Channeling of Carbon Monoxide during Anaerobic Carbon Dioxide Fixation†

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ABSTRACT: Carbon monoxide is an intermediate in carbon dioxide fixation by diverse microbes that inhabit anaerobic environments including the human colon. These organisms fix CO_2 by the Wood−Ljungdahl pathway of acetyl-CoA biosynthesis. The bifunctional CO dehydrogenase/acetyl-CoA synthase (CODH/ACS) catalyzes several key steps in this pathway. CO_2 is reduced to CO at a nickel iron−sulfur cluster called cluster C located in the CODH subunit. Then, CO is condensed with a methyl group and coenzyme A at cluster A, another nickel iron−sulfur cluster in the ACS subunit. Spectroscopic studies indicate that clusters A and C are at least 10−15 Å apart. To gain a better understanding of how CO production and utilization are coordinated, we have studied an isotopic exchange reaction between labeled CO_2 and the carbonyl group of acetyl-CoA with the CODH/ACS from *Clostridium thermoaceticum*. When solution CO is provided at saturating levels, only CO_2-derived CO is incorporated into the carbonyl group of acetyl-CoA. Furthermore, when high levels of hemoglobin or myoglobin are added to remove CO from solution, there is only partial inhibition of the incorporation of CO_2-derived CO into acetyl-CoA. These results provide strong evidence for the existence of a CO channel between cluster C in the CODH subunit and cluster A in the ACS subunit. The existence of such a channel would tightly couple CO production and utilization and help explain why high levels of this toxic gas do not escape into the environment. Instead, microbes sequester this energy-rich carbon source for metabolic reactions.

CO is produced anthropogenically by the incomplete combustion of organic materials. CO also is produced naturally by bacteria (*4*−*6*), plants (*7*), and animals (*8*). Although CO is highly toxic to all oxygen-respiring organisms, in animals, heme-oxygenase-derived CO acts as a neurotransmitter by activating neuronal guanylyl cyclase (*9, 10*). Despite the many sources of CO, its atmospheric concentration remains at approximately 100 parts per billion. This is due to several processes, including metabolism by bacteria (*11*).

Certain anaerobic microbes, including methanogenic Archaea and acetogenic bacteria, produce carbon monoxide as an intermediate during carbon dioxide fixation. These organisms have the potential to generate significant quantities of CO by the high CO_2 reductase activity of CODH (*12*); however, they release only low levels of CO during growth (*13, 14*). This is presumably because they convert CO_2-derived CO into acetyl-CoA by the Wood−Ljungdahl pathway (*15, 16*). In acetogenic bacteria, the catalysts for CO_2 reduction and for forming acetyl-CoA from CO, CoA, and a methyl group (donated by a vitamin B_{12} protein) are located on separate subunits of the bifunctional protein CO dehydrogenase/acetyl-CoA synthase (CODH/ACS)[1] (*17*). CO_2 reduction takes place at a nickel iron−sulfur cluster called cluster C on the small 78 kDa subunit (*18, 19*), whereas acetyl-CoA synthesis occurs at a different nickel iron−sulfur cluster called cluster A, which is housed in the large 79 kDa subunit (*19*). In methanogenic Archaea, these sites are located within a five-protein CODH/ACS complex (*20*−*23*).

It is clear that CO is a true intermediate in acetyl-CoA synthesis by acetogenic bacteria whether they grow on inorganic substrates such as H_2/CO_2 or organic substrates such as glucose (*24*). For example, when they metabolize pyruvate, pyruvate:ferredoxin oxidoreductase converts pyruvate to acetyl-CoA and CO_2; CODH reduces CO_2 to CO; and ACS condenses CO with CoA and the methyl group to form acetyl-CoA (*24*). Then, acetyl-CoA is used for biosynthesis of cell material or is converted to acetate, thus generating ATP by substrate-level phosphorylation. Two alternative scenarios for the utilization of CO by CODH/ACS can be considered. One possibility is that CO derived from CO_2 could migrate from cluster C in the small subunit through a channel in the interior of the protein to the large subunit. There it would react with cluster A to form a metal−carbonyl species that condenses with the methyl group and CoA to form acetyl-CoA (*25*−*27*). Another possibility is that CO, produced at the CODH active site, escapes into solution and then binds to cluster A at the ACS active site where it is incorporated into acetyl-CoA.

