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Kinetic Characterization of the [3′-^{32}P]Coenzyme A/Acetyl Coenzyme A Exchange Catalyzed by a Three-Subunit Form of the Carbon Monoxide Dehydrogenase/Acetyl-CoA Synthase from *Clostridium thermoaceticum*†

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ABSTRACT: The ability of acetyl coenzyme A synthesizing carbon monoxide dehydrogenase isolated from *Clostridium thermoaceticum* to catalyze the exchange of [3′-^{32}P]coenzyme A with acetyl coenzyme A is studied. This exchange is found to have a rate exceeding that of the acetyl coenzyme A carbonyl exchange also catalyzed by CO dehydrogenase ([1-^{14}C]acetyl coenzyme A + CO ⇌ acetyl coenzyme A + ^{14}CO). These two exchanges are diagnostic of the ability of CO dehydrogenase to synthesize acetyl coenzyme A from a methyl group, coenzyme A, and carbon monoxide. The kinetic parameters for the coenzyme A exchange have been determined: K_m(acetyl coenzyme A) = 1500 μM, K_m(coenzyme A) = 50 μM, and V_{max} = 2.5 μmol min^{-1} mg^{-1}. Propionyl coenzyme A is shown to be a substrate ($K_m \approx 5$ mM) for the coenzyme A exchange, with a rate $^1/_{15}$ that of acetyl coenzyme A, but is not a substrate for the carbonyl exchange. CO dehydrogenase capable of catalyzing both these two exchanges, and the oxidation of CO to CO_2, is isolated as a complex of molecular weight 410 000 consisting of three proteins in an $\alpha_2\beta_2\gamma_2$ stoichiometry. The proposed γ subunit, not previously reported as part of CO dehydrogenase, copurifies with the enzyme and has the same molecular weight on sodium dodecyl sulfate–polyacrylamide gel electrophoresis as the disulfide reductase previously separated from CO dehydrogenase in a final chromatographic step.

Recent work has outlined the biosynthetic pathways by which acetogenic and methanogenic bacteria conduct artful chemistry (including C–C bond assembly) with single-carbon units, enabling these organisms to grow with CO_2 as their sole source of carbon (Wood et al., 1986; Ljungdahl & Wood, 1982; Diekert & Thauer, 1978; Diekert et al., 1979). In acetogenic bacteria, the enzyme responsible for acetyl-CoA[1] synthesis catalyzes the formation of a key carbon–carbon bond between carbon monoxide (enzymically generated from CO_2) and a methyl group (transferred from a corrinoid protein). This extremely oxygen-labile enzyme has been isolated from *Clostridium thermoaceticum* (Drake et al., 1980; Ragsdale et al., 1983) and characterized as containing nickel, iron, and zinc with two subunits of 78 and 71 kDa (Ragsdale et al., 1983). As this protein also belongs to a large class of oxidoreductases known to catalyze the oxidation of CO to CO_2, it has commonly been called carbon monoxide dehydrogenase (CODH), but we have suggested it also be termed an acetyl-CoA synthase to indicate its physiological C–C assembly role.

CO dehydrogenase/acetyl-CoA synthase from *C. thermoaceticum* has been shown by Wood and colleagues to catalyze two diagnostic isotopic exchange reactions, indicative of the unique ability of the enzyme to assemble the acetyl group of acetyl-CoA from one-carbon units (Ragsdale & Wood, 1985; Pezacka & Wood, 1986):

$$CH_3{}^{14}CO\text{—}SCoA + CO \rightleftharpoons CH_3CO\text{—}SCoA + {}^{14}CO \quad (1)$$

$$CH_3CO\text{—}SCoA + {}^*CoASH \rightleftharpoons CH_3CO\text{—}S{}^*CoA + CoASH \quad (2)$$

The exchange of [1-^{14}C]acetyl-CoA with CO (eq 1), first demonstrated by Ragsdale and Wood (1985) (but not well

