Evidence That Carbon Monoxide Is an Obligatory Intermediate in Anaerobic Acetyl-CoA Synthesis†

Saurabh Menon and Stephen W. Ragsdale*

Department of Biochemistry, Beadle Center, University of Nebraska, Lincoln, Nebraska 68588-0664

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ABSTRACT: Carbon monoxide is produced by several biological reactions. It is proposed to act as an intracellular signaling molecule and can serve as the carbon and electron source for certain bacteria. Direct evidence for a new biological role for CO is presented here. The results strongly indicate that CO is produced as an obligatory intermediate during growth of the acetogenic bacterium *Clostridium thermoaceticum* on glucose, H_2/CO_2, or aromatic carboxylic acids. Our results are consistent with earlier hypotheses of the intermediacy of CO during growth of acetogenic bacteria on CO_2 and hexoses [Diekert, G., & Ritter, M. (1983) *FEMS Microbiol. Lett. 17*, 299−302] and methanogenic *Archaea* on CO_2 [Stupperich, E., Hammel, K. E., Fuchs, G., & Thauer, R. K. (1983) *FEBS Lett. 152*, 21−23]. Therefore, CO production is a key step in the Wood−Ljungdahl pathway of acetyl-CoA synthesis. The carbonyl group of acetyl-CoA is shown to be formed from the carboxyl group of pyruvate by the following steps. (i) Pyruvate undergoes decarboxylation by pyruvate:ferredoxin oxidoreductase to form acetyl-CoA and CO_2. (ii) CO_2 is reduced to CO by the CODH site of the bifunctional enzyme CO dehydrogenase/acetyl-CoA synthase (CODH/ACS). (iii) CO generated *in situ* combines with the ACS active site to form a paramagnetic adduct that has been called the NiFeC species, and (iv) the bound carbonyl group combines with a bound methyl group and CoA to generate acetyl-CoA. To our knowledge, this paper represents the first demonstration of a pathway in which CO is produced and then used as a metabolic intermediate.

Carbon monoxide is produced from the incomplete combustion of organic compounds. CO is also generated biologically during methionine production from methylthioadenosine (Wray & Abeles, 1993) and aromatic amino acid metabolism by bacteria (Hino & Tauchi, 1987), aldehyde decarbonylation by plants (Cheesbrough & Kolattukudy, 1984), and heme degradation by heme oxygenase (Tenhunen *et al*., 1969). There is no evidence that CO generated by these reactions undergoes further metabolism other than oxidation to CO_2. CO has been proposed to serve as a neurotransmitter (Verma *et al*., 1993) and to regulate vascular cGMP levels (Morita *et al*., 1995). Although it is toxic to most aerobic organisms, CO can serve as a carbon and energy source for certain aerobic and anaerobic bacteria (Meyer & Schlegel, 1983). The aerobes convert CO to CO_2, which is assimilated into cell carbon. The role of CO in the metabolism of anaerobes is controversial. CO can serve as the precursor of the carbonyl group of acetyl-CoA (Hu *et al*., 1982) in the Wood−Ljungdahl or reductive acetyl-CoA pathway [for review, see Ljungdahl (1986), Ragsdale (1991), and Wood and Ljungdahl (1991)]. This pathway allows acetogenic bacteria and methanogenic *Archaea* to grow autotrophically. It was proposed that CO can serve as an intermediate during growth of acetogenic bacteria on CO_2 and hexoses (Diekert & Ritter, 1983) and methanogenic *Archaea* on CO_2 (Stupperich *et al*., 1983). However, it has been argued that, although CO can be used *in vitro*, it is not a physiological substrate during growth of these bacteria (Grahame & Demoll, 1995).

The Wood−Ljungdahl pathway of acetyl-CoA formation is summarized in Figure 1. The earliest experiments performed on acetogenic bacteria demonstrated that glucose was converted to 3 mol of acetic acid (Fontaine *et al*., 1942). Glucose is converted to 2 mol of pyruvate by the Embden−Meyerhof pathway (step 1). Pyruvate is then metabolized by two branches: one leading to the carbonyl (the b branch) and the other to the methyl group (the a branch) of acetyl-CoA. On the methyl branch, pyruvate undergoes decarboxylation in a reaction catalyzed by pyruvate:ferredoxin oxidoreductase (PFOR)[1] to form acetyl-CoA and CO_2 (Drake *et al*., 1981) (step 2a). Then, CO_2 is reduced by formate dehydrogenase (Yamamoto *et al*., 1983) and enzymes of the tetrahydrofolate (H_4folate) pathway (Ljungdahl, 1986; Ragsdale, 1991) to form CH_3-H_4folate (step 3a). The methyl group of CH_3-H_4folate then undergoes transfer to a corrinoid iron−sulfur protein (CFeSP) to form methylcobamide (Ragsdale *et al*., 1987) (step 4a) and then to the Ni site on CODH/ACS to form a methylnickel intermediate (Kumar *et al*., 1995) (step 5).

The metabolic branch leading to the carbonyl group of acetyl-CoA has not been as well-understood as the methyl branch. Figure 1 shows several possible intermediates. PFOR catalyzes the decarboxylation of pyruvate to form acetyl-CoA and either free CO_2 or an enzyme-bound [CO_2] species (step 2b). The former is the expected product of the PFOR reaction, while the latter is suggested by experiments indicating that the carboxyl group of pyruvate can be channeled directly into the final steps of acetyl-CoA synthesis without equilibrating with solution CO_2 (Schulman *et al*.,

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* Address correspondence to Stephen W. Ragsdale. Phone: 402-472-2943. Fax: 402-472-7842. E-mail: sragsdale@unlinfo.unl.edu.