Evidence described here strongly suggests that CO production is tightly coupled to CO utilization. This coupling is proposed to be achieved by a channel connecting the two subunits of the bifunctional enzyme, CODH/ACS. CO produced at the active site for CO_2 reduction is channeled to the site of acetyl-CoA synthesis. In very recent studies, it was found that hemoglobin did not inhibit the synthesis of acetyl-CoA from CO_2, CH_3-H_4folate, and CoA, also supporting the concept of a CO channel (*28*).

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[1] Abbreviation: CODH/ACS, CO dehydrogenase/acetyl-CoA.

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EXPERIMENTAL PROCEDURES

Materials. N_2 (99.98%) and CO (99.99%) were obtained from Linweld (Lincoln, NE). N_2 was deoxygenated by passing through a heated column containing BASF catalyst. Reagents were of the highest purity available. Titanium(III) citrate was prepared as described (*29*). All other materials were of the highest purity available.

Organism and Enzyme. C. thermoaceticum (now named *Moorella thermoacetica*) strain ATCC 39073 was grown on glucose and CO_2 as previously described (*30*). CODH/ACS (*31*) was purified as described except that the ammonium sulfate precipitation step was omitted. The protein was purified under strictly anaerobic conditions at 17 °C in a Vacuum Atmospheres chamber maintained below 1 ppm oxygen (Teledyne Oxygen Analyzer). Protein concentrations were determined by the Rose Bengal method (*32*).

Enzyme Assays. The CODH specific activity of CODH/ACS was 360 units mg^{-1} with 1 unit defined as 1 μmol of CO oxidized min^{-1} at 55 °C using an assay mixture that contained 10 mM methyl viologen, 2 mM dithiothreitol (DTT), and 50 mM Tris-HCl buffer, pH 7.60 (*31*). The isotope exchange reaction between CO and the carbonyl group of acetyl-CoA was performed at pH 6.0 as described (*33*). The specific activity of CODH/ACS in this reaction at 55 °C was 300 nmol min^{-1} mg^{-1}.

To evaluate the coupled CODH and ACS activities, we studied an isotopic exchange reaction between CO_2 and the carbonyl group of acetyl-CoA. The total volume of the assays was 500 μL, and the assays were carried out in a sealed 1.5 mL V-shaped vial from Wheaton. The initial radioactivity was present in $^{14}CO_2$ at a specific activity between 30 and 43 disintegrations per minute (dpm) $nmol^{-1}$. At given times, 40 μL of the reaction mix was withdrawn and quenched into scintillation vials (7 mL) that contained 40 μL of 0.5 M HSO_4^-/SO_4^{2-} buffer, pH 2.2. The quenched samples were then flushed with dry nitrogen to remove CO_2 from solution. The amount of radioactive CO_2 exchanged into acetyl-CoA was quantified using a Packard scintillation counter. After subtraction of the residual radioactivity at the beginning of the reaction, the concentration of acetyl-CoA ($1-^{14}C$) was calculated using the specific activity of $^{14}CO_2$ at time zero. The CO_2 concentration was estimated by the Henderson−Hasselbalch equation assuming a pK_a of 6.3 for the CO_2/bicarbonate couple.

RESULTS AND DISCUSSION

CO_2 is reduced to CO at cluster C of the CODH subunit of the bifunctional enzyme, CODH/ACS. During acetyl-CoA synthesis, CO combines with a methyl group and CoA at cluster A of the ACS subunit of this bifunctional enzyme. One scenario for the utilization of CO_2-derived CO is that it is channeled to the ACS subunit. Another possibility is CO escapes into solution and then binds to cluster A at the ACS active site where it is incorporated into acetyl-CoA.

One way to distinguish between the two possible mechanisms of CO entry into the ACS active site is by performing an isotopic exchange reaction between CO_2 and the carbonyl group of acetyl-CoA. This is a two-step reaction (Scheme 1A). In the first step, CODH catalyzes CO_2 reduction to CO. In the second step, ACS catalyzes an exchange reaction between CO and the carbonyl group of acetyl-CoA (*17*). The

