microbes (e.g., sulfate reducers and methanogens) use the Wood–Ljungdahl pathway for cell carbon synthesis. Important enzymes in this pathway that are covered in this review are pyruvate ferredoxin oxidoreductase, CO dehydrogenase/acetyl-CoA synthase, a corrinoid iron-sulfur protein, a methyltransferase, and the enzymes involved in the conversion of carbon dioxide to methyl-tetrahydrofolate.

Key words: acetogenic bacteria; carbon dioxide fixation; carbon monoxide; cobalamin

Introduction

Some anaerobic microbes, including acetogenic bacteria and methanogenic archaea, convert CO₂ to cellular carbon by the Wood–Ljungdahl pathway (FIG. 1).^{1,2} The global importance of acetogens is covered fully in Harold Drake's chapter.³ Acetogenic bacteria use this pathway as their major means of generating energy for growth. *Moorella thermoacetica*, isolated in 1942,⁴ is the model acetogen and is the organism on which most studies of the Wood–Ljungdahl have been performed; its genome was recently sequenced. Methanogens growing on H₂/CO₂ use the pathway for generating cell carbon; however, those that can grow on acetate, essentially run the pathway in reverse and generate energy by oxidizing acetate to 2 mol of CO₂.⁵ Acetoclastic methanogens also convert acetate into acetyl-CoA for cell carbon synthesis through the combined actions of acetate kinase^{6,7} and phosphotransacetylase.⁸

The Wood–Ljungdahl pathway contains an *Eastern* (in red) and a *Western* (in blue) branch (FIG. 1), as originally described.⁹ The Eastern branch is essentially the folate-dependent one-carbon metabolic pathway that is present from bacteria to humans and recapitulated with methanopterin in methanogens. The West-

ern branch is unique to acetogens, methanogens, and sulfate reducers, and exhibits novel mechanistic features. Acetogenic bacteria (e.g., acetogens or homoacetogens) synthesize acetic acid as their sole or primary metabolic end-product. Globally, acetogens produce over 10¹³ kg (100 billion U.S. tons) of acetic acid annually,¹⁰ which dwarfs the total output of the world's chemical industry.

FIGURE 1 depicts growth of acetogens on glucose. However, these organisms can use a variety of substrates, including the biodegradation products of most natural polymers, such as cellulose, lignin (sugars, alcohols, aromatic compounds), and inorganic gases (CO, H₂, CO₂). When acetogens grow on H₂/CO₂, carbon enters the Wood–Ljungdahl pathway at the CO₂ reduction step, with H₂ serving as the electron donor. Acetogens are important in the biology of the soil, of extreme environments, and of organisms that house them in their digestive tract, such as humans, termites, and ruminants.^{11–13}

Pyruvate Ferredoxin Oxidoreductase

Pyruvate ferredoxin oxidoreductase (PFOR) was reviewed relatively recently.¹⁴ Catalyzing the oxidative decarboxylation of pyruvate to form acetyl-CoA and CO₂, PFOR links heterotrophic metabolism to the Wood–Ljungdahl pathway (FIG. 1). PFOR is also key to cell carbon synthesis since, besides its catabolic function, PFOR catalyzes pyruvate formation by reductive carboxylation of acetyl-CoA.^{15,16} Pyruvate can then

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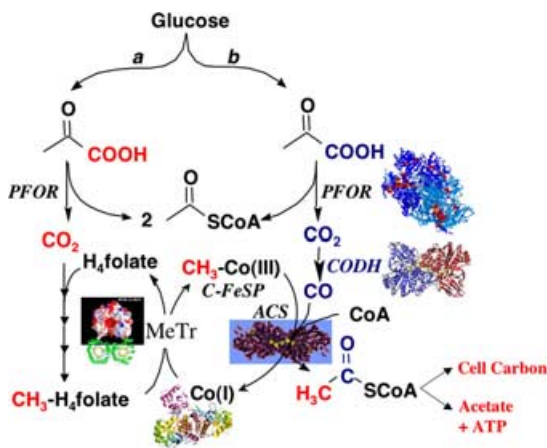
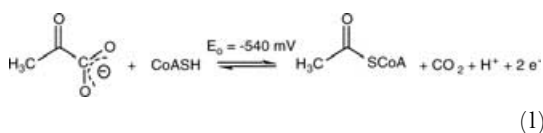