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[1] Abbreviations: CODHACS, CO dehydrogenase/acetyl-CoA synthase; PFOR, pyruvate:ferredoxin oxidoreductase; EPR, electron paramagnetic resonance; TPP, thiamin pyrophosphate; H_4folate, tetrahydrofolate; MES, 2-(*N*-morpholino)ethanesulfonic acid.

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1973). The next step involves a two-electron reduction to form an intermediate species at the redox level of CO (step 3b). This species has been referred to as the "carbonyl-level", "[CO]", or "formate-level", "[HCOOH]", intermediate (Drake *et al*., 1981, 1994; Hu *et al*., 1982; Diekert & Wohlfarth, 1994; Müller & Gottschalk, 1994; Grahame & Demoll, 1995). One possibility is that this intermediate is CO *per se*, which can be formed by the reduction of CO_2 by CODH/ACS (eq 1). It was demonstrated over a decade ago that CO could replace pyruvate or CO_2 as the donor of the carbonyl group of acetyl-CoA (Hu *et al*., 1982) (eq 2). However, it has been argued that, although CO can be used *in vitro*, it is not a physiological substrate (Grahame & Demoll, 1995).

$$2H^+ + *CO_2 + 2e^- \rightarrow *CO + H_2O \quad (1)$$

$$*CO + CH_3\text{-}H_4\text{folate} + CoA \rightarrow CH_3\text{-}*CO\text{-}SCoA + H_4\text{folate} \quad (2)$$

Another possible intermediate in the synthesis of the carbonyl group of acetyl-CoA is a paramagnetic adduct between CO and CODH/ACS (step 4b). This species exhibits an electron paramagnetic resonance (EPR) spectrum with *g* values of 2.08, 2.07, and 2.03 and has been called the NiFeC signal (Ragsdale *et al*., 1982). Studies of the CO-reacted enzyme by electron nuclear double resonance (Fan *et al*., 1991), EPR (Ragsdale *et al*., 1985; Fan *et al*., 1991; Xia *et al*., 1995), X-ray absorption (Cramer *et al*., 1987; Bastian *et al*., 1988; Xia *et al*., 1995), and Mössbauer (Lindahl *et al*., 1990b) spectroscopy have demonstrated that this species is an unusual heterometallic cluster in which a nickel ion is bridged to a $[Fe_4S_4]^{2+/1+}$ cluster, e.g., [Ni-X-Fe_4S_4], where X is an unknown bridge between the Ni ion and one of the irons in the cubane cluster. Questions have been raised about whether this adduct is a catalytic intermediate in the pathway of acetyl-CoA synthesis (Gorst & Ragsdale, 1991) or is formed in a side reaction resulting from high concentrations of the "powerful, nonphysiological reducing agent CO" (Grahame & Demoll, 1995). A variety of biochemical studies, including rapid freeze−quench EPR and resonance Raman spectroscopy, stopped flow, rapid chemical quench, and isotope exchange, have provided support for the intermediacy of the [Ni-X-Fe_4S_4]-C≡O species during acetyl-CoA synthesis from CO (Ragsdale *et al*., 1985; Shanmugasundaram *et al*., 1988; Gorst & Ragsdale, 1991; Kumar *et al*., 1993; Qiu *et al*., 1994).

The methyl and carbonyl branches converge at CODH/ACS which catalyzes C−C bond formation to generate a bound acetyl intermediate and C−S bond formation to generate the third mole of acetyl-CoA from the six carbons of glucose. Acetyl-CoA then is utilized for cell carbon synthesis during autotrophic growth or for energy as cleavage of the thioester bond is coupled to ATP synthesis.

The work described here focused on the mechanism of acetyl-CoA synthesis from pyruvate, CH_3-H_4folate, and CoA. It was aimed at answering several important questions. Do the pathways of acetyl-CoA synthesis from CO, CO_2, and the carboxyl group of pyruvate have the same intermediates? Is CO a physiological intermediate in formation of the carbonyl group of acetyl-CoA? What is the nature of the [CO] or [HCOOH] intermediate? Can the NiFeC intermediate be formed from substrates other than CO? It was demonstrated that both CO and the NiFeC species are intermediates that are generated from CO, CO_2, and the carboxyl group of pyruvate. The Wood−Ljungdahl pathway was, thus, identified as the first example of a biological pathway that generates and assimilates CO as a metabolic intermediate.

MATERIALS AND METHODS

Organism and Enzyme Purification. *Clostridium thermoaceticum* strain ATCC 39073 was grown on glucose at 55 °C (Andreesen *et al*., 1973). PFOR was purified as described (Wahl & Orme-Johnson, 1987) except that ion exchange chromatography was performed on a DEAE Sephacel column and the enzyme was eluted with a 2 L linear gradient from 0 to 0.4 M NaCl in 50 mM 3-(*N*-morpholino)-propanesulfonate buffer (pH 7.5). CODH/ACS (Ragsdale *et al*., 1983a), the CFeSP (Ragsdale *et al*., 1987), ferredoxin (Elliot & Ljungdahl, 1982), and methyltransferase (Zhao *et al*., 1995) were purified as described under strictly anaerobic conditions at 17 °C in a Vacuum Atmospheres chamber maintained below 1 ppm oxygen. Protein concentrations were determined by the Rose Bengal method (Elliott & Brewer, 1978).