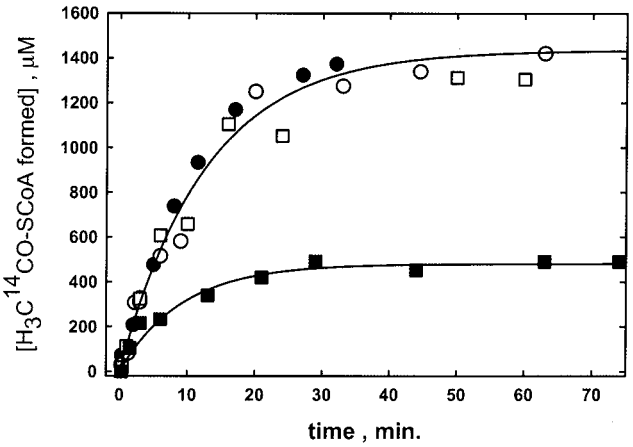
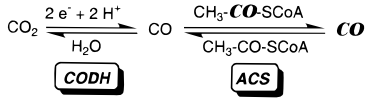


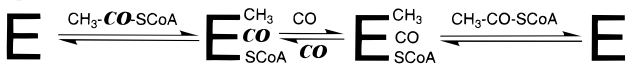
FIGURE 1: Effect of hemoglobin and myoglobin on the isotopic exchange between $^{14}CO_2$ and the carbonyl group of acetyl-CoA. The assays were performed at 35 °C in a reaction mixture containing 2.5 mM acetyl-CoA, 34 mM CO_2, 26 mM HCO_3^-, 0.1 M MES buffer, pH 6.0, 4.5 mM titanium(III) citrate, 0.1 mM methyl viologen, and 8 μM CODH/ACS. The following concentrations of heme proteins were present: (●) no heme proteins; (○) 0.1 mM myoglobin; (□) 0.1 mM hemoglobin; and (■) 1.0 mM hemoglobin. The upper curve is the fit to a single-exponential increase with an amplitude of 1440 μM and a rate constant of 0.079 ± 0.023 min^{-1}. The lower curve is the fit with an amplitude of 454 μM and a rate constant of 0.109 ± 0.021 min^{-1}.

Scheme 1

A



B



CO/acetyl-CoA exchange reaction involves the disassembly of acetyl-CoA into bound carbonyl, methyl, and CoA moieties; exchange of the bound carbonyl group with CO; and reassembly of acetyl-CoA (Scheme 1B). The CO/acetyl-CoA exchange reaction, first discovered by Hu et al. (*34*), has been an extremely important diagnostic tool for understanding the Wood−Ljungdahl pathway. Studies of this reaction led to the discovery that CODH and ACS activities are catalyzed by the same enzyme (*17*).

In a recent study, it was found that hemoglobin did not inhibit CO_2 reduction to acetyl-CoA (*28*). Hemoglobin and myoglobin bind CO with extremely high affinity and thus function as CO sponges.[2] Figure 1 shows that when these proteins are present at a concentration of 0.1 mM, there is no inhibition of the exchange reaction between CO_2 and acetyl-CoA. However, in these experiments, one must be cognizant of the amount and rate of CO production (which is rapid under these conditions). Under these conditions, the heme protein was added at a concentration sufficient to bind 25% of all of the CO that is produced during the exchange reaction.[3] Thus, at least 25% inhibition by heme proteins

[2] Myoglobin and hemoglobin bind CO rapidly ($k_{on} \sim 10^6$ M^{-1} s^{-1}) and tightly ($K_d \sim 50$ nM) (*1*). The K_d for the myoglobin−CO complex under these conditions was 100 nM. The affinity of CODH for CO is much weaker. The K_d for CO in CO oxidation is 6 μM (*2*), and the K_m of CO in the CO/acetyl-CoA exchange reaction is 10 μM (*3*).

would have been expected if the CO in solution was required for exchange. With a higher level of hemoglobin (1 mM) that is sufficient to bind all the CO formed during the reaction, 66% inhibition of the exchange is observed. Thus, even when all of the solution CO is trapped by the heme sponge, 33% is still channeled from cluster C to cluster A. Furthermore, there is no lag phase under any of these conditions. This indicates that, even when CO is produced rapidly enough to escape from the putative channel, solution CO is not required for the CO/acetyl-CoA exchange reaction. Invoking a channeling mechanism helps to explain the effect of hemoglobin observed earlier on the total synthesis of acetyl-CoA from pyruvate, CH_3-H_4folate, and CoA (*24*).[4] In these experiments, we were able to account for the inhibition by a model in which hemoglobin and ACS compete for CO. What we neglected to discuss was why hemoglobin did not fully inhibit CODH/ACS. It was found that "infinite" levels of hemoglobin[5] could only inhibit approximately 60% of the acetyl-CoA synthesis activity from pyruvate, CH_3-H_4folate, and CoA (*24*). This question of incomplete inhibition was one of the first hints that some of the CO was inaccessible to hemoglobin. Thus, high levels of hemoglobin do inhibit acetyl-CoA synthesis and the CO_2/acetyl-CoA exchange reaction; however, significant amounts of CO_2-derived CO are sequestered from the heme sponges in solution.

