# Assembly of an Exchange-Coupled [Ni:$Fe_4S_4$] Cluster in the α Metallosubunit of Carbon Monoxide Dehydrogenase from *Clostridium thermoaceticum* with Spectroscopic Properties and CO-Binding Ability Mimicking Those of the Acetyl-CoA Synthase Active Site

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*Received August 18, 1995*

Carbon monoxide dehydrogenases are one of only four known types of naturally-occurring Ni-containing enzymes.[1−3] The enzyme from *Clostridium thermoaceticum* (CODH) catalyzes two types of reactions: the reversible oxidation of CO to $CO_2$ and the synthesis of acetyl coenzyme A from CO, coenzyme A, and the methyl group of a corrinoid/iron−sulfur enzyme. These reactions are part of the Wood/Ljungdahl pathway used by these bacteria to grow autotrophically on $CO_2$ and $H_2$.[4,5]

CODH has an $\alpha_2\beta_2$ quaternary structure[6] ($M_r(\alpha) = 81\,730$ Da; $M_r(\beta) = 72\,928$ Da[7]) and contains *ca*. 4 Ni, 22−26 Fe, and ∼30 $S^{2-}$ ions, organized into three types of clusters. One of these, called the *A-cluster*, is almost certainly the active site for the synthesis of acetyl-CoA.[8−12] The exact structure of the A-cluster is unknown, but a plethora of spectroscopic studies have shed light on many of its properties. When reduced and bound with CO, the A-cluster has $S = {}^1/_2$ and exhibits the *NiFeC* EPR signal (Figure 1A). This signal, with $g_\perp = 2.08$ and $g_\parallel = 2.03$, is so-named because it exhibits hyperfine broadening when samples are enriched in either $^{61}$Ni ($I = {}^3/_2$) or $^{57}$Fe ($I = {}^1/_2$), or when samples are reduced with $^{13}$CO ($I = {}^1/_2$).[13−15] The broadening indicates that the A-cluster consists of a Ni-and-Fe exchange-coupled cluster that binds CO. The CO appears to bind end-on to one of the irons.[16] The Ni is probably coordinated to S and N/O donors[17,18] and is linked through a bridge to an Fe−S moiety that has Mössbauer spectroscopic properties of $[Fe_4S_4]^{2+}$ clusters.[19,20] The Ni in the A-cluster can be selectively removed with the chelator 1,10-phenanthroline (phen) and reinserted by incubating phen-treated enzyme with $Ni^{2+}$.[8−10] Removing this Ni destroys the enzyme's NiFeC signal and CO/acetyl-CoA exchange activity, and replacing it restores this signal and activity. The NiFeC signal quantifies to a maximum of only ∼0.3 spin/$\alpha\beta$.[19] This result, along with the fact that only ∼0.3 Ni/$\alpha\beta$ are removed by the phen treatment, suggests that only about 30% of $\alpha\beta$ dimers contain A-clusters with labile Ni sites and that the remaining dimers lack A-clusters but contain nonlabile Ni ions.[10]

