

Demonstration of Carbon-Carbon Bond Cleavage of Acetyl Coenzyme A by Using Isotopic Exchange Catalyzed by the CO Dehydrogenase Complex from Acetate-Grown *Methanosarcina thermophila*

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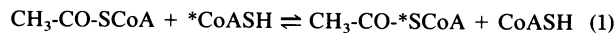
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The purified nickel-containing CO dehydrogenase complex isolated from methanogenic *Methanosarcina thermophila* grown on acetate is able to catalyze the exchange of [1-¹⁴C] acetyl-coenzyme A (CoA) (carbonyl group) with ¹²CO as well as the exchange of [3'-³²P]CoA with acetyl-CoA. Kinetic parameters for the carbonyl exchange have been determined: K_m (acetyl-CoA) = 200 μ M, V_{max} = 15 min^{-1} . CoA is a potent inhibitor of this exchange (K_i = 25 μ M) and is formed under the assay conditions because of a slow but detectable acetyl-CoA hydrolase activity of the enzyme. Kinetic parameters for both exchanges are compared with those previously determined for the acetyl-CoA synthase/CO dehydrogenase from the acetogenic *Clostridium thermoaceticum*. Collectively, these results provide evidence for the postulated role of CO dehydrogenase as the key enzyme for acetyl-CoA degradation in acetotrophic bacteria.

Acetogens and methanogens possess novel pathways for the synthesis of acetyl-coenzyme A (acetyl-CoA) from single carbon units which allow these organisms to grow on CO₂ as the sole carbon source (5). Other strains of methanogenic bacteria such as *Methanosarcina* species are able to grow on analogous C1 feedstocks including methanol and methylamine by using variations of this same pathway for carbon assimilation. The key enzyme in these cases is carbon monoxide dehydrogenase, a complex nickel enzyme so named for its ability to catalyze the reduction of CO₂ to CO. However, the enzyme has been implicated in a number of cases as the catalyst for the carbon-carbon bond formation from suitable methyl donors and enzymatically generated CO from CO₂ to give acetyl units.

In the acetogenic *Clostridium thermoaceticum*, one molecule of CO₂ is reduced to formate and formate is reduced to a methyl group by using tetrahydrofolate as a carrier; enzymatic transfer to a corrinoid iron/sulfur protein yields the methylated organocobalt species (19). Nickel-containing CO dehydrogenase (more appropriately called acetyl-CoA synthase) then acts as the site of carbon-carbon bond coupling by accepting the methyl group and catalyzing its carbonylation in the presence of CoA to give acetyl-CoA as product. The enzyme from *C. thermoaceticum* is the most well characterized acetogenic enzyme containing 2 Ni, 11 Fe, and 1 Zn atoms per active site; there is some discussion about the subunit composition of the functional complex as isolated: $\alpha_3\beta_3$ [78, 71 kDa] (6); $\alpha_2\beta_2$ [78, 71 kDa] (3); or $\alpha_2\beta_2\gamma_2$ [78, 71, 50 kDa] (10).

The pivotal role of CODH has been revealed by the ability of the clostridial enzyme to catalyze two mechanistically revealing isotope exchange reactions first noted by Wood and colleagues (7, 8) and studied extensively by us (10-12):



The exchange of [γ -³²P]CoASH with acetyl-CoA minimally requires the enzyme be competent in C-S bond cleavage with formation of an acetyl-enzyme intermediate. The exchange of [1-¹⁴C]acetyl-CoA with free CO demands that the enzyme be capable of both C-S bond cleavage and the key C-C bond cleavage as well as reversible storage of methyl, CO, and HSCoA fragments on the enzyme and release of CO into solution. We examined this reaction with the clostridial enzyme in detail, finding that reversible decarbonylation and recarbonylation of both R and S chiral methyl (H, D, T) acetyl-CoA proceeds with complete retention of configuration (12). This result is consistent with carbon-carbon bond formation occurring as a metal-based CO insertion into a metal-methyl bond, which is known to proceed with retention of stereochemistry. The presence of a unique Ni-Fe-C center has been detected by Ragsdale et al. by using electron paramagnetic resonance spectroscopy (9) and supported by EXAFS studies performed by us (2). Additional electron paramagnetic resonance evidence shows that both CO and CoASH interact with this nickel site (9). These studies led us to postulate that biosynthesis of acetate occurs as a nickel (or nickel-iron)-based carbonylation via methyl-nickel and acetyl-nickel species (12).

