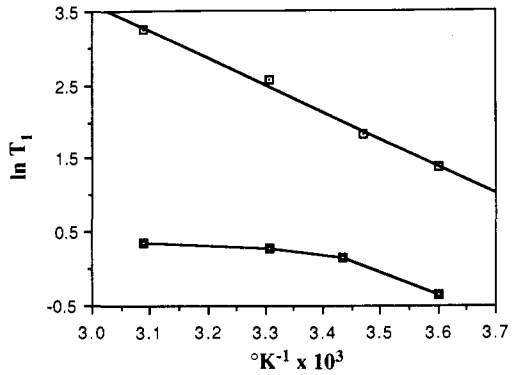

Figure 3. Plots of ln T_1 vs. inverse temperature for d[CGT(^{15}N^1)ACG] (■) and d(^{15}N^1)A (□).

than the corresponding monomer ^{15}N resonance, and the N^6 relaxes faster than the N^1 for both the hexamer and the monomer. The N^6, with two directly bonded protons, undergoes efficient ^{15}N–^{1}H dipolar relaxation. For example, a greater than 4-fold increase in the d(^{15}N^6)A T_1 was observed on changing the solvent to 100% D_2O. The N^1, which lacks a directly bonded proton, may have a more complicated relaxation mechanism. Pyridine ^{15}N relaxation occurs by a combination of dipolar, chemical shift anisotropic, and spin–rotation interactions,[16] each of which, by analogy, could be significant for the deoxyadenosine N^1. However, the contribution of spin–rotation relaxation in this case was shown to be negligible by the linear relationship between ln T_1 and T^{-1} for d(^{15}N^1)A, shown in Figure 3. The spin–rotation relaxation rate, unlike both the dipolar and chemical shift anisotropic relaxation rates, *increases* with temperature. The result for pyridine is that plots of ln T_1 vs. T^{-1} are nonlinear.[16] In an extreme case such as *n*-butyl nitrite, where spin–rotation relaxation predominates over the entire temperature range observed, such a plot is once again linear but has an opposite (positive) slope. Spin–rotation relaxation is most important for small molecules at high temperature,[16-19] and it therefore may not be surprising that it does not contribute to relaxation of the deoxyadenosine N^1.

Figure 3 also shows a plot of ln T_1 vs. T^{-1} for the ^{15}N^1 hexamer relaxation times. In this case the nonlinearity displayed is most likely a result of the changes in size and shape of the molecule which accompany the helix-to-coil transition. The rotational correlation time, τ_r, and hence the relaxation rate, are directly related to both size and shape.[10,18,19] In the duplex state the hexamer has approximate dimensions of 24 × 24 × 20.4 Å and can be treated as a sphere.[10] The single strand is not necessarily well described by a sphere at all and certainly not by a sphere of the same radius as the duplex. Thus, from these limited data, it appears that the ^{15}N T^1 also may be used to follow oligonucleotide structural changes.

We have shown for the first time that ^{15}N NMR of an oligonucleotide specifically ^{15}N labeled at either an H-bond acceptor nitrogen (N^1) or an H-bond donor nitrogen (N^6) is a sensitive structural monitor. Moreover, the T_m and thermodynamic data obtained for the helix-to-coil transition from the ^{15}N NMR agree well with those obtained by other methods. Unlike most other methods, however, ^{15}N NMR of specifically labeled molecules has the potential to probe otherwise inaccessible local structural phenomena in large molecules. These could range from thermally induced local melting, and the behavior of mismatched base pairs, to the structural changes that accompany enzyme recognition or drug binding.

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Supplementary Material Available: Tables of ^{15}N^1 chemical shifts, ^{15}N^6 chemical shifts, and ^{15}N relaxation times (2 pages). Ordering information is given on any current masthead page.