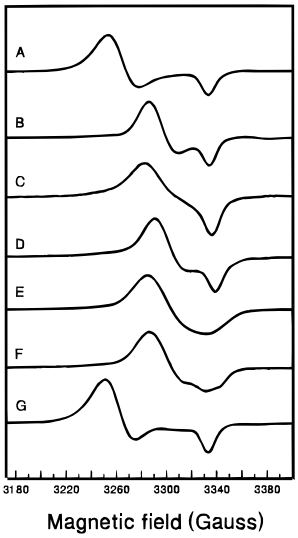


**Figure 1.** EPR of CODH, α, and FM-CODH after incubation with Ni. (A) Native CODH with CO (0.25 spin/$\alpha\beta$). (B) α incubated in $Ni^{2+}$ and CO (0.12 spin/α). (C) α incubated in $^{61}Ni^{2+}$ and CO (0.03 spin/α). (D) $^{61}$Ni-enriched α (i.e., prepared from CODH grown on $^{61}$Ni-enriched media) incubated in natural-abundance $Ni^{2+}$ and CO (0.09 spin/α). (E) $^{57}$Fe-enriched α incubated in $Ni^{2+}$ and CO (0.10 spin/α). (F) α incubated in $Ni^{2+}$ and $^{13}$CO (0.08 spin/α). (G) FM-CODH incubated in $Ni^{2+}$ and CO (0.11 spin/$\alpha_1\beta_2$). Samples were prepared as described.[25] EPR conditions: microwave frequency, 9.43 GHz; microwave power, 80 mW; temperature, 130 K; modulation amplitude, 11.8 G.

Upon treatment with SDS, native $\alpha_2\beta_2$ decomposes into α metallosubunits and a stable $\alpha_1\beta_2$ form called FM-CODH.[21] Isolated α contains 1 Ni and 4 irons.[22] The Ni is coordinated to two S donors at 2.19 Å and two N/O donors at 1.89 Å in a distorted square-planar geometry. The irons in α are organized into an $[Fe_4S_4]^{2+/+}$ cluster that is $S = {}^3/_2$ in its reduced form and exhibits EPR signals in the $g = 4-6$ region.[22] Mounting evidence suggests that α subunits house the A-cluster and function predominantly in acetyl-CoA synthesis.[21] Thus, the Ni and $Fe_4S_4$ centers in α may be decomposition products of the A-cluster.[22]

In this report, we show that incubating α with $Ni^{2+}$ affords an exchange-coupled cluster that closely mimics the spectroscopic properties and CO-binding ability of the A-cluster. Moreover, a similar incubation with FM-CODH yields *functional* A-clusters.

A sample[23] of isolated α was incubated with $NiCl_2$, CO, and a trace amount of native CODH (to reduce the sample). The solution exhibited an EPR signal (0.12 spin/α) with $g_\perp = 2.04$ and $g_\parallel = 2.02$ (Figure 1B) that was present at 130 K and started to saturate at ∼5 $\mu$W, 10 K. This signal had *g*-values and

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(23) Four batches of CODH were used in these experiments, with CO oxidation activities of 190, 240, 230, and 490 units/mg, and CO/acetyl-CoA exchange activities of 0.17, 0.15, 0.06, and 0.30 unit/mg, respectively. Batches 1 and 2 were enriched in $^{57}$Fe and $^{61}$Ni, respectively. CODH, α, and FM-CODH were purified, isolated, and characterized as described.[6,22,24]
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properties unlike the $g_{av} = 1.94$, 1.82, and 1.86 signals of CODH, but similar to the NiFeC signal. Thus, we call it the *pseudo-NiFeC* signal. If $NiCl_2$ was not added in the incubation, samples afforded less intense pseudo-NiFeC signals (0.04 spin/α). If $Ni^{2+}$ alone was added, no signal was observed.

The pseudo-NiFeC signal could not have originated from the native enzyme added, since the resulting concentration of native enzyme (1 μM) was below the EPR detection limit. Nor could it have originated from a contaminant of native CODH; the α used was ∼95% pure, and the corresponding 10 K spectra lacked the $g_{av} = 1.86$ signal which would have been present had the pseudo-NiFeC signal originated from native CODH. Thus, we conjecture that this signal originates from a cluster in the α subunit which we call the *pseudo-A-cluster*. Given that the signal developed upon incubation with Ni, we further suggest that the labile Ni ion in the A-cluster largely dissociated during isolation of α, and that the added $Ni^{2+}$ inserted into this empty site. A fraction of α subunits appears to have retained their labile Ni ions during the isolation procedure, since a residual pseudo-NiFeC signal was obtained when isolated α was incubated without added $Ni^{2+}$.