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[1] Abbreviations: CODH, carbon monoxide dehydrogenase; CoA, coenzyme A; AcCoA or acetyl-CoA, acetyl coenzyme A; PrCoA or propionyl-CoA, propionyl coenzyme A; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; EPR, electron paramagnetic resonance; HPLC, high-performance liquid chromatography; FPLC, fast protein liquid chromatography; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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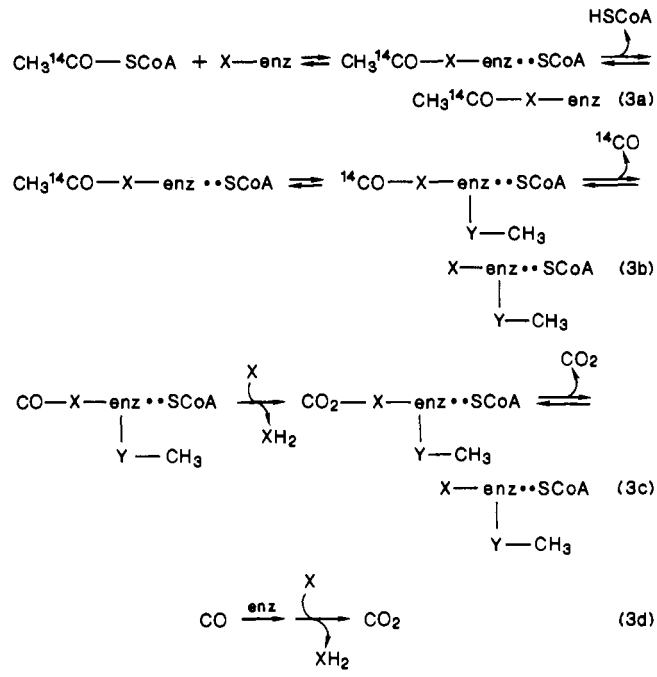
characterized kinetically), clearly establishes the ability of CODH to form and cleave both the carbon–carbon and carbon–sulfur bonds of acetyl-CoA as well as to store methyl, CO, and CoA fragments at the active site. We recently examined this reaction in detail (Raybuck et al., 1988), reporting kinetic parameters for this highly labile assay, and noted that many enzymic preparations capable of oxidizing CO to CO_2 were unable to catalyze the decarbonylation of acetyl-CoA after exposure to the slightest traces of oxygen or prolonged storage. Further kinetic analysis of the carbonyl exchange revealed that CoA is a potent inhibitor, $K_I = 7\ \mu M$, the high affinity of CoA being consistent with the role of CODH in acetyl-CoA synthesis. Investigation of the ability of CoA analogues to inhibit the carbonyl exchange demonstrated the necessity of the thiol group of CoA for binding to CODH. Extension of the carbon chain of AcCoA to determine substrate specificity of the carbonyl exchange showed no enzymic exchange of [1-^{14}C]-propionyl-CoA with CO.

The exchange of labeled CoA with acetyl-CoA (eq 2) minimally requires that the enzyme be competent in carbon–sulfur bond cleavage, and therefore has been demonstrated by other proteins which act as acyl transferases. Thiolase, for example, catalyzes the CoA-dependent cleavage of acetoacetyl-CoA (and other 3-ketoacyl-CoA esters) but can be assayed in the reverse as an enzyme-catalyzed Claisen formation of acetoacetyl-CoA from two molecules of acetyl-CoA. That synthesis occurs via two parts: (1) generation of an enzyme-bound acetyl thioester from acetyl-CoA with release of CoA and (2) transfer of the acetyl group to a second molecule of acetyl-CoA yielding acetoacetyl-CoA. In this case, the CoA/acetyl-CoA exchange exhibited a V_{max} identical with that for overall acetoacetyl-CoA synthesis, the kinetic data suggesting that the formation of the acetyl-enzyme intermediate from acetyl-CoA was rate limiting for the overall process (Gilbert et al., 1981).

Pezacka and Wood (1986) reported the existence of the CODH-catalyzed exchange of [^{3}H]CoA with AcCoA, demonstrating the required ability of CODH to form acetyl intermediates enroute to the biosynthesis of AcCoA. However, in their initial report this isotope exchange reaction proceeds at best to 10% completion in 30 min at approximately 1% of the rate of the carbonyl exchange. The requirement of an additional enzyme component, a disulfide reductase of 225 kDa, was also reported. We suggested the slow rate and low conversion of the exchange could be a reflection of very slow dissociation of CoA from the active site, given the high affinity of CoA for CO dehydrogenase/acetyl-CoA synthase (Raybuck et al., 1988). Demonstration of the kinetic relevance of the CoA/acetyl-CoA exchange would provide an additional mechanistic tool into substrate binding and carbon–sulfur bond cleavage, presumably the first steps of the more demanding carbonyl exchange assay (eq 3a and 3b). Such information would be particularly useful given the potential alternate substrates (e.g., propionyl-CoA) which are unreactive toward enzymic decarbonylation/recarbonylation.