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Nickel-Containing CO Dehydrogenase Catalyzes Reversible Decarbonylation of Acetyl CoA with Retention of Stereochemistry at the Methyl Group

Scott A. Raybuck,† Neil R. Bastian,† Lynne D. Zydowsky,‡ Koji Kobayashi,‡ Heinz G. Floss,‡ William H. Orme-Johnson,† and Christopher T. Walsh*†

Department of Chemistry, MIT Cambridge, Massachusetts 02139
Department of Chemistry, The Ohio State University Columbus, Ohio 43210

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Acetogenic and methanogenic bacteria can grow chemolithoautotrophically on CO_2 and H_2.[1] They do not have a number of the common carboxylases and thus make acetate by a distinctive route from two one-carbon fragments. A large body of incisive experimental work[2-6] has demonstrated that the methyl group of acetate in acetogens derives from N_5-methyltetrahydrofolate and that the carboxyl group of acetate derives from carbon monoxide, generated from CO_2 at the active site of the enzyme CO dehydrogenase. This enzyme appears to be the catalyst for C–C bond formation.

CO dehydrogenase has been best characterized from the acetogenic *Clostridium thermoaceticum*[7-9] where it is an $\alpha_3\beta_3$ structure containing zinc, iron–sulfur clusters, and tightly bound nickel ions. The nickel site of this metalloenzyme is incompletely characterized but will bind CO as detected by ^{13}CO hyperfine broadening of the ^{61}Ni EPR signal,[10] and the nickel site may be part of a mixed iron–nickel cluster.[11] This air-sensitive enzyme is routinely assayed anaerobically by CO dehydrogenation to CO_2 with passage of electrons to the artificial electron acceptor methylviologen, but it also catalyzes two mechanistically diagnostic isotope exchange reactions: (a) a *CoA ⇌ acetyl-*CoA exchange, suggesting a reversibly formed acetyl enzyme and (b) an exchange of *CO ⇌ *acetyl-CoA (carbonyl group).[12] The latter led Wood and colleagues[9] to propose an additional cleavage between the acetyl C_2–C_1 bond, producing a species with CH_3, CO, and SCoA

† MIT.
‡ The Ohio State University.
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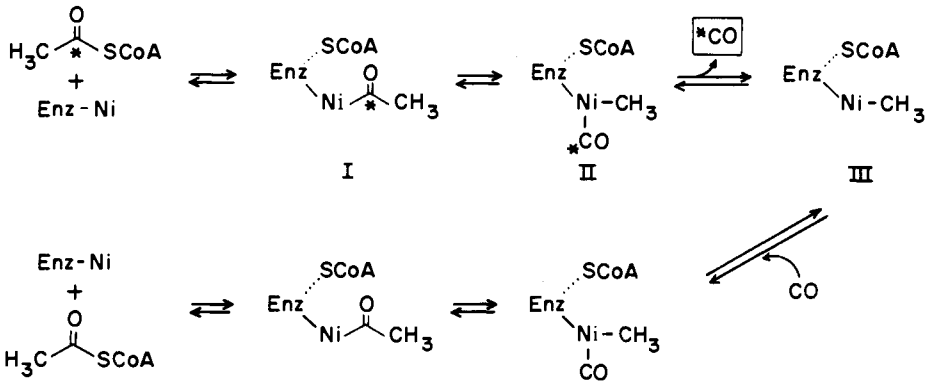
Scheme I



fragments stored reversibly on the enzyme.

A particularly attractive mechanistic hypothesis for this enzyme is use of the active-site nickel in a biocarbonylation sequence. Thus, in the acetyl-CoA cleavage direction an acetyl nickel species could fragment to a methyl nickel with an adjacent CO ligand. Exchange of the CO with free CO in solution and then insertion of the liganded CO back into the nickel–CH_3 bond would constitute a carbonyl insertion in the synthesis direction. The key steps for C–C bond fragmentation, reformation, and CO exchange would involve structures I–III of Scheme I. This carbonylation route to carbon–carbon bond formation would be novel biologically but is well precedented in organotransition-metal chemistry. For example, Yamamoto et al.[13] have prepared crystalline acetylnickel complexes by stoichiometric carbonylation of the corresponding methylnickel precursors; reductive elimination between the acetyl group and ligated *p*-cyanophenolate gave *p*-cyanophenyl acetate as the organic product, in analogy to the acetyl-CoA thiol ester formation proposed in Scheme I.