To test these ideas, a sample of isolated α was briefly incubated with phen (to remove labile Ni), then freed of phen chromatographically, and incubated with $^{61}NiCl_2$ as described.[25] The resulting signal exhibited substantial hyperfine broadening (Figure 1C), demonstrating that the added $^{61}Ni$ incorporated into the α subunit and is a constituent of the pseudo-A-cluster. To test whether the pre-existing Ni center in isolated α was part of the pseudo-A-cluster, $^{61}Ni$-enriched α (i.e., obtained from *C. thermoaceticum* grown on $^{61}Ni$-enriched media) was incubated with natural-abundance $NiCl_2$ (without pretreatment with phen). The resulting pseudo-NiFeC signal exhibited almost no broadening (Figure 1D), suggesting that the pre-existing Ni center in isolated α is not part of the pseudo-A-cluster. The very slight broadening obtained is almost certainly due to the fraction of α that retained $^{61}Ni$ in the labile center during isolation (the signal could be simulated by mixing signal 1B at 60% and 1C at 40% intensities).

Given the correlation between NiFeC signal intensity and labile Ni,[10] the intensity of the pseudo-NiFeC signal (∼0.1 spin/mol) suggests that only 10% of isolated α subunits have pseudo-A-clusters and labile Ni sites. The other 90% would appear to contain nonlabile Ni ions. This is consistent with the metal content of isolated α (1.0 ± 0.1 Ni/α).[22] However, quantitation of these proportions using radioactive $^{63}Ni$ is needed to establish this relationship.

To determine whether the $[Fe_4S_4]^{2+/+}$ cluster in α was a constituent of the pseudo-A-cluster, $^{57}Fe$-enriched α was incubated as above. The resulting signal exhibited substantial hyperfine broadening (Figure 1E), demonstrating that the $Fe_4S_4$ cluster in α is indeed part of the pseudo-A-cluster. To examine whether the pseudo-A-cluster binds CO, $^{13}CO$ was used in the reaction. The resulting signal was hyperfine-broadened (Figure 1F), demonstrating that this cluster has CO bound in its $S = {}^1/_2$ state. Thus, the spectroscopic properties and CO-binding ability of the pseudo-A-cluster mimic those of the true A-cluster. However, Ni-incorporated α did not exhibit CO/acetyl-CoA exchange activity, indicating that isolated α lacked a factor required for catalytic activity.

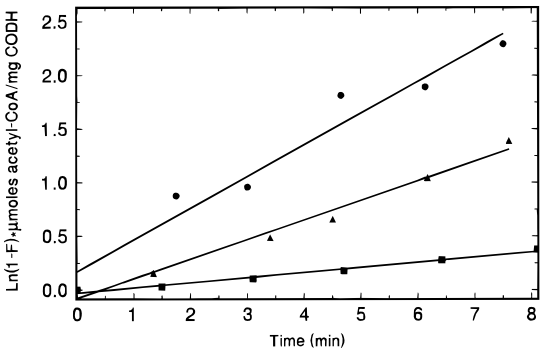


**Figure 2.** CO/acetyl-CoA exchange activity of native CODH (●), FM-CODH (■), and FM-CODH after incubation in $Ni^{2+}$ (▲). Samples were from batch 4. Native CODH (0.24 mg), FM-CODH (0.45 mg), and FM-CODH after incubation with 1 mM $NiCl_2$ for 15 h (0.45 mg) were assayed as described.[26,27] Solid lines are least-squares best fits to the data.

Whether α required the β subunits for activity was examined by incubating FM-CODH ($\alpha_1\beta_2$) with $Ni^{2+}$. In this case, EPR signals (0.08−0.14 spin/$\alpha_1\beta_2$) with the same *g*-values and properties of the true NiFeC signal resulted (Figure 1G), and samples exhibited substantial CO/acetyl-CoA exchange activity. One FM-CODH sample (∼85% pure) exhibited 0.05 unit/mg CO/acetyl-CoA exchange activity prior to adding $Ni^{2+}$ (Figure 2, ■), and 0.18 unit/mg thereafter (Figure 2, ▲). The native CODH sample from which that FM-CODH sample had been obtained exhibited 0.30 units/mg (Figure 2, ●). The activity prior to adding $Ni^{2+}$ probably arose because a portion of FM-CODH molecules retained their labile Ni ions during isolation.