FIGURE 1. The Wood-Ljungdahl pathway with the Eastern and Western branches depicted in red and blue, respectively. (In color in *Annals* on line.)

enter the incomplete tricarboxylic acid cycle^{17,18} to generate intermediates for cell carbon synthesis. PFOR is found in archaea, bacteria, and even anaerobic protozoa like *Giardia*.¹⁹



The structure of the *Desulfovibrio africanus* PFOR reveals seven domains.²⁰ Thiamine pyrophosphate (TPP) and a proximal [4Fe-4S] cluster (Cluster A) are deeply buried within the protein, while the other two clusters (B and C) lead to the surface (FIG. 2). The TPP binding site consists of two domains that bear strong structural similarity to those in other TPP enzymes²¹ and contains conserved residues that interact with Mg²⁺, pyrophosphate, and the thiazolium ring. Rapid kinetic studies indicate that the initial steps in oxidative decarboxylation of pyruvate by PFOR are similar to those of other TPP enzymes.²² Deprotonation of TPP yields the active ylide, which forms an adduct with pyruvate. Then, CO₂ is released, forming the 2 α -hydroxyethylidene-TPP adduct (HE-TPP).²³ In acetogens, the CO₂ feeds into the Wood-Ljungdahl pathway^{24,25} (FIG. 1) and, based on isotope-exchange studies,²⁶ may be channeled directly to CO dehydrogenase/acetyl-CoA synthase (CODH/ACS).

After forming the HE-TPP intermediate, the negative charge on C-2 of HE-TPP promotes one-electron reduction of a proximal FeS cluster, forming an HE-TPP radical intermediate (FIG. 2). Based on the crystal structure, this intermediate was proposed to be a novel sigma-type acetyl radical²⁷; however, recent stud-

ies show that it is a pi radical with spin density delocalized over the aromatic thiazolium ring, as shown in the figure.^{28,29}

In the next step of the PFOR mechanism, the HE-TPP radical transfers an electron to the Fe-S electron-transfer chain, presumably through the oxidized A-cluster (FIG. 2). The rate of this electron-transfer reaction is CoA dependent.³⁰ In the absence of CoA, the half-life of the HE-TPP radical intermediate is ~ 2 min; however, in the presence of CoA, the rate of radical decay increases 100,000-fold.²² By studying various CoA analogues, it was shown that the thiol group of CoA alone lowers the transition state barrier for electron transfer by 40.5 kJ/mol. The final step in the PFOR mechanism is electron transfer through Clusters A, B, and C (FIG. 2) to ferredoxin,²⁰ the terminal electron acceptor for PFOR. This electron-transfer reaction occurs extremely rapidly, with a second-order rate constant of $2-7 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$.^{22,31}

CODH/ACS

CODH/ACS was recently reviewed,³² so this part of the Wood-Ljungdahl pathway will be treated rather briefly. As shown in FIGURE 1, when acetogens are grown heterotrophically, the CO₂ and electrons generated by the PFOR reaction are utilized by CODH/ACS and formate dehydrogenase to generate CO and formate, respectively. When they are grown on CO, CODH generates CO₂, which is then converted to formate in the Eastern branch of the pathway and CO is incorporated directly as the carbonyl group of acetyl-CoA. Both CO and CO₂ are unreactive without a catalyst, but the enzyme-catalyzed reactions are fast, with turnover numbers as high as 40,000 s⁻¹ reported for CO oxidation by the Ni-CODH from *Carboxydotherrmus hydrogenoformans* at its physiological growth temperature.³³ Even the least active CODHs catalyze CO oxidation at rates of $\sim 50 \text{ s}^{-1}$.³⁴ There are two major classes of CODHs: the aerobic Mo-Cu-Se CODH from carboxydobacteria and the Ni-CODHs. Found in aerobic bacteria that oxidize CO with O₂,^{35,36} the Mo-CODH contains FAD,³⁷ Fe/S centers, Cu, and 2 Mo atoms bound by molybdopterin cytosine dinucleotide, and its structure has been solved.³⁸ This enzyme will not be discussed further in this chapter, since only the Ni-CODH/ACS is involved in the Wood-Ljungdahl pathway. Ni-CODHs are divided into two classes: the monofunctional nickel CODH, which catalyzes the reaction shown in Equation 2, and the bifunctional CODH/ACS, which couples Equation 2 (CO formation) with Equation 3 (acetyl-CoA synthesis).