Therefore, as part of our ongoing studies to elucidate the mechanism of this unique biocarbonylation enzyme we have examined CO dehydrogenase to optimize the conditions for the exchange of [3′-^{32}P]CoA with AcCoA. We now find that the exchange goes to 100% completion at a rate *exceeding* that of the carbonyl exchange. Propionyl-CoA, which is not a substrate for the carbonyl exchange, has been found to undergo CoA exchange at a rate $^1/_{15}$ that of AcCoA. We also present evidence that CODH capable of catalyzing CO oxidation, rapid carbonyl exchange, and rapid CoA exchange is an $\alpha_2\beta_2\gamma_2$ hexamer with molecular weight of 410 000. The γ subunit appears to be the previously reported disulfide reductase separated away from CO dehydrogenase in the last purification step in previous reports (Pezacka & Wood, 1986).



Materials and Methods

General. [1-^{14}C]Acetyl coenzyme A (50 mCi/mmol) was purchased from NEN Research Products. Adenosine 5′-[γ-^{32}P]triphosphate (3 Ci/mmol) was obtained from Amersham. All other reagents were purchased from Sigma Chemicals. [3′-^{32}P]CoA was prepared from 3′-dephospho-CoA and [γ-^{32}P]ATP with dephosphoCoA kinase according to Miziorko and Behnke (1985) and purified by HPLC to greater than 95% radiochemical purity. DephosphoCoA kinase was isolated according to Worrall and Tubbs (1983) or was a gift from Pharmacia. HPLC was done with a Waters Associates liquid chromatography system and a Vydac (Separations Group) 5-μm C_{18} reverse-phase protein/peptide column (4.6 mm × 25 cm). Prepurified carbon monoxide was purchased from Matheson and oxygen-free (<0.5 ppm O_2) nitrogen and argon were obtained from Linde Gases. For purification and storage of enzyme, a Braun MB 150M anaerobic chamber (Innovative Technology, South Hamilton, MA) was used with a nitrogen atmosphere. The oxygen content was constantly monitored (Braun oxygen analyzer) and maintained below 0.5 ppm. The anaerobic procedures are critical to the success of these experiments; such methods are described in Beinert et al. (1978). Radioactivity was measured with a Beckman LS1801 scintillation counter using ACS scintillation cocktail (Amersham). An Isco Model 1312 gel scanner was used for densitometry scanning of proteins on SDS–polyacrylamide gels.

Protein Purification. C. thermoaceticum (DSM 521) was grown as described (Diekert & Thauer, 1978); the cells were harvested under argon and frozen in liquid nitrogen. All protein purification was done in the Braun anaerobic glovebox at room temperature with buffers that were degassed under argon prior to sparging with glovebox atmosphere (nitrogen, <0.5 ppm O_2).

Cell Extract. Frozen cells (50 g) were suspended in 100 mL of buffer A (50 mM Tris-HCl, pH 8.0, 5 mM DTT) and thawed. The suspended cells were removed from the anaerobic box in a serum-stoppered Wheaton bottle and broken in a

degassed, anaerobic French pressure cell at 19 000 psi. The broken cells were centrifuged under a positive pressure of argon at 8000 rpm for 3 h.

DEAE-Sephacel. The supernatant (80–90 mL) was applied directly to a DEAE-Sephacel column (2.5 × 10 cm) equilibrated in buffer A. The column was washed with 100 mL of buffer A and then successively with 200 mL each of buffer A containing 0.1 M NaCl, 0.2 M NaCl, and then 0.3 M NaCl. The CODH activity eluted in the 0.3 M NaCl wash.

Phenyl-Sepharose. The fractions with CODH activity were combined, made 10% saturated in $(NH_4)_2SO_4$, and applied to a phenyl-Sepharose column (2.5 × 10 cm) equilibrated with 4% $(NH_4)_2SO_4$ in buffer A. The column was washed with 50 mL of 4% $(NH_4)_2SO_4$ and eluted with a linear gradient from 4% $(NH_4)_2SO_4$ to buffer A (100 mL each). The fractions with CODH activity were combined and concentrated to 4 mL on an Amicon filtration unit with a PM50 membrane.

Sephacryl S-300. The concentrated enzyme solution was applied to a Sephacryl S-300 column (1.5 × 65 cm) equilibrated and eluted with buffer A containing 0.2 M NaCl. CODH-active fractions were combined and concentrated by Amicon filtration. This protein, one band on nondenaturing PAGE, consisted of three major protein bands on SDS–PAGE and was used for all kinetic analysis. Enzyme of this purity was stored at 4 °C under N_2 at approximately 4 mg/mL. The enzyme solution remained in the anaerobic chamber and was aliquoted daily as needed. When stored in this manner, CODH retained kinetically viable acetyl-CoA synthase activity for greater than 4 weeks.

