Tight Coupling of Partial Reactions in the Acetyl-CoA Decarboxylase/Synthase (ACDS) Multienzyme Complex from Methanosarcina thermophila

ACETYL C–C BONDFragmentationAT THE A CLUSTER PROMOTED BY PROTEIN
CONFORMATIONAL CHANGES

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Direct synthesis and cleavage of acetyl-CoA are carried out by the bifunctional CO dehydrogenase/acetyl-CoA synthase enzyme in anaerobic bacteria and by the acetyl-CoA decarboxylase/synthase (ACDS) multienzyme complex in Archaea. In both systems, a nickel- and Fe/S-containing active site metal center, the A cluster, catalyzes acetyl C–C bond formation/breakdown. Carbonyl group exchange of [1-14C]acetyl-CoA with unlabeled CO, a hallmark of CODH/ACS, is weakly active in ACDS, and exchange with CO2 was up to 350 times faster, indicating tight coupling of CO release at the A cluster to CO2 oxidation at the C cluster in CO dehydrogenase. The basis for tight coupling was investigated by analysis of three recombinant A cluster proteins, ACDS β subunit from Methanosarcina thermophila, acetyl-CoA synthase of Carboxydothema thermodynamoformans (ACSCh), and truncated ACSCh lacking its 317-amino acid N-terminal domain. A comparison of acetyl-CoA synthesis kinetics, CO exchange, acetyltransferase, and A cluster Ni2+ EPR characteristics demonstrated a direct role of the ACS N-terminal domain in promoting acetyl C–C bond fragmentation. Protein conformational changes, related to “open/closed” states previously identified crystallographically, were indicated to have direct effects on the coordination geometry and stability of the A cluster Ni2+-acetyl intermediate, controlling Ni2+-acetyl fragmentation and Ni2+-acetyl condensation. EPR spectral changes likely reflect variations in the Ni2+-CO equatorial coordination environment in closed buried hydrophobic and open solvent-exposed states. The involvement of subunit-subunit interactions in ACDS, versus interdomain contacts in ACS, ensures that CO is not released from the ACDS β subunit in the absence of appropriate interactions with the α5ε2 CO dehydrogenase component. The resultant high efficiency CO exchange explains the low rate of CO exchange relative to CO2.

Acetyl-CoA decarboxylase/synthase (ACDS) is a 5-subunit-containing multienzyme complex of ~2,000 kDa found exclusively in methanogens and certain other Archaea (1–3). The complex, an (α5ε2)4(β)6γ8 oligomer, catalyzes the direct synthesis and cleavage of the acetyl C–C and C–S bonds of acetyl-CoA with steps that involve one- and two-carbon organometallic intermediates. Cleavage of acetate is a major role of the ACDS complex in methanogens such as Methanosarcina and Methanosaeta species carrying out disproportionation of acetate to methane and CO2 (4, 5). In the reverse direction, ACDS is used to synthesize acetyl groups needed for autotrophic growth on one-carbon substrates (6, 7). In the overall process (Reaction 1),

\[
\text{acetyl-CoA + H}_2\text{SPt + H}_2\text{O + 2Fd}_{\text{ox}} \rightleftharpoons \text{CoASH + CH}_3\text{H}_2\text{SPt + CO}_2 + 2\text{Fd}_{\text{red}} + 2\text{H}^+ \\
\text{REACTION 1}
\]

the substrate tetrahydroserotonin (H5SPt, a tetrahydrofolate analog, and where CH3-H5SPt is N5-methyltetrahydroserotonin) functions as the acceptor for methyl groups derived from acetyl C–C bond cleavage, and ferredoxin acts to take up electrons produced from oxidation of the carbonyl group to CO2 (2, 6). Reaction 1 is composed of several partial reactions (Scheme 1), which include acetyl group transfer, decarboxylation, methyl group transfer, and CO oxidation, catalyzed by different metalloprotein/enzyme subcomponents that can be released in active forms by partial proteolytic digestion of the complex (8–10). Evidence indicates that the β subunit contains an intact A cluster (11–13), which encompasses a binuclear Ni-Ni center bridged by a cysteine thiolate to an [Fe3S4] cluster (14, 15). The nickel atom proximal to the [Fe3S4] cluster, designated Niγ, is coordinated by three μ-cysteine thiolates, one of which is shared with iron in the [Fe3S4] cluster and with the other two in shared coordination with the distal Niδ. Coordina-

