Discovery of a Labile Nickel Ion Required for CO/Acetyl-CoA Exchange Activity in the NiFe Complex of Carbon Monoxide Dehydrogenase from *Clostridium thermoaceticum*

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Received August 5, 1992

Acetogenic bacteria such as *Clostridium thermoaceticum* use CO dehydrogenase (CODH) to grow autotrophically.[1-5] CODH catalyzes the reversible oxidation of CO to CO_2,[6-8] the synthesis of acetyl coenzyme A,[9-12] and various exchange reactions[9,11-17] including that of the carbonyl group of acetyl coenzyme A with free CO (CO/acetyl-CoA exchange). The synthase activity can be estimated by measuring CO/acetyl-CoA exchange since both reactions are mechanistically similar.

CODH has an $(\alpha\beta)_3$ quaternary structure.[7,18] Each $\alpha\beta$ dimer contains two Ni atoms, 11-13 Fe atoms, and ~14 sulfides,[7] organized into various complexes and clusters.[19-27] The most studied of these is the *NiFe complex*. The three to four irons associated with it[26] exhibit Mössbauer parameters typical of $[Fe_4S_4]^{2+}$ clusters.[25] The Ni associated with it is coordinated predominantly by S ligands and appears not to be incorporated into a cubane-type Fe-S cluster.[22,23,25,26] The complex binds CO and can be reduced by one electron ($E_m' = -540$ mV vs NHE[27]) to yield the so-called NiFeC EPR signal ($g = 2.08$, 2.075, and 2.028).[20,26] For an unknown reason, the intensity of this signal corresponds to only 0.1-0.35 spin/$\alpha\beta$.[24,25]

We recently found that adding 1,10-phenanthroline (phen) to CODH causes complete loss of CO/acetyl-CoA exchange activity but no loss of CO oxidation activity.[28] Adding phen also eliminates the NiFeC signal, but none of the enzyme's other EPR signals. Such behavior demonstrates that there are two active sites in CODH and that the NiFe complex is required for the synthase/exchange activities but not for CO oxidation.

Since phen is a bidentate ligand known to coordinate tightly to metal ions, we conjectured that it might act by removing metal ions from CODH. To test this, we added Ni^{2+} to phen-treated CODH. Before any treatment, the enzyme[29] exhibited the NiFeC EPR signal (Figure 1A) with an intensity corresponding to 0.21 spin/$\alpha\beta$ and had 0.31 unit/mg CO/acetyl-CoA exchange activity. After being treated with phen, it exhibited no NiFeC signal (Figure 1B) and had no (0.01 unit/mg) exchange activity. The phen-treated enzyme was separated from the phen-containing products of the reaction, and $NiCl_2$ was added.[30] The resulting sample exhibited the NiFeC EPR signal (Figure 1C) with an intensity corresponding to 0.19 spin/$\alpha\beta$ and had 0.29 unit/mg exchange activity. Thus, adding aqueous Ni^{2+} to the phen-treated enzyme restored the NiFeC signal and exchange activity near to those levels observed with the original sample.

To determine whether the added Ni^{2+} was actually the agent responsible for the regenerated activity and signal, a phen-treated sample was reactivated with $^{61}Ni^{2+}$ ($I = {}^3/_2$). The resulting NiFeC signal (Figure 1D) was significantly broader than that obtained when natural-abundance Ni^{2+} was used. The broadening is undoubtedly the result of hyperfine interactions between the ^{61}Ni nuclear spin and the electronic spin ($S = {}^1/_2$) of the NiFe complex. The magnitude of the broadening was similar to that observed in spectra of enzyme prepared from bacteria grown on ^{61}Ni-enriched media, an example of which is shown in Figure 1E. This experiment demonstrates that Ni^{2+} can be incorporated into phen-treated enzyme. To provide even more evidence of this, the ^{61}Ni-enriched sample used to generate the hyperfine-broadened signal in Figure 1E was treated with phen and reactivated with natural-abundance Ni^{2+}. The resulting NiFeC signal (Figure 1F) showed no sign of hyperfine broadening, indicating that the ^{61}Ni originally associated with the NiFe complex in that sample had been replaced with natural-abundance Ni.

Phen apparently reacts with CODH by removing Ni^{2+} from the NiFe complex, creating a structure with a vacant Ni coordination site. The inability of phen-treated CODH to catalyze exchange suggests that NiFe complexes with such vacant sites cannot function in catalysis. The ability of phen-treated CODH to catalyze CO oxidation and to elicit other EPR signals (g_{av} = 1.94 and 1.86)[28] suggests that loss of Ni from the NiFe complex does not alter the properties of the species involved in these phenomena. It also suggests that the enzyme's overall protein structure is not changed significantly when Ni is removed.