Analysis of Protein. Protein was determined by the method of Bradford (1976) with bovine serum albumin as the standard. SDS–PAGE was done according to Laemmli (1970); nondenaturing PAGE used the same method but omitted the SDS and sample treatment. Molecular weight determination of native protein was done with a Sephacryl S-300 column (2.5 cm × 78 cm) and 50 mM KP_i, pH 7.3, containing 0.2 M KCl. FPLC was done aerobically with a Pharmacia FPLC system and Superose 6 column. The elution buffer was 50 mM KP_i, pH 7.0, containing 0.15 M NaCl, and the flow rate was 0.4 mL/min. Molecular weight standards were thyroglobulin, ferritin, catalase, and aldolase obtained from Pharmacia.

Assays. CO dehydrogenase and carbonyl exchange activities were measured as described previously (Ragsdale & Wood, 1985; Raybuck et al., 1988). The exchange of [3′-^{32}P]CoA with acetyl-CoA was measured under conditions similar to those of the carbonyl exchange assay with a CO atmosphere in anaerobic exchange vessels fitted with tandem septum seals (Raybuck et al., 1989; Beinert et al., 1978). The assay was routinely done in a total volume of 0.9 mL of buffer (150 mM KP_i, 1 mM DTT, 0.2 mM methylviologen, pH 6.0) with acetyl-CoA, [3′-^{32}P]CoA (1×10^6 cpm), and 60 μg of CODH. Aliquots (100 μL) were removed at desired intervals, immediately frozen in liquid nitrogen, and analyzed within 4 h[2] by HPLC using an isocratic solvent system (14:86 methanol/50 mM KH_2PO_3, pH 4.5); CoA elutes at 4.8 min and acetyl-CoA at 7.5 min. The corresponding peaks were collected and analyzed for radioactivity. The kinetic parameters of the [3′-^{32}P]CoA exchange with propionyl-CoA were determined at 45 °C and a CoA concentration of 100 μM.

For the determination of the carbonyl exchange activity of [1-^{14}C]propionyl-CoA, assays with [1-^{14}C]propionyl-CoA and with [1-^{14}C]acetyl-CoA were run in parallel. These experiments were performed in anaerobic exchange vessels at 45 °C under a CO atmosphere in a volume of 0.8 mL containing 150 mM KP_i, pH 6.0, 1.0 mM DTT, 0.2 mM methylviologen, and 0.38 mM [1-^{14}C]AcCoA or [1-^{14}C]PrCoA (400 000 dpm). For AcCoA, 0.13 mg of CODH was used, and for PrCoA, 0.33 mg was added. Time points were obtained over 10 min. The [1-^{14}C]PrCoA showed no indication of exchange, and the standard deviation of the dpm of the six time points was less than 1.0%.

Kinetic Analysis. Exchange velocities were calculated from the equation derived by Segel (1975):

$$v^* = -\frac{[\text{CoA}][\text{AcCoA}]}{[\text{CoA}] + [\text{AcCoA}]}\frac{1}{t}\ln(1 - F) \qquad (4)$$

where F is the fraction of isotopic equilibrium at time t. Plots of t vs ln $(1 - F)$ were linear to isotopic equilibrium, and the slopes were used to determine the velocity. Kinetic parameters were determined from initial velocity data analyzed by the methods of Cleland (1979).

Results and Discussion

Enzyme Activity. CO dehydrogenase with acetyl-CoA synthase activity, measured by assaying for carbonyl exchange, can be routinely isolated under rigorously anaerobic conditions by use of modifications of previously described procedures (Ragsdale et al., 1983; Diekert & Ritter, 1983). When the enzyme is stored under N_2 with complete exclusion of O_2, the carbonyl exchange activity is maintained for greater than 4 weeks. As a further probe to the enzymic ability to generate acetyl-enzyme intermediates via carbon–sulfur bond cleavage, we have examined the exchange of [3′-^{32}P]CoA with acetyl-CoA. We find that this CoA exchange activity is also extremely labile to oxygen and the stability of this activity parallels that of the carbonyl exchange but not $CO \rightleftharpoons CO_2$ activity. It is clear that CODH is capable of catalyzing two independent reactions, the $CO \rightleftharpoons CO_2$ conversion (measured by the transfer of electrons to methylviologen) and the synthesis of acetyl-CoA (measured by the carbonyl and CoA exchanges).