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§ The abbreviations used are: ACDS, acetyl-CoA decarboxylase/synthase; CODH/ACS, bacterial α5ε2 bifunctional enzyme containing acetyl-CoA synthase (ACS) and carbon monoxide dehydrogenase (CODH) with subscripts Mt and Ch used to designate the bacterial source Moorea terrae- moactaea or Carboxydothema thermodynamoformans; H5SPt, tetrahydroserotonin; MES, 2-(N-morpholino)ethanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; NTA, nitrilotriacetic acid; HPLC, high pressure liquid chromatography.
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In contrast to the ACDS complex in Archaea, anaerobic bacteria use the CO dehydrogenase/acetyl-CoA synthase (CODH/ACS) enzyme system for anaerobic oxidation of acetate (18) and for de novo synthesis of acetyl CoA in autotrophic species and acetogens (18–20). The CODH/ACS system includes several individual enzymes, CODH/ACS, corrinoid-Fe/S protein, and methyltransferase, which, unlike ACDS, are isolated separately and not present in the form of a high molecular mass complex (21). In the acetogen *Moorella thermoacetica*, the A cluster-containing acetyl-CoA synthase corresponds to the α subunit of a bifunctional CODH/ACS αβ2 heterotetramer, in which CO dehydrogenase activity resides on the β subunit (14, 15, 22, 23). In *Carboxydotothermus hydrogenoformans*, CODH/ACS exists as a heterotetramer as well, but the same ACS protein also can be isolated in monomeric form separate from CODH (24).

Other structural differences between the ACDS and CODH/ACS systems include the absence of an ε subunit in bacteria (25), substantial lack of homology in N- and C-terminal regions of various subunits, and the presence of a unique domain that harbors two additional [Fe₄S₄] clusters in the archaeal CODH α subunit. A prominent difference between bacterial and archaeal ACS proteins is the existence of an ~317-amino acid N-terminal region (~45% of the bacterial ACS α subunit) not present in the ACDS β subunit.

Enzymatic functional differences between the ACDS and CODH/ACS systems are also observed. For example, methyl group transfer to the corrinoid protein (Scheme 1) requires a separate methyltransferase enzyme in bacteria, but the activity is intrinsic to the γδ subcomponent in ACDS. Furthermore, the isotope exchange reaction (Reaction 2),

\[ [1-^{14}C]acetyl-CoA + CO \rightleftharpoons \text{acetyl-CoA} + ^{14}CO \]

which takes place as a consequence of C–S and C–C bond cleavage activity (26), typically exceeds the rate of overall acetyl-CoA synthesis by as much as 3- and 17-fold in experiments with CODH Mt/ACS Mt and the monomeric ACS Ch, respectively (24, 27, 28), but exhibits low (sometimes variable) rates with the ACDS complex (10, 29). Unpublished data from our laboratory suggest that ACDS catalyzes CO exchange much more slowly than the overall synthesis and cleavage of acetyl-CoA (Reaction 1) or any of the individual partial reactions (Scheme 1). Observations made with buffer-soluble extracts of *M. barkeri* also indicate an exceedingly low rate of CO exchange (30).

Crystallographic structures of the CODH/ACS heterotetramer show the presence of an extended intersubunit tunnel (14, 15, 31), which, as indicated by kinetic channeling effects and results from site-directed mutagenesis (32–34), is functional to transfer CO, produced by reduction of CO₂ at the C cluster in the β subunit, to the α subunit A cluster for synthesis of acetyl-CoA. A major portion of the tunnel runs through the 317-amino acid N-terminal domain of the ACS α subunit that interfaces with the CODH β subunit. In addition to the N-terminal domain, the α subunit possesses two other principal domains formed from the central and C-terminal regions of the protein (Scheme 2) (14, 15, 24).