The Ni^{2+} added to phen-treated CODH binds at the vacant site of the NiFe complex, restoring the enzyme's full catalytic abilities. Thus, the Ni in the NiFe complex can be removed and replaced reversibly. This reversibility provides further evidence that loss of Ni does not significantly change the protein's structure. That this Ni ion so sensitively controls the exchange activity suggests that it has a critical function in catalysis. We are presently investigating the possibility that it is the binding site

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(29) Purified CODH[7,15] was homogeneous by SDS-PAGE, contained 1.8 Ni and 9.4 Fe/$\alpha\beta$, and had 250 units/mg CO oxidation activity.
(30) In an Ar atmosphere (0.5 ppm O_2) at ~28 °C, phen was added (130 μM final) to dithionite-free CODH (11 mg/mL). After 1 day, enzyme was separated from phen by gel filtration (Sephadex G-25, 1.6 × 15 cm, in 50 mM Tris pH 8) and incubated with dithiothreitol (10 mM final). After 1 h, enzyme was separated from dithiothreitol chromatographically (as above) and reacted with $NiCl_2$ (0.5 mM final). After 2 days, samples were reduced with CO and frozen.

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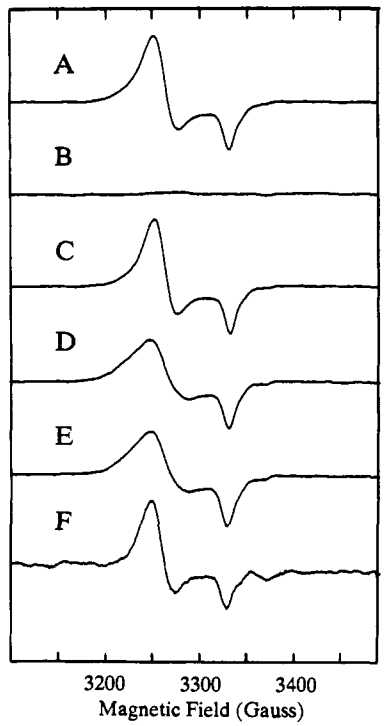

Figure 1. EPR spectra of CODH before and after addition of 1,10-phenanthroline and $NiCl_2$: A, CODH before addition of phen; B, CODH (sample used in A) after phen; C, phen-treated CODH (sample used in B) after addition of Ni^{2+}; D, phen-treated CODH after addition of $^{61}Ni^{2+}$; E, CODH from bacteria grown in ^{61}Ni-enriched media; F, phen-treated CODH (sample used in E) after addition of natural-abundance Ni^{2+}. Sample concentrations: A-D, 2.7 mg/mL; E and F, 1.8 mg/mL. Samples were reduced by CO. EPR conditions: temperature, 130 K; microwave power, 80 mW; microwave frequency, 9.45 GHz.

for one or more of the substrates used in acetyl-CoA synthesis.

Acknowledgment. We thank Mark E. Anderson for preparing the ^{61}Ni-enriched CODH. This research was supported by the Robert A. Welch Foundation and the Center for Energy and Mineral Resources at Texas A&M University.

Rigid-Rod Molecules: Carborods. Synthesis of Tetrameric *p*-Carboranes and the Crystal Structure of Bis(tri-*n*-butylsilyl)tetra-*p*-carborane

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Received August 21, 1992

The design and synthesis of rigid-rod molecules has recently attracted increasing attention, because of their potential application in nanoarchitecture[1] and the preparation of novel materials.[2] Rod-like molecules containing two to five polycyclic units such as bicyclo[1.1.1]pentane,[3] bicyclo[2.2.2]octane,[4] and cubane[5] have been reported recently. The recognition of the need for small and rigid molecular structures has led us to the synthesis of rods, rings, and related motifs based upon the use of exceedingly stable carborane cages as building blocks. New and efficient synthetic methods have been developed in our laboratory for the synthesis of carborane-containing rigid-rod molecules (carborods) capable of functionalization at their C-H termini. We report here the first successful synthesis of tetrameric *p*-carborane and the crystal structure of bis(tri-*n*-butylsilyl)tetra-*p*-carborane.

Our synthesis of carborane-containing rigid-rod molecules starts with the coupling of two 1,12-$C_2B_{10}H_{12}$ (*p*-carborane) (**1**) cages forming a C-C bond. This was achieved by the reaction of 1-Li-1,12-$C_2B_{10}H_{11}$ with CuCl or $CuCl_2$.[6] Accordingly, we attempted to prepare the tetrameric *p*-carborane in a single reaction by coupling dilithiated **1** with 2 equiv of $CuCl_2$ in refluxing diethyl ether (Scheme IA). The tetrameric **3** was formed in 16% yield and the dimer **2** in 80% yield after a 3-day reaction. We suggest that the dimeric **2** anion is first formed and further coupling gives the tetrameric **3** anion. Acidic workup led to **3** as an insoluble white solid.[7] **2** is soluble in Et_2O, THF, and benzene and was characterized by NMR spectroscopy.[8] The ^{11}B NMR spectrum of **2** contains two peaks corresponding to two types of boron atoms in **2**. The EIMS of **3** exhibits the molecular ion at m/z 570.[7] The poor solubility of **3** in organic solvents precluded its complete characterization by NMR spectroscopy. An attempt to functionalize **3** by introducing R_3Si groups was also unsuccessful due to the poor solubility of **3**.