To address the issue of whether a metal-centered biocarbonylation sequence occurs in CO dehydrogenase catalysis, we turned to a stereochemical probe at C_2 of acetyl-CoA. Insertion of CO into alkylmetal species is known to proceed with retention of configuration at the alkyl center[14] in organoiron[15] and organopalladium[16] CO insertions. By analogy, retention would be anticipated with nickel, both in the acetyl-CoA decarbonylation and in the microscopic reverse, carbonylation of the methyl nickel species with labeled CO to regenerate acetyl-CoA. We have therefore used (*R*)-[*methyl*-^{1}H,^{2}H,^{3}H]- and (*S*)-[*methyl*-^{1}H,^{2}H,^{3}H]acetyl-CoA samples with purified CO dehydrogenase to analyze the stereochemical outcome at the C_2 methyl group of acetyl-CoA after reversible enzyme decarbonylation.

When (*R*)-[^{1}H,^{2}H,^{3}H]- or (*S*)-[^{1}H,^{2}H,^{3}H]acetyl-CoA samples were mixed with [1-^{14}C]acetyl-CoA and incubated with purified enzyme,[17,18] 75% of the ^{14}C label exchanged over a 45-min interval, so three-quarters of the chiral [^{1}H,^{2}H,^{3}H]acetyl-CoA molecules had also undergone reversible fragmentation between C_2 and C_1. The reisolated [^{1}H,^{2}H,^{3}H]acetyl-CoA was subjected to chirality analysis by the method of Cornforth et al.[19] and Arigoni and co-workers,[20] using the procedure described by Floss and Tsai.[21] As shown in Table I, (*R*)-[^{1}H,^{2}H,^{3}H]acetyl-CoA with initial *F* values of 73–75 (80–84% ee) gave after enzymic decarbonylative incubation, values of 73.8 and 74.5 on reisolation, a quantitative retention of configuration. Inversion would have given *F* values of 38 while a racemization mechanism at 70–72% exchange would have lowered *F* values to ca. 56. Corresponding retention results were obtained with chiral (*S*)-[^{1}H,^{2}H,^{3}H]acetyl-CoA (*F* = 27.6, 77% ee) after enzymic decarbonylation ^{14}C isotopic exchange.

Table I. Stereochemical Fate of the Methyl Group of Chiral Methyl Acetyl-CoA in the Exchange Reaction with CO Catalyzed by CO Dehydrogenase from *Clostridium thermoaceticum*[a]

	F	ee	config
(*R*)-[*methyl*-^{1}H,^{2}H,^{3}H]Acetyl Coenzyme A			
starting material	74.0, 75.0	av 84%	*R*
incubation without enzyme	73.3	80%	*R*
70% exchange	73.8	82%	*R*
72% exchange	74.5	84%	*R*
(*S*)-[*methyl*-^{1}H,^{2}H,^{3}H]Acetyl Coenzyme A			
starting material	27.3	78%	*S*
incubation without enzyme	27.6	77%	*S*
76% exchange	26.8	80%	*S*
78% exchange	27.8	77%	*S*

[a] Incubation conditions are described in the text[18] and the percent exchange value monitors the exchange of [1-^{14}C]acetyl-CoA with CO.