Two different conformational forms of the ACS α subunit have been observed, designated open and closed, that differ in how the N-terminal domain is positioned relative to the central and C-terminal domains and in whether or not nickel was present instead of nickel at the position proximal to the [Fe₄S₄] cluster (Scheme 2) (15). In the closed conformation, the tunnel passes through the α subunit N-terminal domain and opens at an axial position above the proximal metal in the A cluster. In this state, the A cluster is buried within the interface formed between the N-terminal domain and the central and C-terminal domains. However, in the open form, blockage of the tunnel occurs within the α subunit N-terminal domain at a point ~20 Å away from the A cluster; the A cluster becomes readily accessible to solvent, and there is a substantial loss of interdomain contacts. Solvent exposure of the A cluster increases upon transition to the open form; however, specific access of CO to the axial position above Niₐ becomes shielded by movement of a nearby conserved phenylalanine residue side chain (Phe-512 in ACS Mt that corresponds to Phe-195 in the ACDS β subunit). A gating mechanism has been postulated in which delivery of CO to the A cluster would occur only in the closed conformation (tunnel

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3 D. A. Grahame, unpublished data.
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SCHEME 2. **Open and closed conformations of ACS<sub>Mt</sub> and corresponding A cluster metal center geometries.** X-ray crystallographic structures of the *M. thermoacetica* α subunit (ACS<sub>Mt</sub>) show a large change in the orientation of the N-terminal domain (magenta) relative to the central and C-terminal domains (blue). An increased number of interdomain contacts is found in the closed form, including contacts with the N-terminal domain in the vicinity of the A cluster, which is exposed to solvent in the open form and buried in the closed conformation. The corresponding A cluster structures contain nickel (green) as the metal ion at the position distal to the [Fe<sub>4</sub>S<sub>4</sub>] cuboid, in square-planar geometry with S<sub>N</sub><sup>2</sup> coordination in both forms. The proximal metal (N<sub>1</sub><sup>p</sup>) is square-planar nickel in the open conformation, but so far the closed form has been observed only with copper or zinc (cyan) in tetrahedral geometry at the proximal metal site. Data are from Darnault et al. (15), Protein Data Bank code 1OA0.

open). Upon transition to the open form, occlusion of the tunnel would prevent leakage of CO produced at the C cluster and allow for entrance of the larger substrates (corrinoid-Fe/S protein and CoA) to react to form acetyl-CoA (15, 35).

The objective of this study is to provide a better understanding of the structural and catalytic differences between the CODH/ACS and ACDS systems. A comparison of the acetyl-CoA carboxyl exchange activities of the ACDS complex with CO and CO<sub>2</sub> was carried out to examine the basis for tight coupling between acetyl C–C bond fragmentation at the A cluster and CO oxidation at cluster C. A previously unrecognized role of the N-terminal ACS domain in producing conformational effects linked to catalysis at the A cluster was revealed by characterization of three different A cluster proteins, the recombinant ACDS β subunit, recombinant full-length ACS, and N-terminally truncated ACS from *C. hydrogenoformans* (DSM 6008) was expressed as a full-length protein, designated ACS<sub>Ch</sub> (732 amino acids), and as a form lacking the 317-amino acid N-terminal domain, designated ACS<sub>Ch</sub><sub>ΔN</sub> (415 amino acids). Forward Ndel and reverse Xhol primers were used in PCR amplifications of the full-length and shortened versions of the *C. hydrogenoformans* acsB gene. The PCR fragments were digested, eluted from agarose gels, and cloned between the Ndel and Xhol sites of the pETDuet-1 vector (Novagen). Following sequence verifications, the plasmids were transformed into *E. coli* NM522 that had been lysogenized with the λDE3 phage. The conditions described previously for anaerobic growth of *E. coli* and induction with isopropyl β-D-thiogalactopyranoside (11) were used for all three proteins.