Initial attempts to demonstrate [3′-^{32}P]CoA exchange under conditions (130 μM [3′-^{32}P]CoA, 260 μM AcCoA, 5 mM DTT, argon atmosphere) essentially identical with those previously described with [^{3}H]CoA (Pezacka & Wood, 1986) resulted in complete exchange within 30 s of initiation of the reaction. This represents a rate estimated to be more than 2 orders of magnitude faster than previously reported (10% exchange in 30 min; Pezacka & Wood, 1986). With the goal of obtaining initial rate data and kinetic parameters which could be directly compared with our previous studies, the conditions of the assay were changed to be identical with those of carbonyl exchange. Under the conditions of CO atmosphere, 55 °C, 0.2 mM methylviologen, and 1 mM DTT, no decrease in rate was observed. Control experiments in which CODH was omitted or inactivated with oxygen demonstrated that AcCoA and CoA are stable to the reaction conditions and that nonenzymatic exchange does not occur. The presence of CO did not inhibit the CODH-catalyzed exchange, in contrast to the previous report where 80% inhibition was observed (Pezacka & Wood, 1986). We find the presence of CO and methylviologen, though not required for CoA exchange, actually increases the reproducibility of the assay, possibly because the methylviologen, reduced by CODH, scavenges traces of oxygen.

At 55 °C the reaction is too rapid for accurate measurement of initial velocities. Examination of the effect of temperature

[2] Samples of acetyl-CoA stored in the presence of DTT are unstable. Even at −70 °C approximately 4% of the acetyl-CoA is converted to CoA overnight, as determined by HPLC analysis.

Table I: Comparison of Kinetic Parameters of the Three Assays of CODH at 55 °C

	K_m(AcCoA) (μM)	K_m(CoA) (μM)	K_I(CoA) (μM)	V_{max} (μmol min^{-1} mg^{-1})	rel rate
$CO \rightleftharpoons CO_2$			nd[a]	300	250
carbonyl exchange[b]	600		7[c]	1.2	1
CoA exchange[d]	1500	50	nd	7.5[e]	6.3

[a] Not detectable. [b] The CODH-catalyzed carbonyl exchange, [1-^{14}C]AcCoA + CO $\rightleftharpoons$ AcCoA + ^{14}CO. [c] Data from Raybuck et al. (1988). [d] The CODH-catalyzed CoA exchange, [3′-^{32}P]CoA + AcCoA $\rightleftharpoons$ CoA + [3′-^{32}P]AcCoA, as described in the text. [e] Calculated from data obtained at 45 °C.

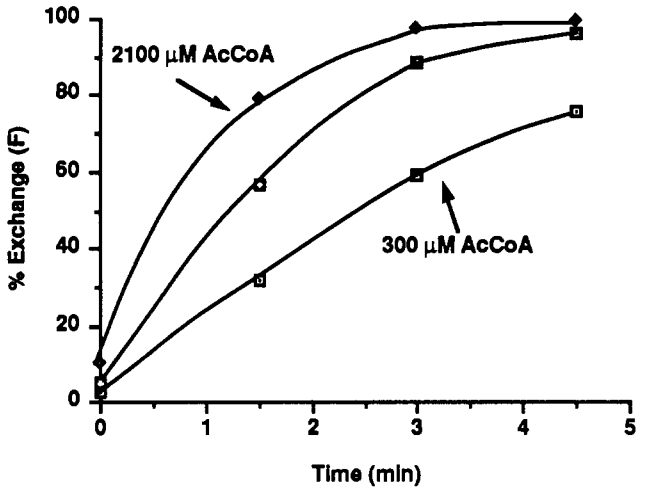


FIGURE 1: Typical time course for the CoA exchange catalyzed by CODH: [3′-^{32}P]CoA + AcCoA $\rightleftharpoons$ CoA + [3′-^{32}P]AcCoA. CoA concentration is 100 μM, AcCoA concentrations are 300, 1300, and 2100 μM, and temperature is 45 °C.

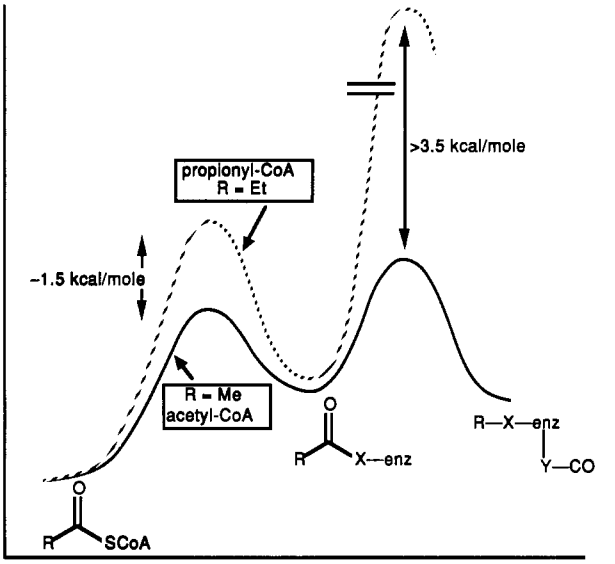


FIGURE 2: Reaction profiles for the carbonyl and CoA exchanges of acetyl-CoA (R = Me) and propionyl-CoA (R = Et).