CO dehydrogenase has also been proposed as a key enzyme in most acetotrophic, anaerobic bacteria that oxidize acetate, catalyzing the carbon-carbon bond cleavage of acetyl-CoA into analogous C1 units (18). Evidence for this postulate comes from the observation of high levels of CO dehydrogenase activity in cell extracts of sulfate-reducing bacteria (14), the absence of key enzymes of the citric acid cycle (13) (normally operating in aerobic organisms), and the ability of cell suspensions to catalyze the exchange between ¹⁴CO₂ and the carboxyl group of acetate (15), demonstrating the existence of a pathway whereby the C-C bond of acetate

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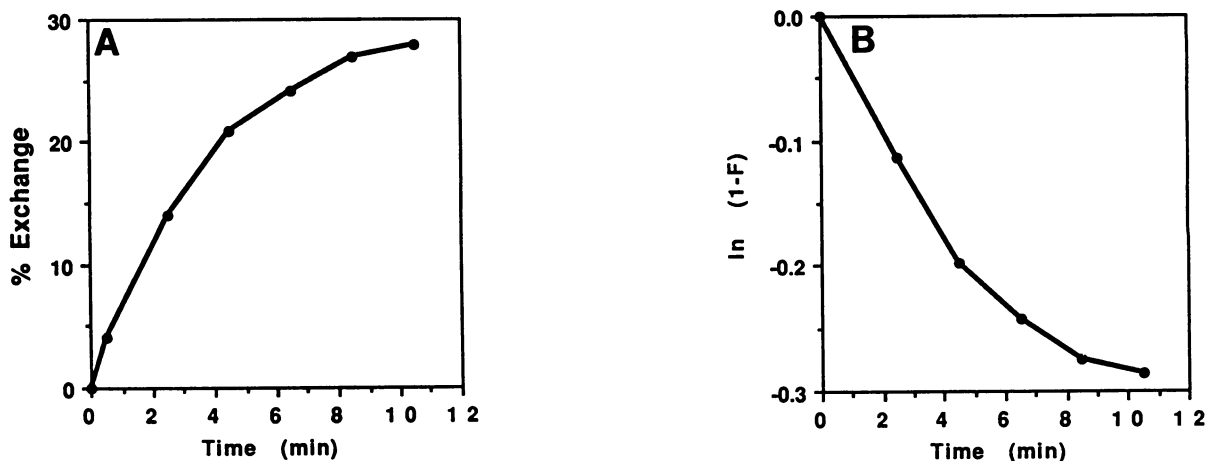


FIG. 1. (A) Time course of the CO dehydrogenase-catalyzed carbonyl exchange, $[1-^{14}\text{C}]\text{acetyl-CoA} + \text{CO} \rightleftharpoons ^{14}\text{CO} + \text{acetyl-CoA}$. The acetyl-CoA concentration is 200 μM . (B) Semilog plot of the data from which initial rates and kinetic constants can be derived.

is reversibly cleaved. It has also been noted that acetate is the only organic compound with a carbon-carbon bond that methanogenic bacteria can degrade for growth (18), again pointing to a unique mechanism for acetate catabolism.

Of the methanogenic bacteria, the CO dehydrogenase from acetate-grown *Methanosarcina thermophila* has been characterized by Terlesky et al. as a five-subunit complex containing acid-labile sulfur, 25 Fe, 3 to 4 Ni, 6 Zn, and a cobalt corrinoid (17). The CO-reduced CODH complex contains a Ni-Fe-C center (shown by electron paramagnetic resonance) analogous to that of the clostridial enzyme (16). The electron paramagnetic resonance signal is perturbed upon addition of acetyl-CoA, supporting the suggestion that acetyl-CoA is a physiological substrate for the enzyme (16). The synthesis of acetyl-CoA from CH_3I , CO, and HSCoA has been reported for CODH from both *C. thermoaceticum* (6) and *M. thermophila* (1). However, the cleavage of acetyl-CoA has not been demonstrated with any purified methanogenic CO dehydrogenase. We report here that the purified CO dehydrogenase from *M. thermophila* is able to catalyze both the acetyl-CoA-CoASH exchange and the CO exchange (demonstrating C-C bond-cleaving ability) and compare the initial rate data with those previously obtained for the clostridial enzyme (11). We were also able to detect acetyl-CoA hydrolase activity for the methanogenic enzyme, not observed for the clostridial enzyme, and suggest that this observation also corroborates the proposed function of the methanogenic enzyme as an acetyl-CoA-cleaving enzyme.