Anion exchange chromatography on Q-Sepharose Fast Flow (GE Healthcare) was employed for initial purification of the nontagged ACS<sub>Ch</sub> and ACS<sub>Ch</sub><sub>ΔN</sub> proteins from supernatants obtained following French pressure cell lysis of resuspended cell pastes, as described previously for the recombinant ACDS β subunit (11). Thereafter, different approaches were used for the final purifications, with anaerobic conditions maintained throughout. ACS<sub>Ch</sub> was subjected to further chromatography on a column of Bio-Gel HTP hydroxyapatite (Bio-Rad), eluted with a 0.01–0.40 M linear gradient of sodium phosphate, pH 7.5. The peak fractions were concentrated, dialyzed for buffer exchange, loaded onto a Mono Q HR 10/10 anion exchange column (GE Healthcare), and eluted with a gradient of 0.05 to 0.80 M KCl in 50 mM Tris-HCl, pH 8.0. For the ACDS β subunit, phenyl-Sepharose 6 Fast Flow (GE Healthcare) was applied, using a buffer containing 0.6 M Na<sub>2</sub>SO<sub>4</sub>, 10% glycerol, 20 mM MOPS, pH 7.2, for binding to the column, with elution by a linear gradient of decreasing Na<sub>2</sub>SO<sub>4</sub> concentration. Following

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**MATERIALS AND METHODS**

Reagents and General Procedures—All chemicals purchased from commercial sources were of the highest purity grade offered. [acetyl-1,14C]Acetyl-coenzyme A was from PerkinElmer Life Sciences. Coenzyme A, disodium salt (>96%, HPLC, Fluka Biochemika), was from Sigma, and methyl viologen dichloride dihydrate, 98%, was from Aldrich. All anaerobic procedures were carried out under an atmosphere of nitrogen containing 1–3% H<sub>2</sub> using a Coy-type anaerobic chamber with O<sub>2</sub> levels in the range of 0.5–2 ppm monitored by a Teledyne model 3190 trace oxygen analyzer. Blue titanium(III) nitritolactate (Ti<sup>3+</sup> NTA) stock solution (~170 mM) was prepared fresh by adding 1 volume of anaerobic 30% (w/w) titanium(III) chloride in 2 N HCl reagent (Acros) to 13.8 volumes of an anaerobic solution containing 0.275 M nitritolactate disodium salt, 0.5 M Tris-HCl, pH 8.0. Further dilutions with water provided working solutions in the range of 2–20 mM Ti<sup>3+</sup> for direct additions to enzyme reaction mixtures. Purple Ti<sup>3+</sup> EDTA and brown Ti<sup>3+</sup> citrate were prepared as described previously (9). Methylcobinamide was prepared from methylcobalamin by the method developed in the laboratory of K. L. Brown (36), as described previously (16).

Recombinant ACDS β Subunit, ACS<sub>Ch</sub> and ACS<sub>Ch</sub><sub>ΔN</sub>—The ACDS β subunit from *Methanosarcina thermophila* TM-1 was expressed in anaerobically grown *Escherichia coli* as a 397-amino acid protein lacking 75 amino acids at the C terminus, as described previously (11). This form of the protein is referred to in this study as the ACDS β subunit and is similar to the native β subunit purified from the ACDS complex after partial proteolysis (8, 9). Acetyl-CoA synthase from *C. hydrogenoformans* (DSM 6008) was expressed as a full-length protein, designated ACS<sub>Ch</sub> (732 amino acids), and as a form lacking the 317-amino acid N-terminal domain, designated ACS<sub>Ch</sub><sub>ΔN</sub> (415 amino acids). Forward Ndel and reverse Xhol primers were used in PCR amplifications of the full-length and shortened versions of the *C. hydrogenoformans* acsB gene. The PCR fragments were digested, eluted from agarose gels, and cloned between the Ndel and Xhol sites of the pETDuet-1 vector (Novagen). Following sequence verifications, the plasmids were transformed into *E. coli* NM522 that had been lysogenized with the λDE3 phage. The conditions described previously for anaerobic growth of *E. coli* and induction with isopropyl β-D-thiogalactopyranoside (11) were used for all three proteins.

