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Reduction and Methyl Transfer Kinetics of the α Subunit from Acetyl Coenzyme A Synthase

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Acetyl-coenzyme A synthase is a bifunctional enzyme found in anaerobic archaea and bacteria that grow autotrophically on simple inorganic compounds such as CO_2/H_2 or CO. The enzyme from *Moorella thermoacetica* (ACS) is an $\alpha_2\beta_2$ tetramer containing two unique Ni−Fe−S active sites connected by a molecular tunnel.[1,2] The $Ni_1Fe_4S_{4-5}$ C-cluster catalyzes the reversible reduction of CO_2 to CO and is located in the β subunit. CO generated at this site migrates through the tunnel to the A-cluster, located in α, where it reacts with CoA and a methyl group to generate acetyl-CoA. During catalysis, the two sites are mechanistically coupled.

The oxidized form of the A-cluster has a Ni^{2+} ion bridged to an $[Fe_4S_4]^{2+}$ cubane, reportedly via a Cu complex.[2] During catalysis, a methyl cation from a corrinoid−iron−sulfur protein (CH_3-Co^{3+}FeSP or Me-CP) transfers, presumably to the Ni. We favor a mechanism in which CO inserts into the CH_3−Ni bond and nucleophilic attack by CoA on the resulting $CH_3C(O)$-Ni group yields acetyl-CoA.[1] Methyl transfer cannot occur unless a site on the enzyme is reduced. This so-called D-site has been suggested to consist of a redox-active cystine/dicysteinate pair closely associated with the Ni.[3] The $2e^-$-reduced D_{red} state transfers those electrons to Ni^{2+} as the methyl cation binds, reaction 1.

$$[Ni^{2+}\ D_{red}] + CH_3\text{-}Co^{3+}FeSP \rightleftarrows [CH_3\text{-}Ni^{2+}\ D_{ox}] + Co^{1+}FeSP \quad (1)$$

The kinetics of this process has been studied by stopped-flow.[4,5] In this technique, solutions of reactants are rapidly mixed, and a reaction-sensitive chromophore is monitored by UV−vis spectroscopy. Reaction 1 can be monitored at 390 or 450 nm, wavelengths sensitive to CoFeSP. Fitting the data required a three-step model involving: (a) docking of ACS and CH_3-Co^{3+}FeSP; (b) methyl transfer, and (c) undocking of the two proteins.[5] Apparent overall forward and reverse second-order rate constants corresponding to reaction 1 ($k_f \approx 12\ \mu M^{-1}\ s^{-1}$; $k_r \approx 2\ \mu M^{-1}\ s^{-1}$) indicate that methyl transfer was reversible ($K_{eq} \approx 6$) and fast.

The α subunit of ACS can be cloned and overexpressed in *Escherichia coli*.[6] Using CO as a substrate, recombinant α catalyzes the synthesis of acetyl-CoA, but at a rate ∼10-times slower than that for ACS. The catalytic mechanism used by α is undoubtedly similar to that used by ACS, allowing mechanistic investigations unfettered by the β subunit. The goals of this study were to investigate the roles of the Fe_4S_4 component of the A-cluster and the D-site, and to understand the relationship between the two, if any. For example, this component could be the D-site itself or an electron-transfer conduit for reducing it.

We wondered whether the methyl group of CH_3-Co^{3+}FeSP also transferred reversibly to isolated α. To evaluate this, α was preincubated in Ti^{3+}citrate (to reduce D_{ox}) and then reacted with CH_3-Co^{3+}FeSP.[7] The data (Figure 1A) demonstrated that the methyl group transferred, albeit slower than with ACS. The reaction was completed within ∼1 s (the equivalent reaction with ACS completes within ∼0.2 s). Nearly identical rates were obtained at 450 nm. The methyl group also transferred in the reverse direction, from CH_3-α to Co^{1+}FeSP (Figure 1B), again at rates significantly slower than with ACS. Experiments were repeated using various protein concentrations (Table 1). Previously described kinetic data-fitting procedures and criteria were employed.[5] Differential equations were generated from models and numerically integrated using a set of candidate rate constants. Simulations were fitted using the Simulated Annealing algorithm. In contrast to the situation with ACS, methyl group transfer data could be simulated assuming a single reaction, namely 1. Optimized k_f and k_r were ∼7 and 14 times smaller, respectively, than with ACS, and K_{eq} (∼11) was ∼2-times larger.