The standard PFOR activity assay during purification [modified from Zeikus *et al*. (1977)] contained 50 mM Tris (pH 7.5), 1 mM thiamin pyrophosphate (TPP), 1 mM CoA, and 10 mM methyl viologen in a final volume of 1 mL. The assay was performed at 25 °C. PFOR concentrations are expressed as the dimer (240 kDa). The average specific activity of CODH was 300 u mg^{-1} (1 u = 1 μmol of CO oxidized per minute) at 55 °C as measured by the standard methyl viologen-linked assay at pH 7.6 (Ragsdale *et al*., 1983a). CODH/ACS concentrations are expressed in terms of the $\alpha_2\beta_2$ tetramer (310 kDa)[2] (Xia *et al*., 1996).

Materials. N_2 (99.98%) and CO (99.99%) were obtained from Linweld (Lincoln, NE). N_2 was deoxygenated by passing it through a heated column containing BASF catalyst. Reagents were of the highest purity available.

Enzyme Assays. Acetyl-CoA formation from carbons 2 and 3 of pyruvate was measured by a coupled malate dehydrogenase/citrate synthase assay. The reaction mixture contained 200 nM PFOR, 10 mM pyruvate, 1 mM CoA, 1 mM TPP, and 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6.0) in a total volume of 1 mL. The electron carriers and their concentrations were varied. At different times, 30 μL aliquots were removed and assayed as described (Pearson, 1965). CO_2 concentrations were measured by a continuous spectrophotometric assay that couples formation of CO_2 to phosphoenolpyruvate carboxylase and malate dehydrogenase in the presence of NADH as described (Hall *et al*., 1983).

Conversion of the carboxyl of pyruvate to CO was followed at 25 °C by measuring the formation of the hemoglobin−CO complex essentially as described (Kumar *et al*., 1994). Aliquots from a PFOR reaction were removed with a Hamilton gastight syringe into a 1 mL assay mixture containing 2 μM hemoglobin that had been reduced with 20

[2] CODH recently was shown by sedimentation equilibrium (Xia *et al*., 1996) and electron micrographic and chemical cross-linking (Sundaresh *et al*., 1996) studies to be a tetramer. Because it has a large Stokes radius relative to its molecular mass, previously, it was thought to be a hexamer (Ragsdale *et al*., 1983a).

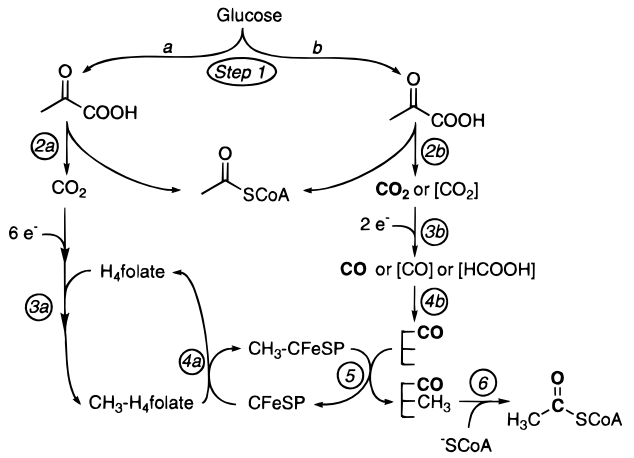


FIGURE 1: Pathway of acetyl-CoA synthesis from the carboxyl group of pyruvate.