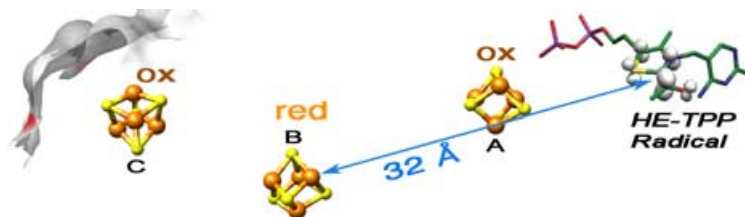
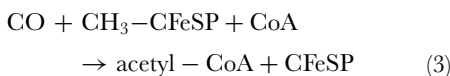
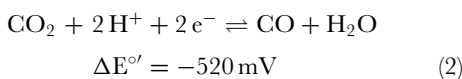


FIGURE 2. PFOR state including the HE-TPP radical, with the structure based on spectroscopic results, showing highly delocalized spin distribution (From Astashkin *et al.*²⁹ Used with permission.), and the distance from HE-TPP radical to coupled cluster. Location of the clusters is based on the structure (PDB 1KEK).

The monofunctional CODH functions physiologically in the direction of CO oxidation, allowing microbes to take up and oxidize CO at the low levels found in the environment, while the CODH in the bifunctional protein converts CO₂ into acetyl-CoA.



The crystal structures of the monofunctional Ni-CODH and the CODH component of the bifunctional enzymes are very similar.^{39–42} These mushroom-shaped, homodimeric enzymes contain five metal clusters per dimer: two C-clusters, two B-clusters, and a bridging D-cluster. The C-cluster is the catalytic site for CO oxidation and is buried 18 Å below the surface. This cluster can be described as a [3Fe-4S] cluster bridged to a binuclear NiFe cluster. The *Rhodospirillum rubrum* CODH structure with a bridging Cys between Ni and a special iron atom called ferrous component II (FCII), while the *C. hydrogeniformans* protein appears to have a sulfide bridge between Ni and FCII.^{43,44} Furthermore, there is evidence for a catalytically important persulfide at the C-cluster.⁴⁵ Issues related to the different structures are discussed in a recent review.⁴⁶ Cluster B is a typical [4Fe-4S]^{2+/1+} cluster, while Cluster D is a [4Fe-4S]^{2+/1+} cluster that bridges the two identical subunits, similar to the [4Fe-4S]^{2+/1+} cluster in the iron protein of nitrogenase.

The CODH mechanism involves a Ping-Pong reaction: CODH is reduced by CO in the “ping” step and the reduced enzyme transfers electrons to an external redox mediator like ferredoxin in the “pong” step. The reduced electron acceptors then couple to other reduced nicotinamide adenine dinucleotide phosphate (NAD(P)H) or ferredoxin-dependent cellular processes that require energy. Details of the CODH reaction have been reviewed.³² Recent studies of CODH linked

to a pyrolytic graphite electrode show complete reversibility of CO oxidation and CO₂ reduction; in fact, at low pH values, the rate of CO₂ reduction exceeds that of CO oxidation.⁴⁷