Table 1. Methyl Transfer Reaction Parameters[a]

	measured, μM		best-fit, μM		best-fit, $\mu M^{-1}\ s^{-1}$		
	[MeCP]	[α_{red}]	[MeCP]	[α-Ni_L]	k_f	k_r	λ_{max} nm
1	5.0	10.0	4.7	3.4	2.0	0.20	390
2	5.0	10.0	5.2	3.5	2.4	0.10	450
3	2.5	5.0	2.9	1.4	1.7	0.19	390
4	2.5	5.0	2.6	1.6	1.8	0.15	450
5	14.0	10.0	14.0	3.1	1.3	0.10	390
6	14.0	10.0	14.2	3.2	1.0	0.10	450
7	6.0	10.0	6.3	2.9	1.1	0.15	390
8	3.0	10.0	3.0	2.9	1.5	0.15	390
av					1.6	0.14	
sd					0.5	0.04	

[a] Results 1−6 are for the forward methyl transfer reaction, while 7 and 8 are for reverse reaction (and where proteins refer to Co^{1+}FeSP and CH_3-α_{ox} rather than to CH_3-Co^{3+}FeSP and α_{red}). Experiments 1−6 used one batch of proteins, while experiments 7−8 used a different batch of proteins. Best-fit [α-Ni_L] refers to the concentration of α containing labile nickel. Listed protein concentrations are those after mixing in the stopped-flow cell.

Next, we explored the role of the Fe_4S_4 component of the A-cluster in catalysis. In previous studies, this component was found to be reducible by low-potential reductants such as dithionite and Ti^{3+}citrate.[8] To examine the kinetics of this process, oxidized α_{ox} was mixed with 1 mM Ti^{3+}citrate, and monitored by stopped-flow at 420 nm, where $[Fe_4S_4]^{2+}$ clusters exhibit a broad absorption shoulder.[9] As these clusters become reduced to the 1+ core state, absorption declines. Reduction occurred slowly and was completed after ∼300 s (Figure 1C). This rate was about 200-fold slower than that of methyl group transfer. The reaction was repeated at various concentrations of α_{ox}; the decline was monophasic and first-order in α_{ox} (k_{app} was invariant at $\sim 0.02\ s^{-1}$). The calculated extinction coefficient for reduction ($\epsilon = 4000\ M^{-1}\ cm^{-1}$) was similar to that reported for ferredoxins and previous α subunit preparations.[6,8,9] The equivalent reaction was performed using α_{ox} that had not been activated with $NiCl_2$. This state, equivalent to phenanthroline-treated ACS, lacks labile Ni, acetyl-CoA synthase activity, the NiFeC EPR signal, and methyl group transfer ability.[1] Reduction was also

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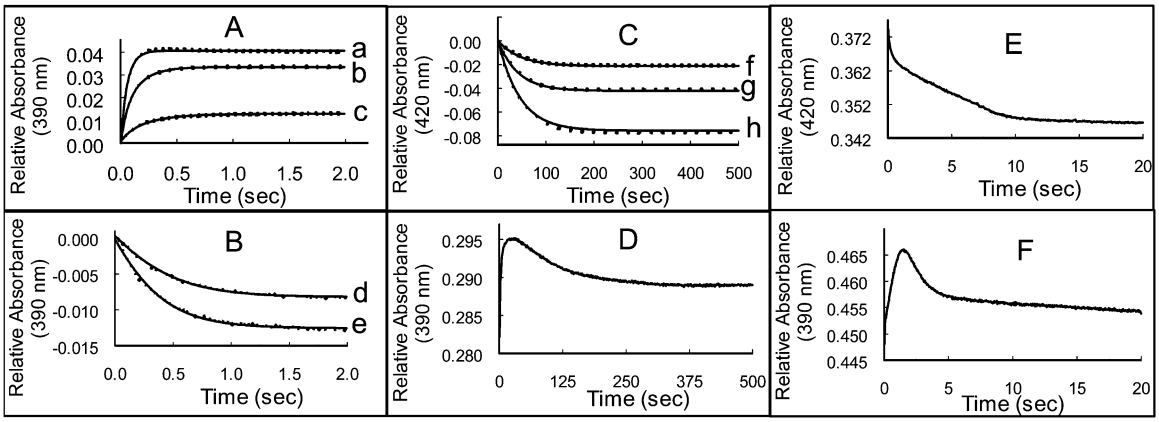

Figure 1. Stopped-flow of ACS and isolated α subunit. (A) Reaction of α with CH_3-Co^{3+}FeSP. The α subunit in 50 mM TrisHCl pH 8.0 (buffer A) was preincubated with Ti^{3+}citrate (500 μM) for 15 min and then mixed with CH_3-Co^{3+}FeSP that had been similarly preincubated with Ti^{3+}citrate (500 μM) for 15 min. Solid lines, experimental curves; dashed lines, best-fit simulations using reaction 1 and parameters in Tables 1. (B) Reaction of CH_3-α_{ox} with Co^{1+}FeSP. Protein concentrations and simulations for a, b, c, d, e refer to entries 5, 1, 3, 8, and 7 of Table 1, respectively. (C) Reduction of α_{ox} with Ti^{3+}citrate (1.0 mM) in buffer A. Traces f, g, h, correspond to $[\alpha_{ox}] = 5$, 10, and 20 μM, respectively. (D) α_{ox} (10.0 μM) mixed with CH_3-Co^{3+}FeSP (5.0 μM) and Ti^{3+}citrate (1.0 mM). (E) Reduction of ACS_{ox} (10.0 μM) with Ti^{3+}citrate (1.0 mM). (F) ACS_{ox} (10.0 μM) mixed with CH_3-Co^{3+}FeSP (5.0 μM) and Ti^{3+}citrate (1.0 mM). For CH_3-Co^{3+}FeSP and Co^{1+}FeSP, extinction coefficients (ϵ) at 390 and 450 nm (in μM^{-1} cm^{-1}) were as reported.[5] For α_{red} (α was reduced by 1.0 mM Ti^{3+}citrate), measured (best-fit) ϵ_{390} and ϵ_{450} were 0.0097 (0.0098 ±0.0007) and 0.0082 (0.0088 ± 0.0005), respectively. For CH_3-α_{ox}, measured (best-fit) ϵ_{390} and ϵ_{450} were 0.0128 (0.0121 ± 0.0005) and 0.0060 (0.0061 ± 0.0005), respectively.