The retention of stereochemistry (and lack of detectable racemization) constrains the mechanism for this nickel enzyme and, while it does not prove the existence of organometallic intermediates, is consistent with an acetylnickel, methylnickel decarbonylation and recarbonylation sequence, proceeding with the same stereochemistry, to explain both CO ⇌ acetyl-CoA exchange and the overall C–C synthetic reaction sequence. Nickel is well suited to be the active metal center for the C–C bond formation in CO dehydrogenase, as EPR evidence indicates both CO and CoASH bind near the nickel site.[10,11,22] While net retention could also be accounted for by two inversion steps (e.g., attack by an enzymic methionine residue to yield an *S*-methylmethionine intermediate), there are no precedents for nucleophilic attack on the methyl group of an acetyl fragment with facile decarbonylation.

The overall sequence in acetyl-CoA formation by acetogenic bacteria involves first a transfer of the methyl fragment from N_5 of methyltetrahydrofolate to a B_{12}-containing protein to yield a methyl–B_{12} enzyme intermediate.[1] This methyl corrinoid is then the actual C_1 donor of the methyl fragment (to the nickel site?) in the CO dehydrogenase mediated acetyl-CoA synthase reaction.

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(17) The specific activity of CO dehydrogenase by the methyl viologen reductase assay[9] was typically 125 μmol/min mg at 55 °C, pH 7.2. Standard conditions for the carbonyl exchange assay: 0.85 mL of KPi/DTT (150 mM/1 mM), pH 6.0, at 55 °C containing 0.2 mM methylviologen, 6.25 nmol of [1-^{14}C]acetyl-CoA (New England Nuclear: specific activity 54 mCi/mmol), 194 nmol of acetyl-CoA (Sigma), 0.5 mg of CO dehydrogenase. All buffers were rigorously degassed under carbon monoxide. The anaerobic assay was initiated by the addition of acetyl-CoA substrate to the reaction vessel containing the enzymic solution under a CO atmosphere (head space of 6 mL which had been preincubated at 55 °C for 10 min.

(18) Assay conditions were the same as noted above,[17] except the substrate mixture contained either (a) 1.35 nmol of [1-^{14}C]acetyl-CoA, 100 nmol of acetyl-CoA, and (*R*)-[*methyl*-^{1}H,^{2}H,^{3}H]acetyl-CoA (specific activity 4.3 mCi/mmol) or (b) 1.35 nmol of [1-^{14}C]acetyl-CoA, 122 nmol of acetyl-CoA, and 74.5 nmol of (*S*)-[*methyl*-^{1}H,^{2}H,^{3}H]acetyl-CoA (specific activity 5.3 mCi/mmol).

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The overall stereochemical outcome of going from chiral [^{1}H,^{2}H,^{3}H]-N_5-methyl tetrahydrofolate to acetate has also recently been determined to be retention,[23] supporting the above sequence.

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Stereochemistry of Acetic Acid Formation from 5-Methyltetrahydrofolate by *Clostridium thermoaceticum*

H. Lebertz,[†] H. Simon,*[†] L. F. Courtney,[‡] S. J. Benkovic,*[‡] L. D. Zydowsky,[§] K. Lee,[§] and H. G. Floss*[§]

Lehrstuhl für Organische Chemie and Biochemie der TU München, D 8046 Garching, West Germany
Department of Chemistry, Pennsylvania State University College Park, Pennsylvania 16802
Department of Chemistry, The Ohio State University Columbus, Ohio 43210

