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Inactivation of Acetyl-CoA Synthase/Carbon Monoxide Dehydrogenase by Copper

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Acetyl-CoA synthases are found in evolutionarily primitive chemoautotrophic anaerobic prokaryotes.[1] The enzyme from *Moorella thermoacetica* (ACS) is an $\alpha_2\beta_2$ tetramer that synthesizes acetyl-CoA from CO, CoA, and a methyl group donated by a corrinoid-iron−sulfur protein (CoFeSP).[2] Two recent X-ray structures of ACS are equivalent except for the conformations of the α subunits and the active site for acetyl-CoA synthesis (the A-cluster).[3,4] In the Doukov et al. structure, this cluster consists of a $Cu(S_{cys})_3$ site in which one cysteinate is μ^2-bridged to an Fe_4S_4 cubane and the other two are μ^2-bridged to a NiN_2S_2 moiety. One α subunit of the Darnault et al. structure contains Zn rather than Cu in this *proximal* site (named relative to the cubane), while the A-cluster of the other α subunit contains Ni in this site (Ni_p). A small fraction of Ni in ACS (0.2−0.3 Ni/$\alpha\beta$) is removed by 1,10-phenanthroline (phen); this abolishes synthase activity and the NiFeC EPR signal otherwise exhibited by this cluster.[5,6] Incubation of phen-treated ACS in $NiCl_2$ restores activity and signal intensity. The geometry of the proximal site, its ability to be occupied by different metal ions, and the proportion of labile Ni removed by phen, taken together, suggest that labile Ni corresponds to Ni_p. A less likely alternative is that labile Ni corresponds to distal Ni_d.

Lurking behind this issue is whether Cu plays a role in ACS's mechanism. Doukov et al. and Seravalli et al. report a correlation between Cu content, acetyl-CoA synthase (technically CO/acetyl-CoA exchange) activity, and NiFeC signal intensity.[4,7] They suggest catalytic mechanisms in which CO and acetyl groups bind to Cu^{1+}. In contrast, Ni_p is the central player in the Darnault et al. mechanism. A subunit from a related enzyme incubated in $NiCl_2$ exhibits a modicum of activity (but not when incubated in $CuCl_2$), suggesting that Cu-containing ACS is inactive and that the Ni form is functional.[8]

We find Cu in our ACS samples at amounts (0.2−0.5 Cu/$\alpha\beta$) similar to those reported.[4,7] However, Cu is abundant in the environment, and we also detect it in solutions of proteins known not to coordinate Cu or require it for catalysis. To address whether Cu is required for ACS activity, we endeavored to prepare a Cu-free ACS sample and determine its activity.[9] Cu was removed from growth and protein purification media and equipment, and Neocuproine (a phen derivative) was added to all purification buffers. The resulting >90% pure ACS sample contained 0.05 Cu/$\alpha\beta$ and exhibited no synthase activity. However, after treating with $NiCl_2$, it exhibited an activity of 105 min^{-1} (Figure 1A, open circles), 52% of the highest activity reported.[10] When exposed to CO, this sample exhibited a NiFeC signal corresponding to 0.15 spin/$\alpha\beta$ (Figure 1C, III). This amount of Cu was insufficient to have been responsible for the observed activity or NiFeC signal intensity, indicating that *Cu is not required for ACS activity or for exhibiting the NiFeC signal*.