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phenyl-Sepharose chromatography, a final step of Mono Q HR 10/10 was employed. After the initial Q-Sepharose purification, ACSD$_2$ΔN was subjected to a single step of chromatography on a HiLoad 16/10 phenyl-Sepharose HP column (GE Healthcare).

Protein concentrations were determined by the method of Perkins (37), with modifications as described previously (11). The concentration of active sites was estimated on the basis of absorbance at 400 nm of the nickel-reconstituted proteins, using a molar absorptivity for the A cluster of 17,900 M$^{-1}$ cm$^{-1}$, determined by inductively coupled plasma–emission spectrometry analysis of iron contents of preparations of the ACDS β subunit found to contain 224 ± 10 µM iron per $A_{400}$ nm. Nickel reconstitution reactions were performed by procedures similar to those described previously (11, 16) in which apoprotein, around 20–25 µM, was incubated overnight in the presence of 150 µM NiCl$_2$ in 50 mM MOPS, pH 7.7. Enzymatic assays were carried out using samples directly removed from the incubation mixtures. For EPR analyses, larger volume nickel reconstitution mixtures were prepared and, after incubation, concentrated to 10-fold and diafiltered using Centricon 30 ultrafiltration units placed under ~1 atm N$_2$ overpressure inside the anaerobic chamber.

**Acetyl-CoA Synthesis**—Assays for the net synthesis of acetyl-CoA from CoA, methylcobinamide, and CO were performed as described previously with minor modifications (16). The reactions were carried out at 25 °C, pH 7.2, in sealed vials under an atmosphere of 50% CO and 50% N$_2$ (to give 0.48 mM dissolved CO, using the α value for CO gas solubility at 25 °C listed in Ref. 38). The enzymes were preincubated for 5 min in the presence of CO and the indicated concentrations of methylcobinamide, with 34 µM Ti$^{3+}$ NTA added as reducing agent. The reactions were initiated by addition of 120 µM CoA, and reaction progress was followed by quantitative HPLC analysis of acetyl-CoA and CoA in aliquots removed over time, with initial rates obtained from fits of the data to the first-order rate equation (16).

**Acetyltransferase**—The ability of the different enzymes to catalyze acetyl group transfer from acetyl-CoA, by activation of the thioester bond, was measured by following the low redox potential-dependent formation of CoA and 3′-dephospho-S-acetyl-CoA from the substrates acetyl-CoA and 3′-dephospho-CoA, by the method used previously (11) with minor modifications. The reaction mixtures (360 µl) were set up at 25 °C and contained 100 µM acetyl-CoA, 100 µM 3′-dephospho-CoA, 100 mM KCl, 50 mM MOPS buffer, pH 6.7, and 50 mM aquacobalamin prereduced with 136 µM Ti$^{3+}$ NTA. All components except the CoA substrates and enzyme were assembled and incubated for 30 min to allow for reduction of B$_{12}$. Thereafter, 3′-dephospho-CoA was added, followed by the enzyme, and the reaction was initiated by addition of acetyl-CoA. For each reaction, a series of aliquots (55 µl) was removed over time. Each aliquot was mixed with an equal volume of stop solution (2 mM TiCl$_3$, 0.5 M sodium citrate, pH 4.0) and frozen. Subsequent HPLC analysis of CoA, acetyl-CoA, 3′-dephospho-CoA, and 3′-dephospho-S-acetyl-CoA in the samples and the calculation of initial rates were performed as described previously (9). It was found that acetyltransferase activity was stimulated ~1.7-fold in the presence of 0.1 M KCl relative to reactions containing either 0.1 M NaCl or no additional salt.

