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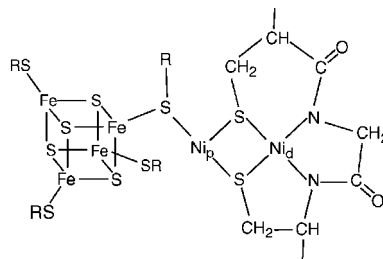
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Acetyl-coenzyme A synthase/carbon monoxide dehydrogenase is an $\alpha_2\beta_2$ tetramer, found in anaerobic archaea and bacteria that grow chemoautotrophically on CO or CO_2 .¹ The active site A-cluster in the synthase α subunit consists of an Fe_4S_4 cluster coordinated through a bridging cysteinate to a $[\text{Ni}_p \text{Ni}_d]$ subcomponent (Chart 1).² The oxidized state of the cluster assembly, A_{ox} , can be formulated as $\{[\text{Fe}_4\text{S}_4]^{2+} \text{Ni}_p^{2+} \text{Ni}_d^{2+}\}$.^{3–5} A_{ox} does not accept, in the presumed first catalytic step, a methyl cation from the corrinoid–iron–sulfur protein ($\text{CH}_3\text{--Co}^{3+}\text{FeSP}$).⁶ However, methyl transfer occurs when A_{ox} is treated with low-potential reductants such as Ti(III) citrate. The electronic configuration for the reductively activated $\text{A}_{\text{red-act}}$ has not been established but the state is probably two electrons more reduced than A_{ox} .⁴ Reduction of the square-planar distal Ni_d^{2+} with amide N coordination is unlikely,⁷ and stopped-flow evidence disfavors the reduction of the $[\text{Fe}_4\text{S}_4]^{2+}$ cluster,⁶ leaving the proximal Ni_p^{2+} as the only site that might accept electrons. Attainment of a Ni_p^0 state has been suggested,⁵ and this idea is supported by a report that a Ni^0 phosphine complex can accept a methyl cation from a $\text{CH}_3\text{--Co}^{3+}$ complex.⁸ Independent DFT studies by Brunold and Field argue against this suggestion and support a $\{[\text{Fe}_4\text{S}_4]^{1+} \text{Ni}_p^{1+}\}$ configuration for $\text{A}_{\text{red-act}}$.^{9,10} This hypothetical state was not observed in our previous Mössbauer study where it was found that Ti(III) citrate-reduced Ni-activated recombinant α subunits exhibited two major species: $\sim 70\%$ of spectral intensity originated from $\{[\text{Fe}_4\text{S}_4]^{1+} \text{Ni}_p^{2+}\}$ centers while $\sim 30\%$ reflected a diamagnetic state containing an $[\text{Fe}_4\text{S}_4]^{2+}$ moiety.⁴ Given that the enzyme is known to be heterogeneous, with $\sim 30\%$ functional and $\sim 70\%$ nonfunctional A-centers,^{3,4} this result was consistent with a $\{[\text{Fe}_4\text{S}_4]^{2+} \text{Ni}_p^0\}$ state in functional subunits.

We recently altered our procedure for preparing recombinant α subunit by adding NiCl_2 to purified apo- α subunit (containing the Fe_4S_4 cluster but lacking the Ni subcomponent) rather than including it in the growth medium.¹¹ Gel filtration FPLC of Ni-activated α subunits indicated Ni-dependent oligomerization into dimers and other forms.¹² Here we report Mössbauer evidence that α dimer samples prepared in this manner, when reduced by Ti(III) citrate, contain A-clusters in the $\{[\text{Fe}_4\text{S}_4]^{1+} \text{Ni}_p^{1+}\}$ state. We also report a new EPR signal that appears to arise from the semireduced $\{[\text{Fe}_4\text{S}_4]^{2+} \text{Ni}_p^{1+}\}$ state.

The 4.2 K Mössbauer spectrum of Ni-activated α dimers reduced with Ti(III) citrate (Figure 1A) exhibits three components. A paramagnetic component ($\sim 20\text{--}30\%$ of Fe), extending from -2 mm/s to $+2$ mm/s Doppler velocity, most probably belongs to $\{[\text{Fe}_4\text{S}_4]^{1+} \text{Ni}_p^{2+}\}$ clusters. The central portion of the spectrum exhibits two doublets (**1** and **2**) with $\Delta E_{\text{Q1}} = 1.21(4)$ mm/s, $\delta_1 =$

Chart 1. Structure of the A-Cluster^a



^a SR represents cysteinyl residues.

$0.56(2)$ mm/s, $51(3)\%$ of Fe, and $\Delta E_{\text{Q2}} = 0.47(2)$ mm/s, $\delta_2 = 0.55(2)$ mm/s, $21(2)\%$. The relative intensities of **1** and **2** are $\sim 2.4:1$ rather than $2:2$ or $3:1$ as one might expect if doublets **1** and **2** would represent subsites in one $[\text{Fe}_4\text{S}_4]$ cluster. The $[\text{Fe}_4\text{S}_4]^{2+}$ cubane of the A-cluster has $\delta = 0.45(2)$ mm/s in all states examined,³ while $[\text{Fe}_4\text{S}_4]^{1+}$ clusters typically have an average $\delta \approx 0.54\text{--}0.59$ mm/s.^{13,14} The $S_c = 1/2$ $[\text{Fe}_4\text{S}_4]^{1+}$ cluster of apo- α has $\delta = 0.55$ mm/s and $\Delta E_{\text{Q}} = 0.92$ mm/s (Supporting Information, Figure S1). Thus, the values for δ_1 and δ_2 indicate the $[\text{Fe}_4\text{S}_4]^{1+}$ state.