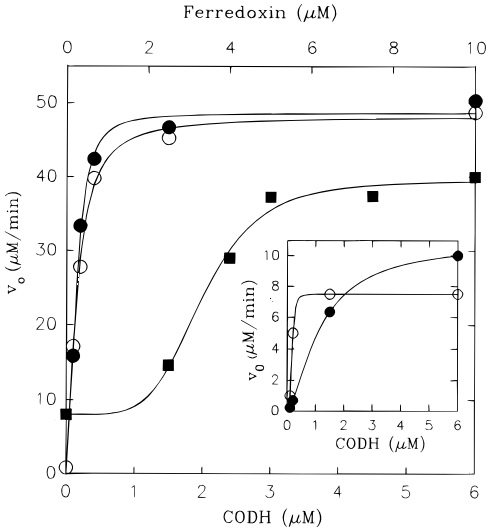


FIGURE 2: Acetyl-CoA and CO synthesis from pyruvate. The assay for the synthesis of acetyl-CoA from pyruvate was performed in 50 mM MES pH 6.0 and contained 200 nM PFOR, 6 μM CODH/ACS ($\alpha_2\beta_2$), and varied concentrations of ferredoxin (■). Acetyl-CoA was quantitated by the malate dehydrogenase/citrate synthase assay (see Materials and Methods). The curve was derived from a fit to the Hill plot which yielded a k_{cat} of 200 ± 4 min^{-1}, a K_m for ferredoxin of 3 ± 0.12 μM, and a Hill coefficient (n_H) of 4 ± 0.5. The synthesis of acetyl-CoA from carbons 2 and 3 of pyruvate (○) and CO from carbon 1 of pyruvate (●) was performed as described above, but at varied concentrations of CODH/ACS and fixed ferredoxin (5 μM). CO was quantitated by the hemoglobin−CO assay. The curves were fit to the Hill equation. For acetyl-CoA synthesis, k_{cat} was 244 ± 6 min^{-1}, K_m for CODH/ACS was 150 ± 10 nM, and n_H was 1.5 ± 0.2. For CO formation, k_{cat} was 243 ± 6 min^{-1}, K_m for CODH/ACS was 140 ± 8 nM, and n_H was 2.0 ± 0.2. (Inset) The synthesis of acetyl CoA (●) from carbons 2 and 3 of pyruvate and CO from carbon 1 of pyruvate (○) with CODH/ACS as the only electron acceptor. The curves were generated from fits to the Hill equation. For acetyl-CoA synthesis, k_{cat} was 37.5 ± 0.1 min^{-1}, K_m for CODH/ACS was 166 ± 1 nM, and n_H was 3.7 ± 0.1; for CO formation, k_{cat} was 54.5 ± 0.4 min^{-1}, K_m for CODH/ACS was 1.2 ± 0.1 μM, and n_H was 1.5 ± 0.1.

μM sodium dithionite in MES buffer (pH 6.0). Formation of the hemoglobin−CO complex was measured at 430 nm where the change in molar extinction coefficient is 0.08 μM^{-1} cm^{-1} (Bonam & Ludden, 1987). In the absence of CODH/ACS, CO was not detected.

Assay of acetyl-CoA synthesis from CH_3-H_4folate, CoA, and pyruvate [modified from Drake *et al*. (1981)] was conducted with MeTr, CODH/ACS, and the CFeSP at saturating levels. The reaction was performed in the dark in a glass V-shaped reaction vial capped with a red rubber serum stopper. The reaction mixture contained 4 μmol of pyruvate, 2 μmol of CoA, 0.4 μmol of TPP, 12 nmol of CODH/ACS, 20 nmol of methyltransferase, 40 nmol of CFeSP, 0.2 nmol of PFOR, and 50 μmol of MES buffer (pH 6.0) in a total volume of 1 mL. After the vial was equilibrated for 2 min in a heating block, the reaction was initiated with 1 μmol of $^{14}CH_3$-H_4folate (100 000 dpm/mmol, from Amersham). At various times, 25 μL aliquots of the reaction mixture were analyzed by Dowex-50W H^+ chromatography.

EPR Spectroscopy. EPR spectra were recorded on a Bruker ESP 300E spectrometer equipped with an Oxford ITC4 temperature controller, a Hewlett-Packard Model 5340 automatic frequency counter, and a Bruker Gaussmeter. The spectroscopic parameters are given in the figure legends.

RESULTS

The multistep synthesis of acetyl-CoA from the carboxyl group of pyruvate, CH_3-H_4folate, and CoA (Figure 1) was studied with purified enzymes from the acetogenic bacterium *C. thermoaceticum*. Decarboxylation of pyruvate by PFOR was performed with different electron acceptors at pH 7.6, which is the optimum pH for the decarboxylation reaction. Electron acceptors used included methyl viologen, ferredoxin, and phenosaphranin. The reaction rate was linear with PFOR concentration, and the steady-state rates of acetyl-CoA and CO_2 formation and electron carrier reduction were identical. Purified PFOR has a specific activity of 17.8 u/mg (k_{cat} = 4200 min^{-1}) at pH 7.6 and 25 °C in the presence of 10 mM methyl viologen. This compares favorably with a specific activity of 12 u/mg at pH 7.5 and 30 °C determined previously for the purified *C. thermoaceticum* PFOR (Wahl & Orme-Johnson, 1987). The k_{cat} for pyruvate decarboxylation at pH 6.0 was found to be 7.2-fold lower than that at pH 7.6.

To establish the mechanism by which the carboxyl group of pyruvate is converted to the carbonyl group of acetyl CoA, the objective was to detect and characterize the intermediates. The reactions were performed (Figure 2) in the presence of ferredoxin, which is the recognized physiological electron acceptor for the clostridial PFOR (Uyeda & Rabinowitz, 1971; Rabinowitz, 1972; Drake *et al*., 1981) and CODH/ACS (Ragsdale *et al*., 1983a,c). They were performed at pH 6.0, which is the optimum pH for CO_2 reduction (Kumar *et al*., 1994) and acetyl-CoA synthesis from CH_3-H_4folate, pyruvate, and CoA (Drake *et al*., 1981). When CODH/ACS was present in the reaction mixture, CO was produced (steps 2b and 3b). The rates of CO production were measured by a hemoglobin-based assay. In this assay, hemoglobin serves as a CO indicator; however, because it has a high affinity for CO, it could also displace the reaction equilibrium to indicate a higher level of CO than that present in the reaction mixture. Thus, the measured rates of CO formation are maximum rates. In the absence of CODH/ACS, CO was not detected. The rates of pyruvate decarboxylation and CO production were ~5-fold faster in the presence (●, ○) than in the absence (Figure 2, inset) of ferredoxin. The data best fit a model that includes a cooperative interaction between CODH/ACS and PFOR. Data treatment according to the Hill equation yielded values for k_{cat} of 244 min^{-1}, a K_m for

CODH/ACS of 0.15 μM, and a Hill coefficient (n_H) of 2. The k_{cat} for CO production was found to equal that of pyruvate decarboxylation, demonstrating that CO was formed as fast as pyruvate was decarboxylated. Since free CO_2 is the decarboxylation product of pyruvate, the rate-limiting step under these conditions appears to be pyruvate decarboxylation, not reduction of CO_2 to CO.