on both the CoA exchange and the carbonyl exchange showed that from 25 to 55 °C the reactions both follow the Arrhenius law giving E_a values of 26.8 and 24.1 kcal/mol, respectively (data not shown). Kinetic parameters were determined under standard conditions at 45 °C, where a wide range of initial velocities could be reproducibly determined. Under these conditions, all acetyl-CoA/CoA exchanges proceeded to isotopic equilibrium in under 10 minutes; typical time course data are shown in Figure 1.

Michaelis constants for AcCoA and CoA were determined by varying the concentration of one substrate at fixed concentrations of the other, and the data were analyzed with the computer programs of Cleland (1979). The K_m values for AcCoA and CoA were determined to be 1500 ± 500 μM and 50 ± 25 μM, respectively, and the V_{max} for the reaction is 2.5 ± 0.5 μmol min^{-1} mg^{-1} at 45 °C. At concentrations as high as 500 μM there is no evidence of substrate inhibition by CoA.

The kinetic parameters obtained from the three assays of CODH are compared in Table I. Inspection of the V_{max} values reveals that the CoA exchange proceeds approximately 6 times faster than the carbonyl exchange, demonstrating the kinetic competence of this assay as a probe for acetyl-enzyme formation and consistent with the sequence in eq 3a and 3b. The relative ratios of K_m(acetyl-CoA)/K_m(CoA) are similar for both exchanges, and the high affinity for CoA in each case is consistent with the role of CO dehydrogenase in *C. thermoaceticum* for acetyl-CoA biosynthesis. The rates of both of the isotopic exchange reactions are at least an order of magnitude slower than the CO $\rightleftharpoons$ CO_2 assay, suggesting that in the back-direction CO oxidation may be faster than exchange to the medium (eq 3b–3d).

Propionyl-CoA. As previously shown (Raybuck et al., 1988), PrCoA is not a substrate for carbonyl exchange, and [1-^{14}C]PrCoA does not undergo carbonyl exchange with an estimated limit to detection of a rate of $^1/_{300}$ that of the [1-^{14}C]AcCoA exchange. In contrast, PrCoA is a substrate for the CoA exchange with a V_{max} of 0.15 μmol min^{-1} mg^{-1}, only a factor of 15 slower than AcCoA (V_{max} = 2.5 μmol min^{-1} mg^{-1}) under the same conditions. The K_m value for PrCoA (5 mM) is only slightly higher than that for AcCoA (1.5 mM). These data clearly indicate that enzymic cleavage of the carbon–sulfur bond of propionyl-CoA occurs, forming a propionyl-enzyme intermediate. Steric or electronic factors may prohibit the enzyme from then proceeding to cleave the C1-(carbonyl)–C2(ethyl) bond at a measurable rate.

The ability of the enzyme to catalyze with propionyl-CoA only one of the two exchanges catalyzed with acetyl-CoA confirms that the reversible formation of an acyl-enzyme species precedes decarbonylative C–C bond cleavage of the acyl-enzyme. From the rate ratios can also be constructed an initial reaction profile for sequential formation of two enzyme intermediates. With acetyl-CoA the formation of acetyl-enzyme (eq 3a) proceeds 6.3 times faster than subsequent C–C bond cleavage and CO exchange (eq 3b) (R = Me, Figure 2). With propionyl-CoA (R = Et, Figure 2) the energy barrier for acyl-enzyme formation is about 1.5 kcal/mol higher than that in the acetyl-CoA case, but the further progress of acyl-enzyme to alkyl-enzyme–CO intermediate has at least a 3.5 kcal/mole higher barrier from propionyl-enzyme than that from acetyl-enzyme and does not proceed detectably.

Stoichiometry of CODH. Pezacka and Wood (1986) recently reported the isolation from *C. thermoaceticum* of a protein termed a disulfide reductase which was required, although it could be replaced by dithioerythritol, for the slow CoA exchange activity of CODH they detected. This enzyme copurifies as a complex with CODH but reportedly can be separated by gel filtration after room temperature treatment with 10 mM dithioerythritol (Pezacka & Wood, 1986). This reductase is reported to show a single band on SDS–PAGE of 50 500 molecular weight and to have an α_4 native form with a molecular weight of 230 000 as determined by gel filtration.