The synthesis of soluble tetrameric *p*-carboranes (**5**) was achieved by functionalizing **2** with a solubility-enhancing trialkylsilyl group to give **4**, which was subsequently monolithiated by *n*-BuLi and coupled by 1 equiv of $CuCl_2$ (Scheme IB; alkyl = *n*-hexyl, *n*-butyl, and isobutyl). **4a-c** were obtained in 52-79% yields and characterized by multinuclear NMR spectroscopy.[9] The ^{11}B NMR spectra of **4a-c** each showed four resonances as expected. **4a-c** are very soluble in common organic solvents, and

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(7) Spectroscopic data for **3**: IR (KBr, cm^{-1}) ν 2612 (B-H), 3101 (C-H); EIMS (70 eV, 150 °C) m/z 570 (M^+, 19, calcd for $C_8H_{42}{}^{10}B_8{}^{11}B_{32}$), 427 (M - $C_2B_{10}H_{11}$, 100).

(8) Spectroscopic data for **2**: mp 330-332 °C; ^1H NMR (500 MHz, C_6D_6, ppm) δ 1.6-2.9 (m, B*H*, 20 H), 1.82 (s, carborane C*H*, 2 H); ^{13}C NMR (90 MHz, C_6D_6, decoupled) δ 83.9 (carborane *CC*), 61.7 (carborane *CH*); ^{11}B NMR (160 MHz, THF, 25 °C, $BF_3{\cdot}Et_2O$ external, decoupled) δ -12.3, -15.8 (1:1); IR (KBr, cm^{-1}) ν 2606 (B-H), 3064 (C-H); EIMS m/z 286 (M^+, 94, calcd for $C_4H_{22}{}^{10}B_4{}^{11}B_{16}$).

(9) Spectroscopic data for **4a**: mp 132-133 °C; ^1H NMR (360 MHz, C_6D_6) δ 2.72 (s, carborane C*H*), 3.4-1.8 (m, B*H*, 20 H), peaks due to *n*-hexyl groups; $^{13}C\{H\}$ NMR (360 MHz, C_6D_6) δ 61.7 (carborane *CH*), 71.8 (carborane *CSi*), 84.2 (carborane *CC*), 87.1 (carborane *CC*), peaks due to *n*-hexyl; $^{11}B\{H\}$ NMR (Et_2O) -15.1, -13.0, -11.5, -10.2; IR (Nujol, cm^{-1}) ν 2612 (B-H), 3026 (C-H); FAB-MS m/z 569 (M^+, 20, calcd for $C_{42}H_{60}{}^{10}B_4{}^{11}B_{16}Si$). **4b**: mp 221-222 °C; ^1H NMR (360 MHz, $CDCl_3$) δ 2.68 (s, carborane C*H*), 3.3-1.4 (m, B*H*, 20 H), peaks due to *n*-butyl groups; $^{13}C\{H\}$ NMR (360 MHz, $CDCl_3$) δ 61.3 (carborane *CH*), 71.3 (carborane *CSi*), 83.8 (carborane *CC*), 86.3 (carborane *CC*), peaks due to *n*-butyl; $^{11}B\{H\}$ NMR (Et_2O) -15.3, -13.2, -11.7, -10.4; IR (Nujol, cm^{-1}) ν 2612 (B-H), 3026 (C-H); FAB-MS m/z 485 (M^+, 20, calcd for $C_{16}H_{48}{}^{10}B_4{}^{11}B_{16}Si$). **4c**: mp 168-169 °C; ^1H NMR (360 MHz, $CDCl_3$) δ 2.68 (s, carborane C*H*), 3.4-1.6 (m, B*H*, 20 H), peaks due to isobutyl groups; $^{13}C\{H\}$ NMR (360 MHz, $CDCl_3$) δ 61.3 (carborane *CH*), 72.5 (carborane *CSi*), 84.0 (carborane *CC*), 87.2 (carborane *CC*), peaks due to isobutyl groups; $^{11}B\{H\}$ NMR (Et_2O) -14.8, -12.7, -11.3, -10.0; IR (Nujol, cm^{-1}) ν 2620 (B-H), 3039 (C-H); FAB-MS m/z 485 (M^+, 20, calcd for $C_{16}H_{48}{}^{10}B_4{}^{11}B_{16}Si$).

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