A sigmoidal kinetic curve was observed (■) when ferredoxin was varied at a fixed concentration of CODH/ACS (6 μM) (Figure 2). Analysis by the Hill equation gave values for k_{cat} of 200 min^{-1}, n_H of 4, and K_m for ferredoxin of 3 μM. Although ferredoxin was stimulatory, significant rates of pyruvate decarboxylation and CO formation were measured in its absence, indicating that CODH/ACS can accept electrons directly from PFOR. The PFOR reaction was then performed with varying concentrations of CODH/ACS in the absence of ferredoxin (Figure 2, inset). At low concentrations of CODH/ACS, acetyl-CoA synthesis lagged behind CO production. Although the k_{cat} value was decreased by ~5-fold when ferredoxin was absent, the k_{cat}/K_m values for ferredoxin and CODH/ACS were similar (6.7×10^7 and 2×10^8 M^{-1} min^{-1}, respectively). This indicates that CODH/ACS and ferredoxin are equally proficient electron acceptors for PFOR. Therefore, CODH/ACS interacts with PFOR to efficiently couple the dearboxylation of pyruvate to the reduction of CO_2 to CO.

Demonstration that CO is produced from pyruvate at kinetically competent rates combined with previous studies using whole cells (Diekert *et al*., 1984) and partially purified (Hu *et al*., 1982) and homogeneous preparations of enzymes (Ragsdale & Wood, 1985) that showed that CO can serve as the carbonyl group of acetyl-CoA provide strong evidence that CO, generated from the carboxyl group of pyruvate, is an intermediate in acetyl-CoA synthesis. However, the latter experiments, which were performed with saturating levels of CO, are open to the criticism that CO is effective only because it is a strong, albeit nonphysiological, reductant (Grahame & Demoll, 1995). Further evidence for the intermediacy of CO was obtained by demonstrating that hemoglobin, which binds CO tightly, inhibits acetyl-CoA synthesis from the carboxyl group of pyruvate, CH_3-H_4folate, and CoA (Figure 3). The data fit a model, described by Scheme 1, in which hemoglobin and ACS compete for CO. This model invokes a mode of inhibition resulting from removal of substrate (CO) from the reaction (Segel, 1975) and is described by eq 3, where v_{inh}/v_{uninh} is the ratio between the rates of acetyl-CoA synthesis determined in the presence

$$v_{inh}/v_{uninh} = \frac{K_S^{CO} + [CO]}{[CO] + K_S^{CO}\left(1 + \frac{[CO][Hb]}{K_{Si}K_O^{CO}}\right)} \quad (3)$$

$$[Hb{-}CO] = 1/2(z \pm \sqrt{z^2 - 4[CO]_t[Hb]}) \quad (4)$$

and absence of hemoglobin. K_S^{CO} is the K_d of ACS for CO; K_{Si} is the dissociation constant for the ternary complex between CO, hemoglobin, and ACS, and K_O^{CO} is the dissociation constant for the binary hemoglobin−CO complex.[3] To determine the steady-state concentration of CO generated *in situ* from the carboxyl of pyruvate and used for acetyl-CoA synthesis, the complex between CO and hemoglobin was monitored by UV−visible spectroscopy. Formation of the hemoglobin−CO complex causes the Soret band to shift, resulting in changes in absorbance at 419 nm ($\Delta\epsilon = +82$ mM^{-1} cm^{-1}) and 430 nm ($\Delta\epsilon = -80$ mM^{-1} cm^{-1}). The hemoglobin−CO concentration was then calculated from difference spectra generated at each hemoglobin concentration. The data were plotted and fit to the quadratic eq 4 (the fit line is shown in Figure 3), which is derived from the Scatchard equation, where $[Hb{-}CO]$, $[CO]_t$, and $[Hb]_t$ are the hemoglobin−CO, total CO, and total hemoglobin concentrations, respectively, and z is $[Hb]_t + [CO]_t + K_O^{CO}$. The steady-state CO concentration derived from the fit was 90 μM. The K_O (or K_d) of hemoglobin for CO under the assay conditions, a parameter in eqs 3 and 4, was obtained in two ways. First, a hemoglobin solution identical to the assay mixture but lacking the enzymes and pyruvate was titrated

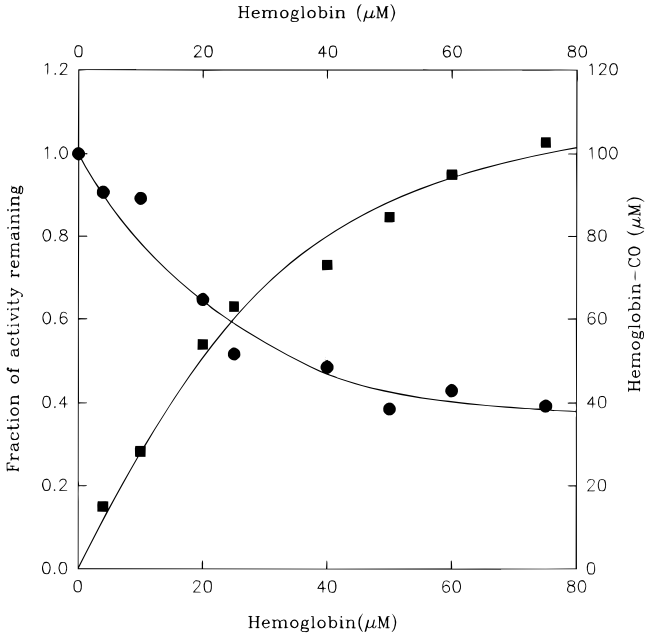
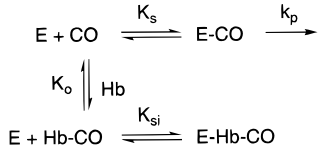