The authors propose that this reductase may function to add and remove CoA from CODH by formation and cleavage of a disulfide bond between the enzyme and substrate and is therefore required for CoA exchange. This protein, in addition to CO dehydrogenase, methyl transferase, and the corrinoid protein, is also necessary for the overall biosynthesis of acetyl-CoA from methyl tetrahydrofolate, CO_2, and CoA.

We consistently isolated CODH which shows three protein bands on SDS–PAGE: the 78- and 71-kDa bands previously reported as the subunits of CODH (Ragsdale et al., 1983; Diekert & Ritter, 1983) and a third band with a molecular mass of ca. 50 kDa. These protein exist in a 1:1:1 ratio as determined by densitometry scanning of the SDS gel, suggesting a minimal molecular mass of 204 kDa for $\alpha_1\beta_1\gamma_1$. This CODH elutes as a single peak upon gel filtration on Sephacryl S-300 (with or without DTT present) or by FPLC on a Superose 6 column and migrates as a single band on nondenaturing PAGE, although some dissociation is observed during PAGE. The molecular mass of the native protein as determined on Sephacryl S-300 or FPLC is 410 kDa, indicating that native active enzyme is an $\alpha_2\beta_2\gamma_2$ dimer of trimers. We have been unable to isolate CODH with only two subunits after repeating the reported purification procedure (Ragsdale & Wood, 1985). Given the published copurification of the disulfide reductase with two-subunit CODH and the similar molecular masses of the disulfide reductase and the γ subunit (50 kDa), we presume that the γ subunit of CODH is identically this "disulfide reductase". Pezacka and Wood (1986) reported this disulfide reductase reduces disulfides on the two-subunit CODH and also could reduce free cystine. In our hands the three-subunit CODH does not reduce exogenous cystine. Pezacka and Wood also note that isolated disulfide reductase is extremely labile and only when added back to the two subunit form of CODH does it form a stable complex that now catalyzes the CoA/AcCoA exchange. We suggest that this was a de facto reconstitution of functional holoenzyme with only 1% of the V_{max} of the enzyme preparations reported here. We have not been able to separate the α, β, and γ subunits without full loss of activity, so a similar study has not been possible.

There have been conflicting reports as to the molecular weight of the multisubunit complex of CODH isolated in its two-subunit form. Gel filtration experiments (Ragsdale et al., 1983) were interpreted to indicate a molecular weight of 420 000, suggesting an $\alpha_3\beta_3$ structure. Sucrose density sedimentation and gradient gel electrophoresis experiments by Diekert and co-workers indicated a molecular weight of 290 000 in accord with an $\alpha_2\beta_2$ form of the enzyme (Diekert, 1988). Our gel filtration experiments on CO dehydrogenase capable of catalyzing all three assays noted in Table I give a molecular weight for the three-subunit form of 410 000, which is most consistent with $\alpha_2\beta_2\gamma_2$ stoichiometry (calculated molecular weight of 408 000). At molecular weights of either 420 000 ($\alpha_3\beta_3$) or 290 000 ($\alpha_2\beta_2$) for CODH and 225 000 (α_4) for native disulfide reductase, our enzyme is clearly not a 1:1 complex of these two native forms.

Concluding Remarks

We report here kinetic parameters for the CO dehydrogenase catalyzed exchange of [3′-^{32}P]CoA with AcCoA, which we find exhibits rates nearly an order of magnitude faster than those of CO dehydrogenase catalyzed exchange of [1-^{14}C]AcCoA with CO. These results establish the CoA exchange as a kinetically competent half-reaction and a useful mechanistic tool for determining substrate binding and the formation of acetyl-enzyme intermediates from CoA thioesters. CODH as isolated in our laboratories contains three subunits of 78, 71, and 50 kDa complexed in a stoichiometric ratio, in contrast to the two subunits previously reported for CODH. We propose that CODH active in all three activities (CO oxidation, carbonyl exchange, and CoA exchange) requires the presence of all three components and therefore suggest acetyl-CoA-synthesizing CO dehydrogenase holoenzyme is composed of three subunits. Furthermore, gel filtration indicates a molecular weight of 410 000 for the native form and therefore a stoichiometry of $\alpha_2\beta_2\gamma_2$ for a fully functional, kinetically competent, acetyl-CoA synthase holoenzyme complex. Such a multisubunit complex is similar to the $\alpha\beta\gamma\delta\epsilon$ composition of the CO dehydrogenase from acetate-grown *Methanosarcina thermophila*, known to contain nickel, iron, and corrinoid (Terlesky et al., 1986, 1987).