monophasic, suggesting that both labile and nonlabile clusters were reduced at essentially the same rate (~0.02 s^{-1} or 2.5 ± 0.5 × 10^{-5} μM^{-1} s^{-1}).

At this point, we knew that $[Fe_4S_4]^{2+}$ reduction was much slower than methyl group transfer but not whether it was slower than D_{ox} reduction. To determine this, *oxidized* α_{ox} was mixed simultaneously with both Ti^{3+}citrate and CH_3-Co^{3+}FeSP, and the reaction was followed at 390 nm where both methyl group transfer and $[Fe_4S_4]^{2+}$ reduction could be monitored. Results (Figure 1D) showed an initial rapid increase in absorbance, due to methyl transfer, followed by a slow decline due to $[Fe_4S_4]^{2+}$ reduction. Thus, the $[Fe_4S_4]^{2+}$ cube need not be reduced to the 1+ state for the methyl group to be accepted, and it is not the D-site or some portion thereof. D_{ox} is reduced far faster than $[Fe_4S_4]^{2+}$, probably via a pathway not involving the cube. Absolute rates for these different processes should be compared cautiously as they may depend differently on reductant or protein concentrations. The redox state of the cube had little, if any, influence on D-site reduction or methyl group transfer; methyl group transfer occurred when the cube was reduced (when α was preincubated in Ti^{3+}citrate prior to methyl group transfer) or oxidized (as just described).

In the final set of experiments, ACS was substituted for α in equivalent reactions. A more complex reduction process involving multiple phases was observed (Figure 1E). Particular redox processes have not been assigned to these phases, but overall reduction was completed well before the $[Fe_4S_4]^{2+}$ component of isolated α would have been reduced (~20 s vs ~300 s). Thus, the presence of the β subunit increases the rate at which the A-cluster cube is reduced. When oxidized ACS was mixed with CH_3-Co^{3+}-FeSP and Ti^{3+}citrate, the reaction was qualitatively similar to that with α_{ox}, in that methyl transfer occurred much faster than reduction of Fe−S clusters (Figure 1F). Absorbance maximized at ~2 s, in contrast to that in α where it maximized at ~20 s. Thus, all related processes (D_{ox} reduction, methyl group transfer, and $[Fe_4S_4]^{2+}$ reduction) must occur faster in ACS than in α.

Our results discount an attractive mechanistic role for the Fe_4S_4 component of the A-cluster—namely as an electron-transfer redox intermediary between the D-site and either external redox agents (e.g., Ti^{3+}citrate) or other clusters in ACS. If it served this role, the rate at which D_{ox} reduced should have been equal to or slower than that at which the $[Fe_4S_4]^{2+}$ component was reduced. Our results for α showed that the rate of D_{ox} reduction was 100 times faster than the rate of $[Fe_4S_4]^{2+}$ reduction. Similar but more complex behavior was observed for ACS. Thus, the role of this component of the A-cluster remains elusive. Mutational studies are underway to assess the possibility that it imparts onto the Ni center critical properties required for catalysis.

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(7) *E. coli* strain JM109(pLHK05) and *M. thermoacetica* cells were grown and harvested as described.[5,6] All subsequent procedures involving the recombinant α, CoFeSP, and methyltransferase were performed as described.[5,6] Final purities for these three proteins were >95%, quantified by SDS-PAGE gels.[5,6] Ti^{3+}citrate was prepared, and Co^{1+}FeSP was methylated using CH_3-THF as described.[5] α was oxidized by thionin and freed from excess thionin by Sephadex G25. Ni-activated[6] α was methylated by adding a 20-fold molar excess of Ti^{3+}citrate, incubating 20 min, and then adding CH_3-THF (5-fold molar excess), Methyltransferase (5% (g/g) of α), and CoFeSP (5% (g/g) of α). After overnight incubation, the extent of reaction was determined by monitoring at 390 nm. The resulting solution was concentrated and subjected to Sephadex G25 chromatography to remove excess Ti^{3+}citrate and CH_3-THF. Stopped-flow experiments were performed as described.[5]
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