Upon incubating CO dehydrogenase from *M. thermophila* with $[1-^{14}\text{C}]\text{acetyl-CoA}$ under a CO atmosphere by using standard conditions as described previously (total volume, 0.85 ml; potassium phosphate $[\text{KPi}]$ -dithiothreitol (150 mM: 1 mM), pH 6.0; 0.2 mM methyl viologen) (11), we were able to detect time-dependent loss of radioactivity from the solution phase (Fig. 1) indicative of the enzymatic formation of ^{14}CO (equation 2). However, the methanogenic CO dehydrogenase-catalyzed exchange proceeded at best to the extent of 30 to 50% in 15 min and not to complete loss of solution radioactivity as would be expected at isotopic equilibrium, given the large excess of CO present and as we had observed for the clostridial enzyme. As the isotopic exchange comprises no net synthesis, the concentration of acetyl-CoA should not change during the assay and we suspected acetyl-CoA degradation as a possible competing

reaction. High-pressure liquid chromatography analysis of the reaction mixture at the end of the assay with a C_{18} column (15% methanol, 0.1 M KPi buffer [pH 5.5]) revealed a considerable amount (10 to 15%) of CoA (identified by retention time) present after 15 min. In addition, 5 to 10% of the initial radioactivity starting as $[1-^{14}\text{C}]\text{acetyl-CoA}$ could be detected in the void volume which would be expected for the second by-product of hydrolysis, i.e., acetate. Nonetheless, by using samples derived from short reaction times of less than 5 min (before substantial substrate degradation occurs), we were able to obtain linear initial velocity kinetics and K_m and k_{cat} values. Kinetic parameters are reported in Table 1 along with the corresponding values previously reported under identical conditions for the clostridial enzyme. The K_m for acetyl-CoA in the CO exchange is 200 μM , in the range of that observed for the clostridial enzyme. The k_{cat} values for the CO exchange are an order of magnitude slower for the methanogenic enzyme, which in turn is three orders of magnitude slower than the corresponding k_{cat} for CO-to- CO_2 interconversion.

Acetyl-CoA hydrolysis was observed at a rate roughly 1/10 that of the CO exchange. This may, in fact, reflect a loss of integrity of the enzyme active site, allowing water access to capture the acetyl-enzyme intermediate, and is probably not a physiological function of the enzyme. Hence, the rates are low, i.e., approximately 5 nmol/min per mg. The depletion of this small amount of acetyl-CoA is by itself not enough to account for the failure of the CO exchange to reach completion. However, our previous studies revealed that CoA is a potent inhibitor of the CO exchange when catalyzed by the enzyme from *C. thermoaceticum*, and we surmised this might be the case for the methanogenic enzyme as well (11). Indeed, analysis of initial velocities for the CO exchange in the presence of micromolar amounts of CoA by a Dixon plot gave a K_i value of 25 μM . This value is similar to that for the clostridial enzyme (7 μM ; Table 1) and agrees well with the K_m for CoASH in the CODH-catalyzed synthesis of acetyl-CoA from CH_3I , CO, and CoASH, which is reported to be $\leq 20 \mu\text{M}$ (1). Thus, competing acetyl-CoA hydrolysis can inhibit the CO exchange because of the buildup of micromolar amounts of CoASH, an effective inhibitor. Additionally, acetyl-CoA hydrolysis and subsequent CoASH release may trap the enzyme in the acetyl-enzyme form, also yielding loss of catalytic activity.