Acetogenic CODH is an interesting acetate-synthesizing, C–C bond assembling enzyme, and the mechanistic details of its catalytic activity are only beginning to be understood. The isolation of CODH as a three-subunit protein now with high catalytic activity for the carbonyl and CoA exchanges is important for mechanistic studies. These two assays also facilitate probing of the mechanism by separating reversible carbon–sulfur bond formation and cleavage from reversible carbon–carbon bond formation.

However, much remains to be determined concerning the active site chemistry and the functional groups responsible for catalysis, particularly the unique role of enzyme-bound nickel. We have previously noted that the distinctive carbonylation chemistry of CO dehydrogenase is easily accounted for by viewing carbon–carbon bond formation as a metal-based CO insertion into a metal–methyl bond (Raybuck et al., 1987). Nickel is well suited as an active site metal for acetate biosynthesis by CO dehydrogenase, given organometallic precedents for nickel-based carbonylations (Kohara et al., 1979) and EPR evidence that both CO and CoA interact with the nickel site (Ragsdale et al., 1988). Recent evidence that CODH methylated by methyl corrinoid enzyme is capable of acetate synthesis and that hydrolysis of the enzyme in 6 M HCl results in the isolation of *S*–methylcysteine points the direction for identification of the methyl binding site of CODH (Pezacka & Wood, 1988). It must be noted, however, that these results do not necessarily prove that the methyl group of methyl-CODH (eq 3b) is attached to a cysteine, since a methyl–metal bond would not be expected to withstand the vigorous hydrolysis in 6 M HCl, but would seem to indicate that a cysteine residue is in or near the active site.

Propionyl-CoA, which we have now shown undergoes carbon–sulfur but not decarbonylative carbon–carbon bond cleavage, may be a useful substrate for the accumulation of acyl-enzyme, perhaps a propionylnickel or perhaps a covalent propionyl-enzyme species. Ultimately, the isolation of covalently bound enzyme intermediates from modified acyl-CoA thioesters (e.g., 2-chloroacetyl-CoA or 3-chloropropionyl-CoA) acting as active site directed inhibitors (as assayed by the [3′-^{32}P]CoA exchange) may help elucidate the binding sites of CODH, provide mechanistic insights, and permit determination of 2 vs 3 molar equiv of inactivator incorporated per 410-kDa species to further assess the stoichiometry of the active carbonylating acetyl-CoA synthase ($\alpha_3\beta_3$ vs. $\alpha_2\beta_2\gamma_2$). Work in this direction is currently under way in our laboratories.

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Calculations of Free Energy Profiles for the Staphylococcal Nuclease Catalyzed Reaction†

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ABSTRACT: Calculations of the free energy profile for the first two (rate-limiting) steps of the staphylococcal nuclease catalyzed reaction are reported. The calculations are based on the empirical valence bond method in combination with free energy perturbation molecular dynamics simulations. The calculated activation free energy is in good agreement with experimental kinetic data, and the catalytic effect of the enzyme is reproduced without any arbitrary adjustment of parameters. The enormous reduction of the activation barrier (relative to the reference reaction in water) appears to be largely associated with the strong electrostatic effect of the Ca^{2+} ion and the two arginine residues in the active site. This favorable electrostatic environment reduces the cost of the general-base catalysis step by almost 15 kcal/mol (by stabilizing the OH^- nucleophile) and then stabilizes the developing negative charge on the 5′-phosphate group in the second step of the reaction by about 19 kcal/mol. The basic features of the originally postulated enzyme mechanism (Cotton et al., 1979) are found to be compatible with the observed activation free energy. However, the proposed modification of the mechanism (Sepersu et al., 1987), in which Arg 87 interacts only with the pentacoordinated transition state, is supported by the simulations. Further calculations on the D21E mutant also give results in good agreement with kinetic data.

Understanding the molecular origin of catalytic reactivity in enzymes is one of the most interesting challenges in theoretical molecular biology and biochemistry today. Progress in this field relies heavily on the availability of high-resolution X-ray structures of relevant enzymes as well as kinetic measurements of the catalyzed reactions, both in the protein and in solution. The recent advances in site-directed mutagenesis of proteins have opened novel possibilities of probing the contributions from single amino acids or groups of residues to the enzyme's total reduction of the activation free energy barrier [e.g., Wilkinson et al. (1984), Craik et al. (1985), Wells et al. (1986), Cronin et al. (1987), and Knowles (1987)]. However, in some cases, the total catalytic effect of the enzyme active site cannot be described simply by additive contributions

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