FIGURE 3: Inhibition of acetyl-CoA synthesis by hemoglobin. Acetyl-CoA synthesis from CH_3-H_4folate, CoA, and pyruvate was performed as described in Materials and Methods. The initial velocity for acetyl-CoA synthesis (●) and the concentration of the hemoglobin−CO complex (■) were measured at varied concentrations of hemoglobin (0−75 μM). Inhibition data were fit to eq 3, and hemoglobin−CO concentrations were fit to eq 4. The total steady-state concentration of CO generated from pyruvate under these conditions was 90 μM. The value of K_{si} obtained from the fit was 21 ± 2 μM.

Scheme 1: Model To Explain Inhibition of Acetyl-CoA Synthesis by Hemoglobin[a]



[a] Equation 4 [from Segel (1975)] is based on this model.

[3] This model includes a step in which a ternary complex is formed between CODH/ACS, hemoglobin, and CO. We attempted to fit the data with a simpler model that includes competition between CODH/ACS and hemoglobin for CO and lacks the ternary complex; however, we were unable to fit the data with realistic values of K_s and K_0. The model described in Scheme 1 allows a good fit to the data, and the calculated dissociation constants for the Hb−CO and the CODH−CO complexes are in accord with values determined previously.

with increasing concentrations of CO and fit to the Scatchard equation. Second, it was derived along with the CO concentration from the fit to eq 4. Within a 10% standard deviation, the same value for the K_O for CO was obtained by both methods.

The excellent fit of the inhibition data to the model in Scheme 1 and the fact that the two lines in Figure 3 mirror each other provide unambiguous evidence that CO is generated from the carboxyl group of pyruvate and is utilized to form acetyl-CoA. In summary, CO is produced and utilized by the following steps. (i) The pyruvate carboxyl group is converted to acetyl-CoA and CO_2 by PFOR (step 2b, Figure 1); (ii) CO_2 is reduced to CO by the CO oxidation site of CODH/ACS (step 3b), and (iii) *in situ*-generated CO is converted to the carbonyl group of acetyl-CoA at the ACS active site (step 6). CODH/ACS contains 12 Fe, 2 Ni, and 14 inorganic acid-labile sulfides that are arranged into three clusters: clusters A and C, both $NiFe_4S_4$ clusters, and cluster B, a Fe_4S_4 cluster (Ragsdale, 1994; Hu *et al*., 1996). CO_2 reduction takes place at cluster C of CODH/ACS (Anderson *et al*., 1993; Kumar *et al*., 1993; Qiu *et al*., 1995; Hu *et al*., 1996).

Since a variety of studies indicate that cluster A of CODH/ACS is the ACS active site (Gorst & Ragsdale, 1991; Shin & Lindahl, 1992; Kumar *et al*., 1993), there must be an intramolecular migration of CO from cluster C to cluster A. A stable adduct between CO and cluster A has been identified. This adduct has been called the "NiFeC species" and has been studied by spectroscopic and kinetic methods [see Ragsdale (1994) for review]. A variety of experiments have provided strong evidence that the NiFeC species is a catalytically competent intermediate in acetyl-CoA synthesis when CO is the donor of the carbonyl group of acetyl-CoA synthesis (Ragsdale *et al*., 1985; Gorst & Ragsdale, 1991; Kumar *et al*., 1993; Qiu *et al*., 1994, 1995). However, its intermediacy during acetyl-CoA synthesis from pyruvate or CO_2 and H_2 has not been studied. It has been contended that the NiFeC species is formed in a side reaction that is not on the main catalytic pathway for acetyl-CoA synthesis (Grahame & Demoll, 1995). We reasoned that, if it is an intermediate, then the NiFeC EPR signal should be formed from pyruvate at rates competent to support acetyl-CoA synthesis. This was a difficult experiment because the product of the reaction is actually an enzyme-bound species. CODH/ACS was used in a 60-fold higher concentration than PFOR so that, early in the reaction, the EPR signal on CODH/ACS would be formed under steady-state conditions. PFOR, thus, was at "catalytic concentrations" and would undergo multiple turnovers. The results shown in Figure 4 demonstrate that the NiFeC species was formed at a rate ($k_{cat} = 47$ min^{-1}) that was 7-fold faster than the rate of acetyl-CoA synthesis from the carboxyl of pyruvate, CoA, and CH_3-H_4folate ($k_{cat} = 6.3$ min^{-1}). Therefore, the NiFeC species can be considered to be a kinetically competent intermediate in acetyl-CoA synthesis from the carboxyl group of pyruvate.