The hemoglobin inhibition experiments suggest the possibility of a CO channel. However, in these experiments, CO production is rapid because the concentration of electron donor is high. Furthermore, having the heme sponge in the reaction mixture has a significant effect on the reaction. It displaces the reaction equilibrium to favor production over utilization of CO and stimulates the rate of CO production ~6-fold. Another way to distinguish between the two possible modes of CO entry to the acetyl-CoA synthesis site is to determine if unlabeled CO inhibits the incorporation of ^{14}C from labeled CO_2 into acetyl-CoA (Figure 2). These experiments are similar to the classical channeling experiments in which the proposed channeled intermediate is competed with the substrate. This is a strict measure of channeling because any solution CO that equilibrates with CO in the putative channel will decrease the rate of exchange of label into product and reduce the final specific activity of the acetyl-CoA. Adding up to 660 μM unlabeled CO has no effect on the rate of the CO_2/acetyl-CoA exchange reaction. There is at most a 6% effect on the amplitude of the reaction. Adding 660 μM CO should have inhibited the exchange reaction by 40% if solution CO were to fully equilibrate with CO_2-produced CO. This is because, at the high levels of Ti(III) used in this assay, 0.4 mM CO builds up (Figure 2, inset). If solution CO is required for the exchange reaction,

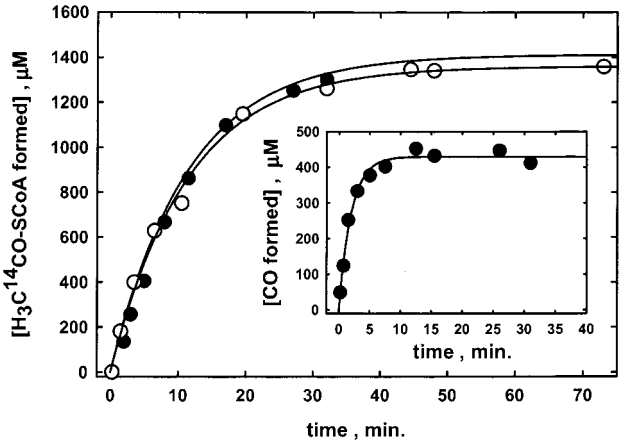


FIGURE 2: Effect of adding unlabeled CO on the $^{14}CO_2$/acetyl-CoA isotopic exchange assay. The conditions are identical to Figure 1 either with no unlabeled CO (●) or with 660 μM unlabeled CO (○). Curves are the fits to a single-exponential increase with amplitudes 1490 μM (no CO) and 1360 μM (660 μM unlabeled CO) and rate constants 0.085 ± 0.006 min^{-1} (no CO) and 0.088 ± 0.006 min^{-1} (660 μM unlabeled CO). Inset: CO formation during the exchange assays. The rate of CO formation was measured by quenching aliquots of the reaction mixture into a separate cuvette containing 20 μM myoglobin and 200 μM titanium(III) citrate to reduce the myoglobin. The heme−CO adduct was observed by following the shift in the Soret band at 434 nm by UV−visible spectroscopy. The curve is the fit to a single-exponential increase with an amplitude of 430 μM and a rate constant of 0.50 ± 0.06 min^{-1}.

the extent of inhibition is expected to be even higher before the *plateau* in free CO is attained. In contrast, high levels of added CO have no effect on the initial exchange velocity.