The association of ACS with CODH forms a bifunctional CODH/ACS machine that is encoded by the *acsA/acsB* genes, respectively, and plays the key role in the Wood–Ljungdahl pathway.^{32,48,49} The CODH component catalyzes the conversion of CO₂ to CO (Eq. 2) to generate CO as a metabolic intermediate.²⁵ The CO₂ comes from the growth medium or from decarboxylation of pyruvate.^{25,30} Then, ACS catalyzes the condensation of CO,⁵⁰ CoA, and the methyl group of a methylated corrinoid iron–sulfur protein (CFeSP) to generate acetyl-CoA (Eq. 3),²⁵ a precursor of cellular material and a source of energy.

CODH/ACS contains a 140-Å channel that delivers CO generated at the C-cluster to the A-cluster.^{40,51,52} The only metalcenter in ACS is the A-cluster, which consists of a [4Fe-4S] cluster bridged to a Ni site (Ni_p) that is thiolate bridged to another Ni ion in a thiolate and carboxamido-type N₂S₂ coordination environment.^{40,42,53} Thus, one can describe the A-cluster as a binuclear NiNi center bridged by a cysteine residue (Cys509) to a [4Fe-4S] cluster, an arrangement similar to the Fe-Fe hydrogenases in which a [4Fe-4S] cluster and a binuclear Fe site are bridged by a Cys residue.^{87,88}

Two mechanisms for acetyl-CoA synthesis have been proposed that differ mainly in the electronic structure of the intermediates: one proposes a paramagnetic Ni(I)-CO species as a central intermediate,² and the other proposes a Ni(0) intermediate.^{54,55} A generic mechanism that emphasizes the organometallic nature of this reaction sequence is described in FIGURE 3.

Step 1, as shown in the figure, involves the migration of CO, derived from CO₂ reduction, through the intersubunit channel to bind to the Ni_p site in the A-cluster. The binding of CO to ACS forms an organometallic complex, called the NiFeC species that has been characterized by a number of spectroscopic approaches.²

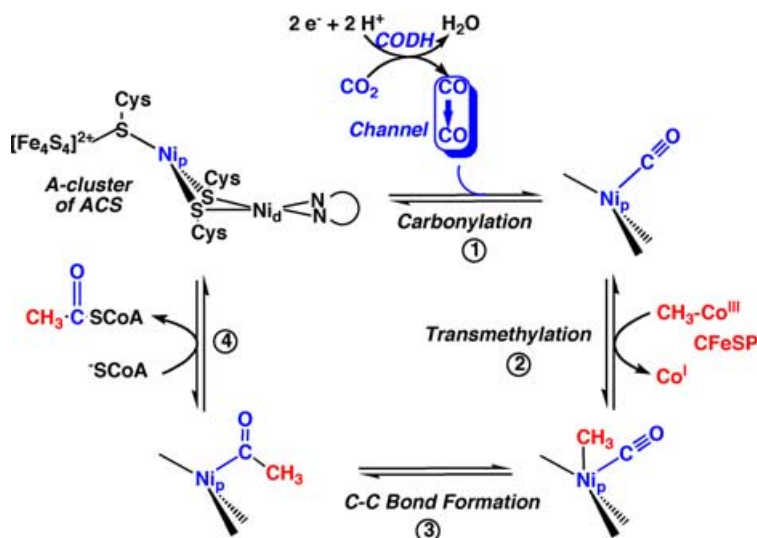


FIGURE 3. ACS mechanism emphasizing the organometallic nature of the reaction sequence and the channel to deliver CO from the CODH active site to the A-cluster.

