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Evidence of a Molecular Tunnel Connecting the Active Sites for CO₂ Reduction and Acetyl-CoA Synthesis in Acetyl-CoA Synthase from *Clostridium thermoaceticum*

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Received June 22, 1999

Autotrophic bacteria and archaea can grow on CO_2 and H_2 as their only source of carbon and energy.[1] The central enzyme responsible for this process is acetyl-CoA synthase (ACS). ACS from *Clostridium thermoaceticum* catalyzes the reversible reduction of CO_2 to CO, and the synthesis of acetyl-CoA from CO, coenzyme A (CoA), and the methyl group of a methylated corrinoid−iron−sulfur protein (CoFeSP).[2] It also catalyzes the exchange of free CO with the carbonyl group of acetyl-CoA, presumably by a similar mechanism. ACS is an $\alpha_2\beta_2$ tetramer containing two active-site Ni-X-Fe₄S₄ clusters.[3−5] The active site for CO₂/CO redox catalysis (the C-cluster) resides in β,[3,6] while that for acetyl-CoA synthesis (the A-cluster) is located in α.[4,5] The two sites appear to function independently, in that they can be separately inactivated.[7]

CO_2 has a significant effect on the enzyme, possibly involving a protein conformational change.[8] Exposure to CO_2 alters the redox and CO-binding properties of the metal centers and *abolishes* the CO/acetyl-CoA *exchange* activity. Since *C. thermoaceticum* grows under a CO_2 atmosphere, the CO_2-altered form of ACS may be physiologically relevant.

To address whether CO_2 affects acetyl-CoA *synthase* activity, we examined initial rates of acetyl-CoA synthesis, using a CO concentration of 3 μM under a balance of Ar or CO_2 (Figure 1, ● and ▲, respectively). Ti(III) citrate (Figure 1A) and methyl viologen (MV) (Figure 1B) were used as reductants.[9] Initial rates were ACS dependent. Under Ar and using MV (Figure 1B, ●), acetyl-CoA was initially synthesized at a rate of 0.04 ± 0.01 μmol min⁻¹ mg⁻¹ (1 μmol min⁻¹ = 1 unit) while under CO_2 (Figure 1B, ▲) the rate was 40 times faster (1.5 ± 0.3 units/mg). Similar initial rates were obtained using Ti(III) citrate (0.07 ± 0.02 units/mg in Ar, Figure 1A ●, and 1.2 ± 0.2 units/mg in CO_2, Figure 1A ▲). More importantly, similar initial rates were obtained in the absence of CO (Figure 1, ■). This indicates that CO *generated from the reduction of CO_2 at the C-cluster* combined with the methyl group and CoA at the A-cluster to form acetyl-CoA.

This raised the issue of whether CO dissociated from the C-cluster into solution and eventually bound the A-cluster once
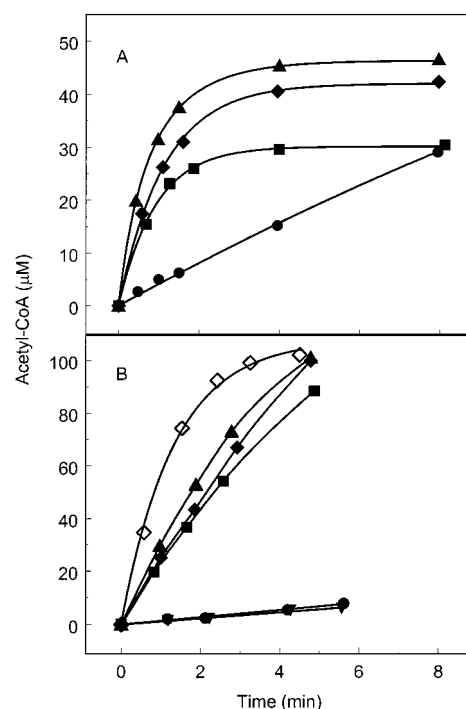
Figure 1. Acetyl-CO synthesis under CO₂, CO, and Ar atmospheres using Ti(III) citrate or viologens as the reductant. Panel A: Acetyl-CoA synthesis using Ti(III) citrate as the reductant. A solution containing 50 mM MES pH 6.3, 2 mM CH₃-tetrahydrofolate, 30 μM CoFeSP, 10 μM methyltransferase, 2 mM Ti(III) citrate, and 40 μM dithiothreitol (all final concentrations) was added to a vessel.[20] After 15 min under various partial pressures of CO, CO₂, and Ar, the reaction was initiated by adding a stock solution of ACS and CoA, affording concentrations of 0.30 μM and 1.0 mM, respectively, and a final volume of 0.50 mL. Samples were removed periodically and analyzed for acetyl-CoA.[21] Conditions: 3 μM CO and a balance of Ar (●); 3 μM CO and a balance of CO₂ (▲); 1 atm of CO₂ (■); 1 atm of CO₂ plus hemoglobin (30 μM final concentration) (◆). Panel B: Acetyl-CoA synthesis using viologens as the reductant. The solution was that described in Panel A except the final ACS concentration was 0.13 μM. Using MV (1.0 mM final concentration of the reduced form[9]), acetyl-CoA was synthesized in the presence of 3 μM CO and a balance of Ar (●); 3 μM CO and a balance of CO₂ (▲); 1 atm CO₂ (■); 1 atm of CO₂ plus hemoglobin (100 μM final concentration) (◆); 1 atm of Ar plus the CO adduct of hemoglobin (100 μM final concentration)[12] (▼); 780 μM CO₂ with a balance of Ar and triquat (1.0 mM reduced-state final concentration) replacing MV (◇).