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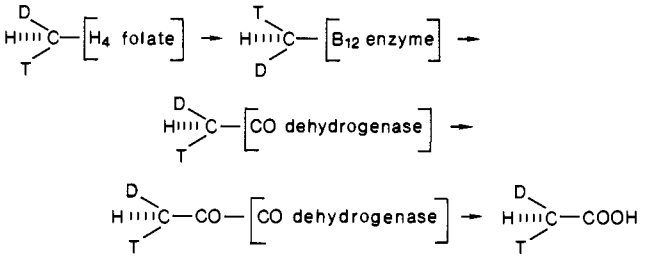
Some acetogenic bacteria ferment hexoses almost stoichiometrically to 3 mol of acetic acid per 1 mol of sugar.[1,2] Two of the three moles of acetic acid arise by decarboxylation of pyruvate formed via the Embden–Meyerhof pathway, and the third mole, remarkably, is produced reductively from 2 mol of CO_2.[3,4] Thus, an organism like *Clostridium thermoaceticum* can synthesize acetic acid entirely from CO or from $CO_2 + H_2$.[5] One mole of CO_2 is reduced via formate, 5,10-methenyltetrahydrofolate, and 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate,[6,7] and another mole is reduced to CO.[8] These two products condense in the presence of coenzyme A to produce acetyl-CoA, which then gives acetate with formation of 1 mol of ATP. The formation of acetyl-CoA from methyltetrahydrofolate requires several enzymes, including CO dehydrogenase,[9,10] a B_{12} enzyme,[11–13] and a methyltetrahydrofolate: B_{12} methyltransferase.[13,14]

The role of CO dehydrogenase was originally thought to be limited to the reduction of CO_2 to CO,[13] and it was assumed that the methyl–B_{12} species might give rise directly to the acetyl group,[8,11–14] by either a carboxylation or a carbonylation of the methyl–cobalamin. Recent studies by Wood and co-workers, however, point to a far more central role of CO dehydrogenase in this process, as the enzyme on which the actual assembly of acetyl-CoA from CoASH, CO, and a methyl group takes place.[5] The most critical piece of evidence is the finding that purified CO dehydrogenase alone is capable of catalyzing exchange between CO and the carbonyl group of acetyl-CoA.[15] This exchange requires that the enzyme can bind the methyl, the carbonyl, and the CoA group of acetyl-CoA. Binding of CO to CO dehydrogenase generates a paramagnetic nickel–iron–carbon center,[16] and EPR studies have shown that acetyl-CoA and CoA bind near this center.[15] This suggests the possibility that the acetyl group may be formed by a "carbonyl insertion" reaction on nickel or iron.

Scheme I. Stereochemical Fate of the Methyl Group in the Conversion of Methyltetrahydrofolate to Acetic Acid by *C. thermoaceticum*



It should be possible to test further the mechanism proposed by Wood and co-workers based on stereochemistry. Intermolecular methyl transfers proceed with inversion of configuration.[17,18] Chemically catalyzed carbonyl insertion, more properly migratory insertion,[19] reactions proceed with retention of alkyl group configuration.[20–23] Wood's mechanism involves two methyl transfers, one from methyltetrahydrofolate to B_{12} and another from methyl–B_{12} to CO dehydrogenase, and an insertion reaction; the predicted overall stereochemical outcome would be net retention of methyl group configuration, if retention of configuration is assumed for the CO dehydrogenase catalyzed insertion reaction, too. A process involving carbonylation on the cobalt of B_{12}, on the other hand, would only include one methyl transfer and the insertion reaction, and should thus proceed with net inversion of methyl group configuration.[24]

To examine this question we incubated (*methyl-R*)- and (*methyl-S*)-[methyl-$^2H_1$,^{3}H]methyltetrahydrofolate (1.0×10^6 and 1.15×10^5 dpm of ^{3}H, respectively, in less than 0.1 μmol), prepared by sequential reduction of 5,10-methenyltetrahydrofolate with deuteriated and tritiated sodium borohydride,[26] anaerobically under a CO atmosphere for 30 min at 55 °C with a cell-free extract of *C. thermoaceticum* in the presence of 0.1 mM CoASH, 4 mM ATP, 1.4 mM Fe^{2+} 16 mM dithiothreitol, and 90 mM potassium phosphate, pH 6.0.[14] Following denaturation with $HClO_4$, acetic acid was isolated, with carrier dilution, in 17.4% and 23.5% yield, respectively, by passage of the reaction mixture through a column of 8 mL of Dowex 50 H^+, neutralization and

[†] Lehrstuhl für Organische Chemie und Biochemie der TU München.
[‡] Pennsylvania State University.
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