The electronic structure of the NiFeC species is described as a $[4\text{Fe-4S}]^{2+}$ cluster linked to a Ni^{1+} center at the Ni_p site, while the other Ni apparently remains redox-inert in the Ni^{2+} state.⁵⁶ Lindahl's group has argued that the NiFeC species is not a true catalytic intermediate in acetyl-CoA synthesis, that it may represent an inhibited state, and that the $\text{Ni}(0)$ state is the catalytically relevant one.^{54,55} See the recent review for a discussion of these issues.³² Step 2 in the ACS mechanism involves methylation of the A-cluster,⁵⁷ which involves the conversion of one organometallic species (methyl-Co) to another (methyl-Ni). There is evidence that the methyl group binds to the Ni_p site of the A-cluster.⁵⁸⁻⁶⁰ Carbon-carbon bond formation, Step 3 in the catalytic cycle, occurs by condensation of the methyl and carbonyl groups to form an acetyl-metal species. In the last step, CoA binds to ACS, triggering thiolysis of the acetyl-metal bond to form the C-S bond of acetyl-CoA, completing the reactions of the Western branch of the Wood-Ljungdahl pathway. FIGURE 3 indicates, for simplicity, that the ACS reaction sequence is ordered with CO binding before the methyl group. Conversely, Lindahl has argued for a strictly ordered binding mechanism, with methyl binding first, then CO, and finally CoA, as described in a recent review.⁵⁵ In the author's opinion, there is insufficient evidence to exclude the 1991 proposal that the carbonylation and methylation steps occur randomly⁶¹ (FIG. 4).

Tetrahydrofolate-Dependent Enzymes

Most of the work on the folate enzymes involved in the Eastern branch of the pathway has been

performed in the Ljungdahl laboratory. The methyl group of acetyl-CoA is formed by the six-electron reduction of CO₂ in the reactions of the Eastern branch of the acetyl-CoA pathway (FIG. 1).^{2,62} First, formate dehydrogenase converts CO₂ to formate,⁶³ which is condensed with H₄folate to form 10-formyl-H₄folate.^{64,65} The latter is then converted by a cyclohydrolase to 5,10-methenyl-H₄folate. Next, a dehydrogenase reduces methenyl- to 5,10-methylene-H₄folate,⁶⁶ which is reduced to (6S)-5-CH₃-H₄folate by a reductase.^{67,68}

Methyltransferase (MeTr, AcSE) and Corrinoid Iron Sulfur Protein (CFeSP, AcsCD)

The methyl group of CH₃-H₄folate is transferred to the cobalt site in the cobalamin cofactor bound to the CFeSP^{69,70} to form an organometallic methyl-Co(III) intermediate in the Wood-Ljungdahl pathway (FIG. 1). This reaction is catalyzed by MeTr, encoded by the *acsE* gene.²⁴ MeTr belongs to the B₁₂-dependent methyltransferase family that includes methionine synthase and related enzymes from methanogens.⁷¹ We have cloned, sequenced, and actively overexpressed MeTr^{72,73} and the CFeSP⁷⁴ in *E. coli*, making them amenable for site-directed mutagenesis studies. In collaboration with Cathy Drennan (MIT, Cambridge, Massachusetts), we also have determined the structure of MeTr and a site-directed variant in its uncomplexed⁷⁵ and CH₃-H₄folate-bound⁷⁶ states.

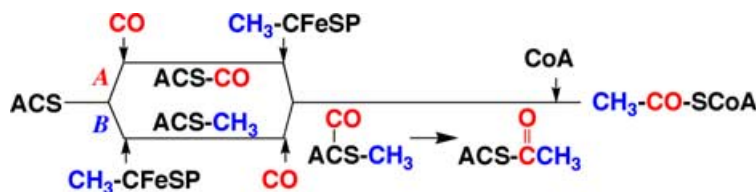


FIGURE 4. Random mechanism of acetyl-CoA synthesis.