In the results described in Figures 1 and 2, only ~60% instead of the expected 96% of the acetyl-CoA underwent exchange with labeled CO_2. Three processes decrease the fractional exchange. The first is incomplete reassembly of the acetyl-CoA after cleavage of the C−C and C−S bonds. It has been shown that most of the acetyl-CoA does undergo reassembly (*34*). The second is the conversion of labeled CO_2 to labeled CO, which enters the gas phase and is effectively removed from the reaction. As shown in the inset to Figure 2, the production of CO from CO_2 during the exchange reaction is rapid. The third is that when unlabeled acetyl-CoA is disassembled by cluster A, CO formed from the carbonyl group migrates to cluster C where it can undergo oxidation to CO_2. Thus, CO_2 derived from acetyl-CoA undergoes exchange more productively than CO_2 in solution.

The CO "chase" results described above agree with the heme inhibition experiments and strongly indicate that CO produced at the CODH active site is utilized in preference to solution CO during acetyl-CoA synthesis. One possible way to explain these results is that cluster A is close enough to cluster C to accept CO_2-derived CO as soon as it is generated and incorporate it into acetyl-CoA. However, that CO_2-derived CO destined for acetyl-CoA synthesis does not

[3] The concentration of myoglobin is 100 μM. The initial rate of CO_2 reduction to CO is 216 μM min^{-1}, which reaches a final CO concentration of 420 μM. Thus, at least 25% inhibition by heme proteins would have been expected if the CO in solution was required for exchange.

[4] A reviewer pointed out that if bicarbonate were the true substrate for CODH, coupling CODH with carbonic anhydrase might explain how "CO_2" (actually bicarbonate) would appear more accessible to the enzyme than CO. However, it has been shown that CO_2, not bicarbonate, is the product of CO oxidation (*35, 36*).

[5] An "infinite" hemoglobin level was defined by the offset of 40% activity obtained by fitting the data to a single-exponential decay curve; i.e., 40% activity remained at infinite levels of hemoglobin.

[6] At distances of 10−15 Å, paramagnetic species experience dipolar interactions, which leads to broad EPR signals with complex morphologies. One of many examples of this type of interaction is with the 8 iron ferredoxins, which have 2 $[4Fe\text{-}4S]^{2+/1+}$ clusters that are separated by ~12 Å. When both clusters are reduced, they are paramagnetic and exhibit complex spectral morphologies, that are characteristic of $S = 1/2$ species undergoing dipolar interactions.

equilibrate with CO in solution strongly suggests some type of molecular sequestrant. Furthermore, although the structure of CODH/ACS has not been determined, spectroscopic results strongly indicate that clusters A and C are not close. When CODH/ACS is reacted with CO, a paramagnetic state of cluster A is generated called the NiFeC species (*26*). Upon CO treatment, clusters B and C in the CODH subunit also become paramagnetic (*25*, *37*). The EPR signals from all of these centers behave like isolated $S = 1/2$ species, which indicates that they are separated by at least 10 Å.[6] X-ray crystallography or further magnetic resonance experiments such as saturation−recovery EPR will be required to measure the actual distances between the two clusters.

To explain these results, we propose that there is a channel leading from cluster C in the small CODH subunit to cluster A in the large ACS subunit of this bifunctional protein. The existence of a channel between the CODH and ACS active sites offers a mechanism to ensure that CO is provided at the levels needed for acetyl-CoA synthesis. Excess CO in solution would undergo oxidation to CO_2. The net effect is to allow very little CO to accumulate in environments harboring these microbes. In the "in vitro" conditions studied here, significant amounts of CO do escape into solution. This is because we have run the reaction under conditions that favor CO production, i.e., at high concentrations of titanium(III) citrate and CO_2. In the growing cell, there is apparently a more equitable balance between the rates of CO_2 reduction and CO utilization. When cells grow on CO_2, H_2 is the electron donor. When they grow on sugars, pyruvate and pyruvate ferredoxin oxidoreductase couple to CODH/ACS to provide CO_2 and electrons.

The existence of a CO channel is advantageous for the microbes since they would not want to waste this rich carbon and energy source. CO is a source of low-potential electrons (midpoint potential at pH 7.0 is −528 mV); it binds well to the low valent states of metal complexes, and is at the same redox state as the carbonyl group of acetyl-CoA. Sequestering CO also is important for animals that harbor these microbes in the digestive tract since even low levels of CO are poisonous. Tight coupling of CO production and utilization ensures that high levels of this toxic gas do not leak into the environment. The importance of maintaining CO in a macromolecular channel provides a rationale for why the ACS and CODH subunits are coexpressed, coregulated, and tightly associated in a bifunctional enzyme.

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