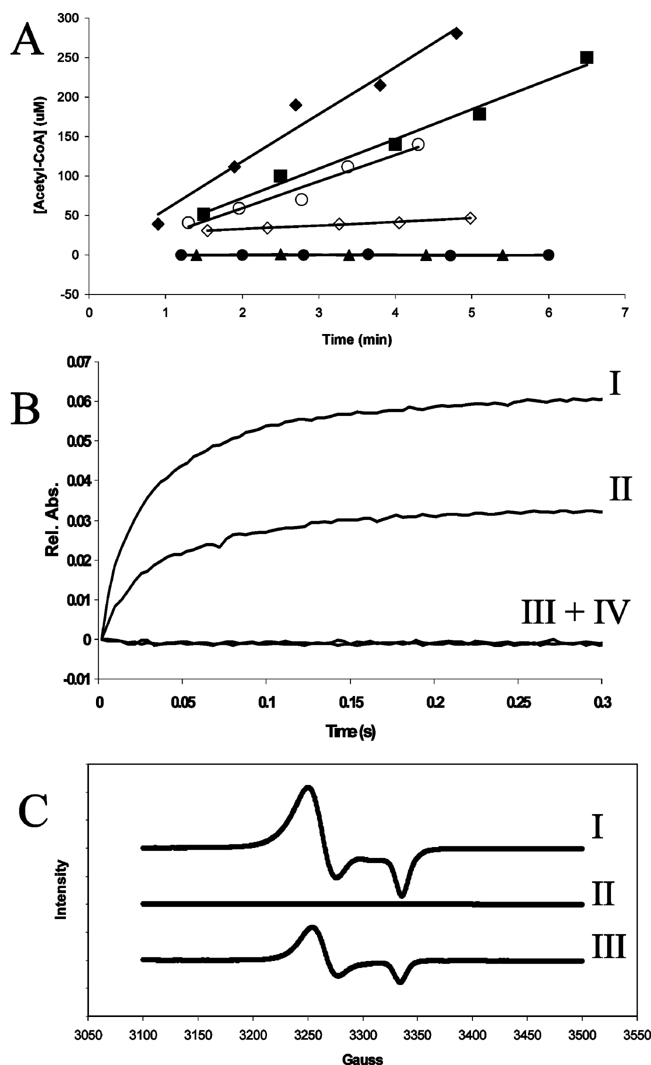

Figure 1. (A) Acetyl-CoA synthase activity of ACS (performed as described[10] using 0.3 μM ACS). (◆) native ACS; (○) Ni-reactivated Cu-depleted ACS; (●) phen-treated ACS; (■) Ni-reactivated phen-ACS (rate = 120 min^{-1}); (▲) Cu-and-phen-treated ACS; (◇) Cu-depleted ACS plus 1.5 equiv of $CuCl_2$ (rate = 15 min^{-1}). (B) Methyl group transfer activity of samples from A.[11] I, native ACS; II, Ni-reactivated phen-treated ACS (k_{app} = 16 s^{-1}); III, phen-treated ACS; IV, Cu-and-phen-treated ACS. (C) 130 K, 80 mW EPR (essentially as described[5]) of ACS. I, native ACS (0.2 spins/$\alpha\beta$); II, ACS plus $CuCl_2$ and Ti^{3+}citrate; III, Ni-reactivated Cu-depleted ACS.

Adding 1.5 equiv of $CuCl_2$ to Ni-reactivated Cu-depleted ACS *lowered* activity 86% (Figure 1A, open diamonds). Treating a native ACS sample that had 197 min^{-1} activity (Figure 1A, closed diamonds) with 10 equiv of Cu lowered activity to 73 min^{-1}. When

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Ti^{3+}citrate was included in an equivalent Cu-treatment (ostensibly to reduce Cu^{2+} to Cu^{1+}) the resulting sample had neither activity nor NiFeC signal (Figure 1C, II). This sample contained 8 Cu/$\alpha\beta$ after passage through a desalting column. Thus, *adding Cu ions to ACS solutions inhibits ACS activity and NiFeC formation*.

We wanted to determine whether the *proximal* site, as defined crystallographically, was the *labile* site as defined by the effect of phen. The activity of an ACS sample was abolished by phen treatment (Figure 1A, closed circles). Aliquots were equivalently incubated in 10 equiv of $NiCl_2$ and $CuCl_2$. The Ni-treated sample exhibited activity (Figure 1A, squares) while the Cu-treated sample remained inactive (Figure 1A, triangles). Portions of these samples were incubated in CO and examined by EPR. Native ACS and Ni-reactivated phen-treated samples exhibited NiFeC signals with intensities of 0.2 (Figure 1C, I) and 0.05 spin/$\alpha\beta$, respectively. Phen-treated and Cu-and-phen-treated samples did not exhibit this signal. The Cu-treated sample was then incubated in $NiCl_2$; no activity developed. This suggests that Cu had bound to the labile site and that it bound more tightly than Ni and blocked Ni from inserting into that site. Since Doukov's structure shows that Cu binds in the proximal site of the A-cluster, we conclude that *the proximal Ni site is the previously identified labile Ni site and that active ACS contains Ni in this site.* We add a cautionary note that Cu might also bind to other sites on ACS, as the Cu-treated sample, after desalting, contained 11 Cu/$\alpha\beta$.