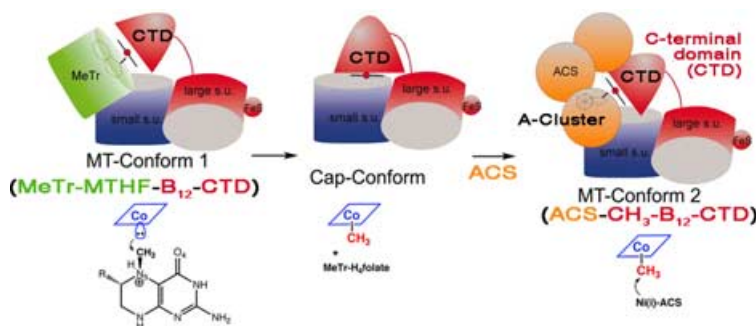


FIGURE 5. Proposed conformers and interactions of the CFeSP. (Modified from Svetlitchnaia *et al.*⁷⁹)

It was concluded that $\text{CH}_3\text{-H}_4\text{folate}$ binds tightly ($K_d < 10 \mu\text{M}$ ⁷⁷) to MeTr within a negatively charged crevice of the triose phosphate isomerase (TIM) barrel.⁷⁸ The structure of the CFeSP was also recently solved.⁷⁹

A key step in the MeTr mechanism is activation of the methyl group of $\text{CH}_3\text{-H}_4\text{folate}$, since the reaction involves displacement at a tertiary amine and because the $\text{CH}_3\text{-N}$ bond is much stronger than the product $\text{CH}_3\text{-Co}$ bond. Of the activation mechanisms that have been considered, protonation at N5 of the pterin seems to be most plausible.⁷¹ Generation of a positive charge on N5 would lower the activation barrier for nucleophilic displacement of the methyl group by the Co(I) nucleophile. There is significant experimental support for protonation at N5 of the pterin, including proton uptake studies,^{77,80} pH dependencies of the steady state, and transient reaction kinetics of MeTr⁸¹ and methionine synthase,⁸² and studies of variants that are compromised in acid–base catalysis.⁷⁶ A question that has not been resolved is whether the proton transfer takes place upon formation of the binary complex, as indicated by studies with MeTr from *M. thermoacetica*,^{49,77} or the ternary complex (with the methyl acceptor), as concluded from studies on *E. coli* methionine synthase.⁸⁰ Recent studies indicate that this protonation step relies on an H-bonding network, instead of a single acid–base catalyst and that an Asn residue is a key component of that network.⁷⁶

As shown in FIGURE 1, the CFeSP interfaces between $\text{CH}_3\text{-H}_4\text{folate/MeTr}$ and CODH/ACS.

This 88-kDa heterodimeric protein contains a $[\text{4Fe-4S}]^{2+/1+}$ cluster and a cobalt cobamide.^{70,74} The Fe-S cluster plays a role in reductive activation of the cobalt to the active Co(I) state.^{83,84} Svetlitchnaia *et al.*⁷⁹ proposed that the C-terminal domain (FIG. 5) of the large subunit is a mobile element that interacts alternatively with the A-cluster domain of ACS and with MeTr. Three major conformers or complexes are described: (1) a methylation complex, in which the Co(I)-CFeSP binds MeTr and accepts the methyl group of $\text{CH}_3\text{-H}_4\text{folate}$; (2) the methylated CFeSP; (3) a complex between ACS and the methylated CFeSP. A fourth conformation, which is not shown here, would be a reductive activation conformer, in which the corrinoid is in the inactive Co(II) state. This molecular juggling proposed for the CFeSP shown in FIGURE 5 has precedent in the related mechanism involving the various domains of methionine synthase, as shown by the elegant structure–function studies of Matthews and Ludwig.^{82,85,86}

Summary

Studies of the enzymes involved in the Wood–Ljungdahl pathway have elucidated new roles of metal ions in biology (including the formation of bioorganometallic intermediates, discovery of new heterometallic clusters, and nucleophilic metal ions), uncovered novel substrate-derived radical intermediates, and revealed channeling of gaseous substrates. These new outcomes and mechanisms will likely be

applicable to other currently less well-studied metal-dependent enzyme systems. Now that detailed structures of PFOR, CODH/ACS, the CFeSP, and MeTr are available to provide a structural framework for these novel and important chemical reactions, mechanistic hypotheses can be posed and tested at a deeper level using a variety of biochemical and biophysical methods.

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Conflict of Interest

The author declares no conflicts of interest.

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