its concentration was sufficiently high. Assuming this and a k_{cat} value of 1.3 s⁻¹ for CO_2 reduction,[10] ~120 h would be required to afford 3 μM CO. The absence of a lag period prior to the onset of acetyl-CoA synthesis (Figure 1, ■) is incompatible with this scenario.

The experiment was repeated in the presence of hemoglobin. Hemoglobin was employed as a CO "sponge" as it binds CO rapidly (2 × 10⁵ M⁻¹ s⁻¹) and tightly (K_d = 50 nM).[11] If CO migrates through solution from the C-cluster to the A-cluster, hemoglobin should scavenge CO and no acetyl-CoA should be synthesized. However, under these conditions, acetyl-CoA *was* synthesized rapidly, with no hint of a lag period (Figure 1, ◆). One explanation is that ACS abstracted CO from CO-bound hemoglobin and used it to make acetyl-CoA. To test this, the

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(9) Reductants were prepared anaerobically. Triquat and Ti(III) citrate were synthesized as described (refs 23 and 24). Viologens (methyl, benzyl, and triquat) were reduced enzymatically in 10 mM MES pH 7.0. Concentrations of the reduced states were determined by titration against a standard potassium ferricyanide solution.

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CO adduct of hemoglobin (100 μM) was prepared[12] and used as the sole source of CO in a control experiment performed under Ar (Figure 1B, ▼). The rate of catalysis (0.03 ± 0.01 units/mg) was within error of that in the absence of CO and CO_2 (0.03 ± 0.01 units/mg), arguing against this explanation.

We conclude that during CO_2-dependent acetyl-CoA synthesis, the CO generated at the C-cluster travels to the A-cluster *without equilibrating into solvent*. The C- and A-clusters are located in different subunits and are not magnetically coupled in their $S = ^1/_2$ states.[13] This suggests that the two clusters are separated by >15 Å. Thus, CO appears to travel through a "molecular tunnel" unifying the two active sites. Other enzymes utilize such tunnels, including carbamoyl phosphate synthetase,[14] tryptophan synthase,[15] and glutamine amidotransferase.[16] These enzymes, including ACS, are multifunctional and use tunnels to transport intermediates from the active site where they are generated to the site where they are consumed.

Under CO_2, the initial rates of acetyl-CoA synthesis were comparable using either Ti(III) citrate or MV as the reductant. However, synthesis halted abruptly after ~3 min with Ti(III) citrate (compare Figure 1A to 1B). We do not fully understand this inhibition, but it prompted us to examine the use of other reductants in this reaction. The rate using triquat ($E° = -0.54$ V vs NHE) was 3.0 ± 0.5 units/mg (Figure 1B, ◇), about three times faster than that obtained with MV ($E° = -0.44$ V) (Figure 1B, ■). The rate using benzyl viologen (BV) ($E° = -0.35$ V) was 10 times slower (0.15 ± 0.03 units/mg). Rate vs potential data are plotted in Figure 2. Data were analyzed assuming two states of a critical redox site, an oxidized state yielding inactive enzyme and a reduced state yielding active enzyme. The rate obtained using BV was subtracted from the others, and a derivative of the Nernst equation was fit to the data. The best-fit line (Figure 2, solid) favored $n = 1$ electron/mol. If n was fixed at 2, the fit (Figure 2, dashed line) was poorer by a factor of 3. Our analysis suggests that an $n = 1$ redox site on either ACS or CoFeSP, with a reduction potential ≤ -0.5 V, must be reduced before the system becomes catalytically active. The site is probably not any of the metal clusters in ACS, as their redox potentials are too high.[8] It does not appear to be the D site, because it would undergo $n = 2$ redox chemistry.[17] The reductive activation step may involve the $Co^{2+/+}$FeSP couple ($E° = -0.50$ V).[18,19]

Although the rate of acetyl-CoA synthesis using MV was only a fraction of V_{max}, we used this reductant (for practical reasons)

(12) A Wheaton vial containing a solution of MV-reduced hemoglobin was sealed with a rubber septum, flushed with CO for 2 min, incubated 5 min, and then purged with Ar for 2 min. The vial was then opened to the glovebox atmosphere for 5−6 h. Successful (i.e., 100%) conversion of hemoglobin to its CO adduct was confirmed spectrophotometrically.