In summary, a cluster with spectroscopic properties and CO-binding ability mimicking those of the A-cluster can be assembled in the isolated α subunit. This cluster is composed of a labile Ni ion exchange-coupled, almost certainly through one or more bridging ligands, to the $Fe_4S_4$ cluster in α. Because the properties of this cluster are so close to those of the A-cluster, we conclude that the A-cluster has fundamentally the same structure and is located in the α subunit. It appears that only ∼10% of isolated α subunits contain a labile Ni site, and that ∼90% contain a Ni center that is not part of the A-cluster and is not labile. Isolated α subunits have no CO/acetyl-CoA exchange activity, but individual α subunits attached to two β subunits (FM-CODH, $\alpha_1\beta_2$) do. This indicates that the β subunits confer activity onto α. It also demonstrates that acetyl-CoA synthase activity does not require the concerted functioning of both α subunits of native $\alpha_2\beta_2$. Thus, the active site for acetyl-CoA synthesis is a single A-cluster.

The ability to isolate the acetyl-CoA synthase active site from the other metal centers in an enzyme as complicated as CODH is remarkable. This achievement should allow us to probe, in some detail, the structure of the active site and the reaction mechanism.

**Acknowledgment.** We thank Mark E. Anderson for providing the $^{61}Ni$-enriched CODH. This work was supported by the National Institutes of Health (GM46441) and the Robert A. Welch Foundation (A-1170).

JA952845U

(25) In A, native CODH (batch 4, 28 μM αβ) was reduced with CO. In B, dithionite-free α (batch 4; 330 μL, 82 μM) was reacted with 30 μL of 10 mM $NiCl_2$, concentrated to 140 μM, reacted with 20 μL of Triton X100 (200 mg/mL) and 2 μL of native CODH (8 mg/mL), and then incubated in a CO-filled EPR tube for 15 h at 26 °C. In C, dithionite-free α (batch 3; 2 mL, 100 μM) was treated with 75 μL of 30 mM 1,10-phenanthroline. After 2 h, the solution was chromatographed on Sephadex G25 equilibrated in 50 mM Tris pH 8.0 and concentrated to 130 μM, 350 μL of which was reacted with 30 μL of 3.3 mM $^{61}NiCl_2$ and 2 μL of native CODH and incubated as above. In D, dithionite-free $^{61}Ni$-enriched α (1.5 mL, 30 μM) was reacted with 100 μL of 10 mM $NiCl_2$, concentrated to 450 μM, reacted with 10 μL of Triton, 2 μL of native CODH, and 10 μL of 10 mM $NiCl_2$, and incubated as above. In E, dithionite-free, $^{57}Fe$-enriched α (1.5 mL, 41 μM) was reacted with 100 μL of 10 mM $NiCl_2$, concentrated to 80 μL, reacted with 8 μL of Triton, 2 μL of native CODH, and 8 μL of 10 mM $NiCl_2$, and then incubated as above. In F, dithionite-free α (batch 4, 1 mL, 73 μM) was reacted with 100 μL of 10 mM $NiCl_2$, concentrated to 170 μL, and reacted with 17 μL of Triton and 17 μL of 10 mM $NiCl_2$. The resulting solution (90 μL) was reacted with 2 μL of native CODH and incubated as above, but in $^{13}CO$. In G, dithionite-free FM-CODH (batch 3, 350 μL, 32 μM $\alpha_1\beta_2$) was incubated with 35 μL of 10 mM $NiCl_2$ for 15 h and then reduced by CO.

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