Isotope substitution experiments were used to demonstrate that the "carbon" in the NiFeC signal was derived from the carboxyl group of pyruvate (Figure 5). A significant broadening of the NiFeC signal was observed (10 and 8 G broadening of the 2.028 and 2.074 resonances, respectively) when [1-^{13}C]pyruvic acid was reacted with PFOR and CODH/ACS. This degree of line broadening is similar to that observed from the reaction of ^{13}CO with CODH/ACS

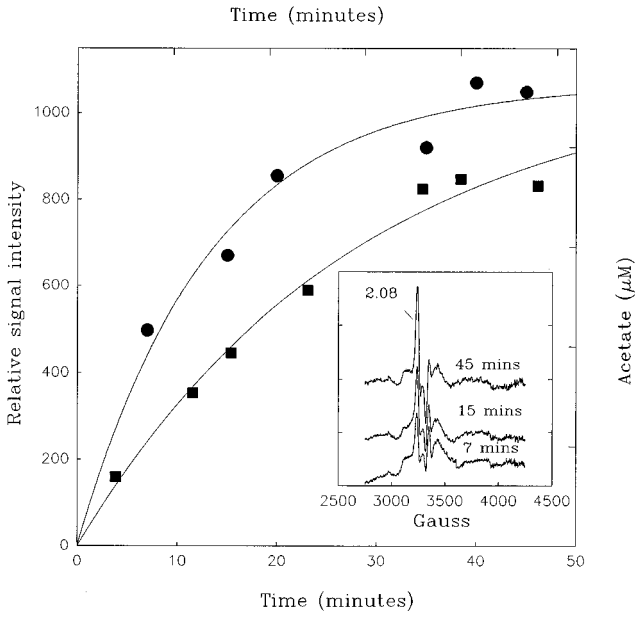


FIGURE 4: NiFeC signal formation and acetyl-CoA synthesis from pyruvate. The synthesis of acetyl-CoA from pyruvate, CH_3-H_4-folate, and CO (■) was performed and assayed at 25 °C as described in Materials and Methods. The value of k_{cat}, obtained from the initial velocity measurement, was 6 ± 0.9 min^{-1}. Acetyl-CoA synthesis was also performed at 55 °C, where the rate was 8-fold higher, as expected for a doubling of rate with every 10 °C increase in temperature. Formation of the NiFeC EPR signal (●) was performed under similar conditions at 25 °C in individual EPR tubes containing CODH/ACS (12 μM), PFOR (0.2 μM), CoA (1 mM), and 50 mM MES buffer (pH 6.0). Pyruvate (10 mM) was added to initiate the reaction, which was quenched at different times by freezing the EPR tube in liquid nitrogen. The data were fit to a single-exponential equation (described by the solid line). Since CODH/ACS was in excess relative to the PFOR concentration, it was treated as a substrate and the initial velocity of NiFeC EPR signal formation was measured from the tangent to the exponential curve. The value of k_{cat}, obtained from the initial velocity measurement was 47 ± 3 min^{-1}. The EPR parameters were as follows: spectral width, 1500 G; field center, 3500 G; modulation frequency, 100 kHz; gain, 2 × 10^4; temperature, 80 K; microwave power, 40 mW; and frequency, 9.447 GHz. (Inset) Representative EPR spectra at 7, 15, and 45 min.

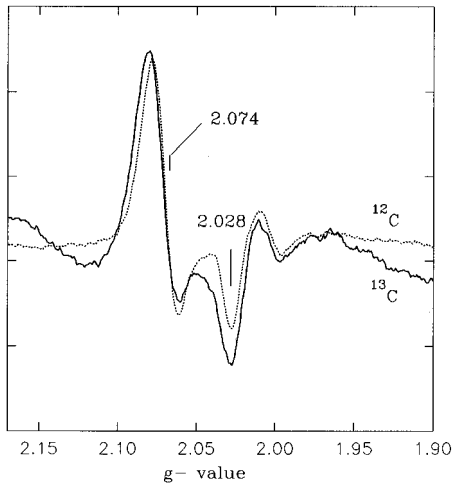


FIGURE 5: Demonstration that the "carbon" in the NiFeC EPR signal is derived from the carboxyl group of pyruvate. CODH/ACS was incubated under conditions identical to those in Figure 4 except that the carboxyl group of pyruvate was labeled with ^{12}C (•••) or ^{13}C (—).

(Ragsdale *et al*., 1983b, 1985; Fan *et al*., 1991). There was no observable increase in line width when [2-^{13}C]pyruvic

acid was reacted under identical conditions. As directed above, since CODH/ACS was in vast excess relative to PFOR, we analyzed the reaction by initial velocity kinetics with CODH as a substrate. The rates of formation of the NiFeC signal using [1-^{12}C]-*versus* [1-^{13}C]pyruvic acids (Cambridge Isotope Labs) were identical (47 min^{-1}). Previous isotope exchange studies demonstrated that the carbonyl group in the NiFeC species can be incorporated into the carbonyl group of acetyl-CoA (Raybuck *et al*., 1987, 1988; Gorst & Ragsdale, 1991; Kumar & Ragsdale, 1992; Qiu *et al*., 1994). Therefore, the combined EPR and kinetic experiments provide strong evidence that the NiFeC species is an intermediate in the conversion of the carboxyl group of pyruvate to the other carbonyl group of acetyl-CoA.