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(20) Assays were performed anaerobically at 30 °C in the dark in a Ar-atmosphere box. Henry's law constants of 31 mM/atm (ref 25) and 0.98 mM/atm (ref 26) were used for CO_2 and CO. Vessels were fabricated from 250 mL Erlenmeyer flasks, each with a 5.0 mL conical-reaction vial fused to the flat bottom and two vacuum stopcocks fused at opposite side walls. A triangular stir bar located at the bottom of the conical vial mixed the solution while a round-tapered stir bar mixed the gas phase.

(21) Aliquots (80 μL) were quenched with 20 μL of 15 mM phenosafranin (Sigma), and analyzed for acetyl-CoA by C_{18} reversed-phase HPLC.
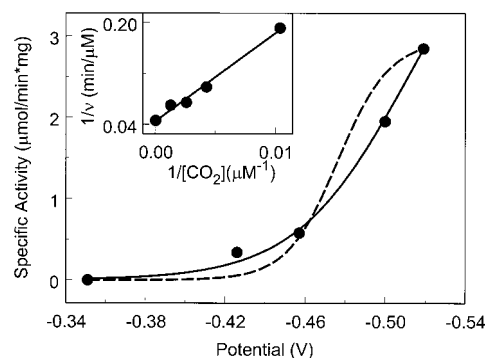
Figure 2. Specific activity of ACS as a function of potential.[22] Activity at $E = -0.35$ V: the solution was that described in the Figure 1 legend except that CoA and ACS, at final concentrations of 1.0 mM and 0.12 μM, respectively, replaced Ti(III) citrate. After 15 min under 0.025 atm (780 μM) CO_2 and a balance of Ar, BV (53% reduced) was added from a stock solution affording a final reduced-state concentration of 1.0 mM in a final volume of 0.50 mL. Samples were removed and analyzed for acetyl-CoA.[21] Activity at $E = -0.43$ V: same as above except that MV (37% reduced) replaced BV. Activity at $E = -0.46$ V: MV (66% reduced) was used. Activity at $E = -0.50$ V: a solution of MV (3.5 mM, 66% reduced) and triquat (6.5 mM, 36% reduced) was added such that the total reduced-state viologen concentration was 1.0 mM. Activity at $E = -0.52$ V: a solution of triquat (31% reduced) was added from a stock solution affording a final reduced-state concentration of 1.0 mM. The equation, Specific Activity = $V_{max}[1 + \exp\{(nF/RT)(E - E°)\}]$, was fit to the data while allowing parameters V_{max}, n and $E°$ to float. Solid line: Best fit to the data, using $V_{max} = 5.1$ units/mg, $E° = -0.51$ V and $n = 0.9$ electron/mol. Dashed line: Best fit obtained by fixing $n = 2$ electron/mol. Inset: Double reciprocal plot of 1/rate vs 1/[CO_2]. Conditions were the same as those described in the Figure 1 legend except that MV (66% reduced, 1.0 mM final reduced-state concentration) replaced Ti(III) citrate. The concentration of CO_2 was varied from 0.1−31 mM. After 15 min, the reaction was initiated by adding ACS and CoA from a stock solution, affording final concentrations of 0.13 μM and 1.0 mM, respectively, and a total volume of 0.50 mL.

to determine initial rates of acetyl-CoA synthesis at CO_2 concentrations ranging from 0.1−31 mM. Plotting the resulting data in double-reciprocal form (Figure 2 inset) yielded $k_{cat(app)} = 170 \pm 20$ min^{-1} and $k_{cat(app)}/K_m = 0.53 \pm 0.07$ μM^{-1} min^{-1}. The linearity of the plot suggests that CO_2 is a classical substrate.

In summary, our experiments indicate that CO_2 is a substrate for the synthesis of acetyl-CoA, and that under these conditions, CO is an intermediate that travels through a tunnel from the C- to the A-cluster. A low-potential one-electron redox site on either ACS or CoFeSP must be reduced at or before the rate-limiting step of catalysis. These results reveal that the two active sites, previously thought to function independently, actually function in concert under physiological conditions in the presence of CO_2.

Supporting Information Available: Experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA992120G

(22) Midpoint potentials for triquat, MV, and BV were taken as −0.54, −0.44, and −0.35 V vs NHE, respectively (ref 27). Potentials were calculated from the ratio of the reduced to oxidized viologen using the Nernst equation.

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