Magnetically isolated $S_c = 1/2$ $[\text{Fe}_4\text{S}_4]^{1+}$ clusters generally exhibit paramagnetic hyperfine structure at 4.2 K (e.g., Figure S1), reflecting hyperfine fields up to ~ 12 T. The observation of doublets **1** and **2** at 4.2 K suggests A-clusters with integer or zero electronic cluster spin. This argument is supported by spectra recorded in applied fields of 0.1, 8, and 7.0 T (Figure 1D), which show that **1** and **2** belong to diamagnetic A-clusters, strongly suggesting exchange coupling between an $S_c = 1/2$ $[\text{Fe}_4\text{S}_4]^{1+}$ cluster and an $S_{\text{Ni}} = 1/2$ Ni_p^{1+} site.

Figure 1C shows a 4.2 K spectrum of Ti(III) citrate-reduced α dimers after incubation with CO. This treatment produces the $S = 1/2$ NiFeC signal, the signature of the exchange coupled, $\{[\text{Fe}_4\text{S}_4]^{2+} \text{Ni}_p^{1+}\text{--CO}\}$, $\text{A}_{\text{red-CO}}$ state.^{3,15} In one preparation, we obtained by EPR 0.39(4) spins/ α together with the corresponding Mössbauer component, representing 34(3)% of the Fe. For a second preparation (Figure 1C) 31(2)% of the A-clusters were in the $\text{A}_{\text{red-CO}}$ state (dashed line drawn above Figure 1C). The remainder of the sample exhibits two doublets, **3** and **4**, again reflecting $[\text{Fe}_4\text{S}_4]^{1+}$ clusters albeit with parameters that differ from the Ti(III) citrate-reduced sample: $\Delta E_{\text{Q3}} = 0.96$ mm/s, $\delta_3 = 0.51$ mm/s, $42(3)\%$ of Fe, and $\Delta E_{\text{Q4}} = 1.88$ mm/s, $\delta_4 = 0.60$ mm/s, $21(2)\%$. Interestingly, addition of CO changed all spectral components. We speculate that **3** and **4** reflect a $\{[\text{Fe}_4\text{S}_4]^{1+} \text{Ni}_p^{1+}\text{--CO}\}$ cluster.¹⁶ The data of Figure 1C also imply that all α subunits in the sample have the proximal site occupied by Ni. Studies at 8.0 T applied field (Figure S3) show that **3** (and probably **4**) originates from a diamagnetic site.

The fractions quoted for **1,2** and **3,4** do not reveal whether doublets **1,2** (and **3,4**) belong to the same cluster; we defer this

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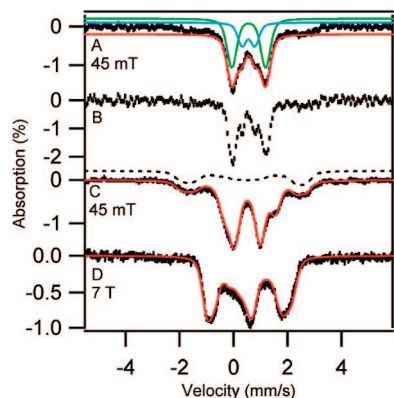


Figure 1. The 4.2 K Mössbauer spectra of α dimer taken in applied fields as indicated. (A) Spectrum of 1 mM Ti(III) citrate-reduced α dimer. Red solid line is a superposition of doublets **1** and **2**, using the parameters quoted in the text; blue and green solid lines show doublets separately. (B) Spectrum A after removing the line width contribution of the ^{57}Co source by a Fourier deconvolution. (C) Spectrum of 1 mM Ti(III) citrate-reduced α dimer after exposure to CO. The solid line is a simulation for doublets **3** and **4** superimposed on a simulation for the $A_{\text{red-CO}}$ species (dashed line above data, using published parameters³); (D) 7.0 T spectrum (parallel applied field) of the sample of spectrum A after removal of a 25% contribution of apo- α . The solid line is a spectral simulation assuming that **1** and **2** are diamagnetic; raw data and all parameters are given in Supporting Information.

problem to future studies. Here we discuss aspects of the electronic structure of the A-cluster implied by the observation of diamagnetic species with $\delta \approx 0.55\text{--}0.60$ mm/s, using **1** as an example.