DISCUSSION

CO was established as an obligatory intermediate in the synthesis of acetyl-CoA from the carboxyl group of pyruvate. This was demonstrated by establishing that CO was formed from the carboxyl group of pyruvate at kinetically competent rates and that acetyl-CoA synthesis was inhibited by hemoglobin, which removes CO from the reaction mixture. Our results are consistent with earlier hypotheses of the intermediacy of CO during growth of acetogenic bacteria on CO_2 and hexoses (Diekert & Ritter, 1983) and methanogenic *Archaea* on CO_2 (Stupperich *et al*., 1983). These proposals were based on the demonstration that *Methanobacterium thermoautotrophicum* produces small amounts of CO during growth on H_2 and CO_2 (Conrad & Thauer, 1983), that the acetogen, *Acetobacterium woodii*, incorporates ^{14}CO specifically into the carboxyl group of acetate (Diekert & Ritter, 1983), and that small amounts of CO are produced by *A. woodii* and *C. thermoaceticum* grown on glucose (Diekert *et al*, 1984).

CO oxidation and CO_2 reduction occur at cluster C of CODH/ACS. The former reaction has been the major focus of attention since it allows organisms to grow on CO as a sole carbon and energy source and since it is ~1000-fold faster than CO_2 reduction. However, the results described here demonstrate that synthesis of CO by cluster C is essential for bacteria to metabolize heterotrophic substrates that generate pyruvate, such as hexoses and pentoses, by the reductive acetyl-CoA pathway. Since CO_2 is the decarboxylation product of pyruvate, our results strongly indicate that CO is also generated as an intermediate during growth on H_2/CO_2 and other substrates that generate CO_2, such as formate and aromatic carboxylates like syringate and vanillate. To our knowledge, the Wood−Ljungdahl pathway is the first that has been shown to produce and assimilate CO as a metabolic intermediates. Since methanogens and other anaerobes also use the Wood−Ljungdahl pathway, have similar CODH and ACS active sites, and grow on CO and H_2/CO_2, our *in vitro* enzyme studies agree with the *in vivo* studies described above and strongly suggest that these autotrophic anaerobes also generate CO as a metabolic intermediate.

CO was found to be produced and assimilated into acetyl-CoA at similar rates. This is significant because, if CO_2 reduction were much faster than CO assimilation, then CO would accumulate in the cell and inhibit hydrogenases and other metalloenzymes. Small amounts of CO are generated during methanogenesis from H_2 and CO_2 (Conrad & Thauer, 1983) and acetogenesis from sugars (Diekert *et al*., 1984); most of the reducing equivalents are shunted into energy and cell carbon production. When acetogens are subjected to high levels of CO, they respond by evolving H_2 and CO_2 (Martin *et al*., 1983) and converting CO to acetyl-CoA.[5] Therefore, it appears that escape of CO from organisms using the acetyl-CoA pathway is negligible. A significant elevation of environmental CO levels would be harmful for most organisms which, like humans, are poisoned by low levels of CO. Thus, evolution of the acetyl-CoA pathway allowed anaerobes to grow on inorganic carbon and enabled them to respond to the quandary of CO toxicity in the early environment.

The results described here demonstrate that, besides CO, another intermediate is formed during acetyl-CoA synthesis from pyruvate, the paramagnetic NiFeC species. This is an adduct between CO and one of the Fe sites in the $[Fe_4S_4]^{2+/1+}$ component of cluster A, which contains a square planar Ni ion bridged to a $[Fe_4S_4]^{2+/1+}$ cluster (Lindahl *et al*., 1990a,b; Fan *et al*., 1991; Xia *et al*., 1995; Xia & Lindahl, 1996). Numerous studies have indicated the intermediacy of this species in acetyl-CoA synthesis from CO. Kinetic studies have shown that it is formed fast enough to support acetyl-CoA synthesis (Gorst & Ragsdale, 1991; Kumar *et al*., 1993; Qiu *et al*., 1995), that the carbon undergoes exchange with the carbonyl group of acetyl-CoA (Gorst & Ragsdale, 1991; Kumar & Ragsdale, 1992; Qiu *et al*., 1994), and that it is formed from acetyl-CoA through a reversal of the pathway (Gorst & Ragsdale, 1991). The EPR signal has also been observed when CODH/ACS was reacted with CO_2 under reducing conditions (Lindahl *et al*., 1990a). The NiFeC species was also shown to disappear when the CO-reacted methanogenic CODH/ACS was treated with the methyl donor, methyltetrahydrometanopterin (Grahame *et al*., 1996). We have shown here that the NiFeC species was formed from the carboxyl group of pyruvate at rates sufficient to support acetyl-CoA synthesis. This is important because it demonstrates that this intermediate not only is formed during acetyl-CoA synthesis from CO but also is formed even when bacteria are grown on glucose. Therefore, we propose that acetyl-CoA synthesis from CO, CO_2, glucose, and carboxylated aromatics includes at least two common intermediates, CO and the paramagnetic NiFeC species. Since anaerobic acetate utilization appears to occur through a reversal of the pathway for acetyl-CoA synthesis, we propose that both intermediates are also formed during aceticlastic methanogenesis and sulfate reduction.

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[4] A kinetic isotope effect was not expected since, even if there were a ^{13}C primary kinetic isotope effect, the difference in rate due to the isotope substitution is predicted to be too small to be detected by initial velocity measurements.

[5] Demonstration of H_2 production from CO is interesting since acetogenic hydrogenases can be among the most CO-sensitive enzymes in nature, with K_i values in the low-nanomolar range (Ragsdale & Ljungdahl, 1984).

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