A native ACS sample accepted methyl groups from CH_3-Co^{3+}FeSP with $k_{app} \approx 20\ s^{-1}$ (Figure 1B, I), while a matching phen-treated ACS sample was unreactive (Figure 1B, III). Ni-reactivated phen-treated ACS had a rate constant corresponding to ~80% of that afforded by native ACS (Figure 1B, II). This proportion was similar to the relative amount of synthase activity exhibited by that sample. The Cu-and-phen-treated sample was unreactive (Figure 1B, IV). This result appears incongruent with Ni_d being the site of methylation; in that case, Cu-and-phen-treated ACS should have accepted a methyl group. We conclude that *Cu in the proximal site cannot accept methyl groups* from CH_3-Co^{3+}FeSP and that *this precludes acetyl-CoA synthesis*.

To determine whether *M. thermoacetica* effectively discriminates between Ni and Cu ions, cells were grown in media containing 1mM $CuCl_2$ (1000× normal). Cells reached a final density corresponding to one-third of normal. Harvested cells were washed to remove an estimated 99.9% of the Cu in the growth media, and ACS was purified without added Cu. The purified protein contained 0.74 Cu/$\alpha\beta$, was catalytically inactive, and did not exhibit a NiFeC signal. That these cells grew at all is surprising, given the effect of Cu on ACS activity and the reported requirement for ACS in the metabolism of this organism.[12] We suspect that Ni_p-containing ACS was synthesized by growing cells but that Cu, present at an unusually high in vivo concentration, gradually and irreversibly replaced Ni_p thereby halting growth. We conclude that *Cu could have been inserted into the samples of Doukov et al. either by the presence of Cu in their growth media or by exposing purified ACS to buffer that contained low concentrations of Cu ions*.

ACS grown in ^{61}Ni ($I = 3/2$), treated with phen and reactivated with natural-abundance $NiCl_2$ afforded a NiFeC signal lacking Ni-hyperfine.[5] In contrast, ACS grown in natural-abundance Ni, treated with phen, and reactivated with ^{61}Ni exhibited a signal *with* Ni-hyperfine.[5] These previous results now suggest that Ni_p but not Ni_d is part of the hyperfine-coupled $S = 1/2$ system and that CO is bound to Ni_p^{1+} in the A_{red}-CO state yielding this signal. This is consistent with the square-planar (i.e., low-spin, diamagnetic) structure of Ni_d^{2+}. The abolition of that signal by Cu binding to the proximal site is likely due to the diamagnetism of Cu^{1+}. The inactivity of Cu−ACS is likely due to the insufficient nucleophilicity of Cu^{1+} for attacking the methyl group of CH_3-Co^{3+}FeSP.

Besides,[4,7] there are no reports of Cu-containing proteins in *any* organism known to contain an ACS or CODH.[13] Taken in conjunction with the results presented here, this raises the possibility that Cu is irrelevant and detrimental to the metabolism of these organisms. ACS/CODH-containing organisms grow at low potentials where Cu ions would be most stable in the Cu(I) state and competing with essential metals (e.g., Ni and Fe) for sulfur ligands. Cu may have become embraced by living systems only after they employed O_2 in respiration and abandoned metabolisms involving O_2-and-Cu(I)-sensitive ACS/CODH's.

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(9) Cells were grown and harvested, and native ACS was purified to 85% purity as described.[11] Cells and Cu-depleted ACS were prepared similarly except that: (a) glassware was soaked in dilute HNO_3, (b) yeast extract was passed through a Chelex column, (c) all buffers contained 0.1 mM neocuproine (Sigma) and, (d) the second DEAE column was replaced with a column of Q-Sepharose Fast Flow (Amersham Biosciences). The resulting protein was 93% pure. ACS was incubated in 10 equiv of $NiCl_2$ for 48 h after dithiothreitol and dithionite were removed by passing through Sephadex G-25 (Sigma). ACS samples were digested overnight in trace-metal grade HNO_3 (Fisher), diluted, and analyzed on a Perkin-Elmer Elan DRC II ICP-MS.
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