$[\text{Fe}_4\text{S}_4]^{1+}$ clusters occur in various spin states, mostly with $S_c = 1/2$ and $3/2$. We ignore $S_c = 3/2$ configurations here as they do not yield a diamagnetic ground-state for $\{[\text{Fe}_4\text{S}_4]^{1+} \text{Ni}_p^{1+}\}$. Frequently, the $S_c = 1/2$ ground-state of $[\text{Fe}_4\text{S}_4]^{1+}$ clusters yields a diferrous and mixed-valence pair, distinguishable by their ΔE_Q and δ values.¹³ However, some $[\text{Fe}_4\text{S}_4]^{1+}$ clusters,¹⁷ like that of apo- α of Figure S1, have the same ΔE_Q and δ for all sites. The exchange-coupling pathway between the $S_c = 1/2$ $[\text{Fe}_4\text{S}_4]^{1+}$ cluster and the $S_{\text{Ni}} = 1/2$ Ni_p^{1+} involves the cysteinate that links one cluster site, previously labeled Fe_D , to the Ni^{1+} ($H = J S_D S_{\text{Ni}}$, where S_D is the local spin of cluster site D).³ The J -term acts in first-order between the two $S = 1/2$ ground manifolds and mixes in second-order excited $[\text{Fe}_4\text{S}_4]^{1+}$ cluster states into the ground manifold. The second-order term is relevant for understanding the $A_{\text{red-CO}}$ state³ but we ignore it here. Coupling then yields a singlet (A-cluster spin $S = 0$) and a triplet state, split by $K_D J$, where K_D is a spin projection factor ($|K_D| \leq 1.5^{18}$) that depends on the internal coupling of the $[\text{Fe}_4\text{S}_4]^{1+}$ cluster and the nature of Fe_D . For $K_D J > 0$ the ground-state is diamagnetic. The solid line in Figure 1D is a spectral simulation assuming that **1** and **2** are diamagnetic.

In a series of preliminary experiments we observed a new EPR signal when samples of Ni-activated α dimers were treated with substoichiometric amounts of Ti(III) citrate. This axial signal (Figure 2A) has $g_{\parallel} = 2.10$ and $g_{\perp} = 2.03$, values typical of Ni^{1+} species. The intensity of the axial signal increases as Ti(III) citrate is initially added (Figure 2B) and maximizes at 0.2–0.4 equivalents/ α of Ti(III) citrate (see also Supporting Information). The qualitative behavior of the titration plot suggests that the signal originates from a semireduced $S = 1/2$ $\{[\text{Fe}_4\text{S}_4]^{2+} \text{Ni}_p^{1+}\}$ state which is then further reduced to the $\{[\text{Fe}_4\text{S}_4]^{1+} \text{Ni}_p^{1+}\}$ state.

After the Mössbauer spectra were collected, the two samples of Figure 1 were analyzed for their ability to accept a methyl group from CoFeSP. In each case, 0.3 methyl groups per α were transferred, indicating that only $\sim 30\%$ of the α subunits in these samples arise from the functional $A_{\text{red-act}}$ state.

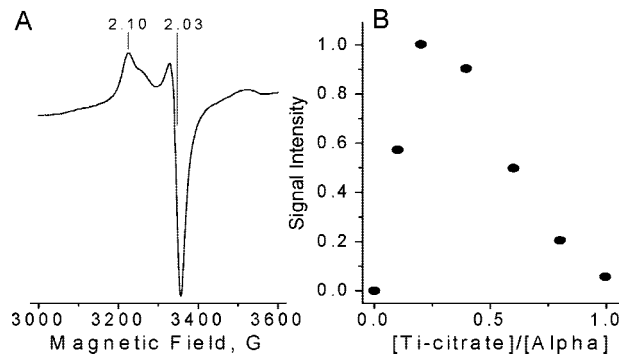


Figure 2. (A) EPR spectrum of Ni-activated α dimer reduced with 0.2 Ti(III) citrate/ α monomer. Conditions: 9.482 GHz; 1 mT modulation; $T = 10$ K; 20 mW microwave power. (B) Plot of EPR signal intensity (amplitude of derivative feature) versus amount of Ti(III) citrate added.

In summary, our results demonstrate that Ti(III) citrate can reduce the isolated Ni-activated α subunit of the title enzyme to form the $\{[\text{Fe}_4\text{S}_4]^{1+} \text{Ni}_p^{1+}\}$ state, and what appears to be the semireduced $\{[\text{Fe}_4\text{S}_4]^{2+} \text{Ni}_p^{1+}\}$ state. The former state has been suggested by DFT calculations, and the latter has been proposed as a catalytic intermediate¹ but here we provide the first experimental evidence for the existence of these states. Although further studies are required to reveal which of the Mössbauer spectral components exhibited by the Ti(III) citrate-treated sample represent the $A_{\text{red-act}}$ state, binding of a methyl cation to $\{[\text{Fe}_4\text{S}_4]^{1+} \text{Ni}_p^{1+}\}$ could result, after internal electron transfer from the $[\text{Fe}_4\text{S}_4]^{1+}$ moiety to Ni_p , in the methyl-bound state $\{[\text{Fe}_4\text{S}_4]^{2+} \text{Ni}_p^{2+-\text{CH}_3}\}$.⁴

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Supporting Information Available: Sample preparations; Mössbauer and EPR spectra of apo- α ; details of Mössbauer analyses and comments on exchange coupling. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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