TABLE 1. Comparison of CO dehydrogenases used in this study

Characteristic	Methanogenic <i>M. thermophila</i>	Acetogenic <i>C. thermoacetikum</i>
Subunit composition	$\alpha\beta\gamma\delta\epsilon$ [89, 71, 60, 58, 19 K]	$\alpha_2\beta_2\gamma_2$ [78, 71, 50 K]
Metals or cofactors per active site	3-4 Ni, 25 Fe, 6 Zn, 1 Co corrinoid	2 Ni, 11 Fe, 1 Zn
Presumed function	Acetate degradation	Acetate biosynthesis
CO \rightleftharpoons CO ₂ (27°C) ^a		
V_{\max} ($\mu\text{mol}/\text{min per mg}$)	6.4	35
k_{cat} (s^{-1})	32	120
CO exchange (55°C) ^b		
V_{\max} ($\mu\text{mol}/\text{min per mg}$)	0.05	1.2
k_{cat} (s^{-1})	0.3	4
K_m acetyl-CoA (μM)	200	600
K_i CoASH (μM)	25	7
CoASH exchange (35°C) ^c		
Observed rate ($\mu\text{mol}/\text{min per mg}$)	2.3	0.4
k_{obs} (s^{-1})	12	1.5
Acetyl-CoA hydrolysis (55°C) ^d		
Observed rate ($\mu\text{mol}/\text{min per mg}$)	0.005	ND ^e
k_{obs} (s^{-1})	0.025	ND ^e

^a Assayed with methyl viologen as an electron acceptor as described previously (11).

^b Assayed under CO atmosphere in a total volume of 0.85 ml of KP₇-dithiothreitol (150 mM:1 mM), pH 6.0, and 0.2 mM methyl viologen as described previously (11).

^c Assayed under CO atmosphere at 35°C by using the same buffer system as for CO exchange, but additionally containing 200 μM CoASH and 1.1 mM acetyl-CoA.

^d Observed rate at 600 μM acetyl-CoA under conditions to CO exchange.

^e Not detected, i.e., less than 0.0005 $\mu\text{mol}/\text{min per mg}$.

Having demonstrated the competence of the CODH complex from *M. thermophila* to engage in both C-S and C-C cleavage of acetyl-CoA and the capture of the intermediate acetyl-enzyme complex by water, we turned to the CoASH-acetyl-CoA exchange (equation 1) to obtain an estimate of the rate of acetyl-enzyme formation. This second exchange merely requires that the enzyme be capable of C-S bond breaking and reformation, and we expected this rate to exceed that for the CO exchange. Under standard assay conditions (200 μM CoASH; 1.1 mM acetyl-CoA; temperature, 45°C), the exchange of [γ -³²P]CoASH into acetyl-CoA was too fast to obtain measurable rates. Lowering the temperature to 35°C allowed us to obtain initial time points, and the rates of the CoASH/acetyl-CoA exchange were measured for both enzymes. A value of 2,300 nmol/min per mg was determined for the methanogenic enzyme, confirming that the *M. thermophila* CO dehydrogenase-catalyzed CoASH-acetyl-CoA exchange was an order of magnitude faster than for the clostridial enzyme (Table 1). This result may be a reflection of the physiological function of the methanogenic enzyme in processing acetyl-CoA as a substrate for degradation into C1 units, whereas the role of the acetogenic enzyme is exclusively for biosynthesis. The CoASH exchange value for the methanogenic enzyme is also the same order of magnitude as the CO dehydrogenase activity, further suggesting that acetyl-CoA degradation and CO-to-CO₂ interconversion are both kinetically relevant physiological processes. Note also that the CoASH exchange is nearly three orders of magnitude faster than the observed rate of acetyl-CoA hydrolysis, implying that less than 0.1% of the acetyl-enzyme intermediates are siphoned off by water attack. Even though the value reported for the CoASH exchange is not a maximal V_{\max} value nor was it adjusted for the temperature differences in the two assays, it is clear that the CO exchange is several orders of magnitude slower than the CoASH exchange. While the methanogenic enzyme is readily competent in C-S bond cleavage and reversible acetyl-enzyme formation, scission of the crucial C1(methyl)-C2(carbonyl) bond occurs with greater difficulty.

It may be that this step can be accelerated when the proper physiological methyl acceptors (pterin?) are present to lower the activation energy for C-C bond cleavage and to remove the stoichiometrically bound methyl group from the active site. To date, direct methyl acceptors for CO dehydrogenase have not been identified.

This first observation of the [1-¹⁴C]acetyl-CoA-CO exchange catalyzed by a methanogenic CO dehydrogenase demonstrates the C-C bond-cleaving ability previously postulated for this enzyme. This work also supports previous observations of ¹⁴CO₂-acetate exchange in cell suspensions of acetate-grown bacteria (4, 15), an activity that can be unequivocally attributed to CO dehydrogenase. We have also highlighted the utility of diagnostic isotope exchange reactions as mechanistic probes, particularly when the total physiological pathway for acetate degradation is not as yet completely ascertained, and suggest these as tools for comparison as the purified enzyme from other sources becomes available.

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