**Carbonyl Exchange Assays**—Analyses of isotopic exchange of CO and CO$_2$ with the carbonyl group of [1-$^{14}$C]acetyl-CoA were based on the general method (26) in which the loss of radiolabel from solution was followed over time during incubation of the enzymes in the presence of excess unlabeled CO or CO$_2$. Reactions for CO exchange were conducted under an atmosphere of 100% CO, which provided dissolved CO concentrations in the range of ~0.94 mM at 26 °C to 0.65 mM at 66 °C (using the α values for CO gas solubility listed in Ref. 38). For exchange with CO$_2$, the atmosphere was 100% H$_2$, and dissolved CO$_2$ (~0.74 mM at pH 7.2) was supplied by addition of 10 mM sodium bicarbonate from a 0.50 M stock solution. All reactions with the ACDS complex (prepared from *M. thermophila* TM-1 as described in Ref. 7) were carried out in 50 mM MOPS buffer, pH 7.2, and contained 12 µM *M.arkeri* ferredoxin plus a reducing agent. The reducing system for CO$_2$ exchange consisted of 50 mM methyl viologen and 50 mM Ti$^{3+}$ EDTA, whereas in CO exchange assays CO from the gas phase served to reduce the ACDS complex (because of intrinsic high activity CO dehydrogenase). For CO exchange, the ACDS complex, 0.12 mg, was preincubated at the indicated temperatures for 5 min in septum-sealed vials in the presence of all components except acetyl-CoA. The reactions, 600 µl, were then initiated using a gas-tight syringe to add [1-$^{14}$C]acetyl-CoA (25 µM final concentration), and aliquots, 50 µl, were subsequently removed over time and mixed with 100 µl of 0.3 M H$_2$SO$_4$ in 5-ml scintillation vials. Approximately 1 h after sample removal, aqueous scintillation mixture was added, and the samples were counted. The CO$_2$ exchange assay reactions, 600-µl total volume, contained the same concentration of acetyl-CoA and the same amount of ACDS complex as in CO exchange reactions; however, preincubation of all components except bicarbonate was performed in sealed vials under H$_2$, and the reactions were subsequently initiated by addition of 10 mM sodium bicarbonate. Aliquot removal and scintillation counting were done as described for the reactions with CO. The initial velocity of exchange reactions (ν$*$) was determined from fits of the data to the equation $ν*$ = −[A]/[P]/([A] + [P])ln(1 − F)/t, where A and P correspond to the concentrations of substrates undergoing exchange of the label, and F is the fraction of isotopic equilibrium attained at time $t$, given by $F$ = (A$_0^*$ − A$^*$)/(A$_0^*$ − A$^*$_), in which A$^*$ represents the label remaining in solution at time $t$; A$_0^*$ is the amount of label initially added as [1-$^{14}$C]acetyl-CoA, and A$^*$_ is the label remaining in A at isotopic equilibrium (39).

**EPR Spectroscopy**—Samples for EPR spectroscopy were prepared by addition of 0.5 mM Ti$^{3+}$ citrate to the concentrated nickel-reconstituted proteins, followed by incubation under an atmosphere of 100% CO for 15 min, transfer to quartz EPR tubes, and freezing in liquid nitrogen. The NiFe signal was generated in the presence or absence of added reducing agent, as observed previously (11, 24); however, the ACDS β subunit preparation used in this study exhibited only about one-fourth of the spin intensity when reducing agent was omitted, and therefore Ti$^{3+}$ citrate was added to all samples prior to reaction with CO. EPR spectra at X-band frequency (9 GHz) were obtained with a Bruker EMX spectrometer fitted with the ER-4119-HS high sensitivity perpendicular mode cavity. Cooling of the sample was performed with an Oxford Instruments
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FIGURE 1. Carbonyl exchange activity of the ACDS complex from M. ther- momphila: The effects of cosubstrate CO or CO₂ on the rates of isotopic exchange of [1-¹⁴C]acetyl-CoA at different temperatures. The rate of exchange of label from the carbonyl group of acetyl-CoA with unlabeled CO was followed in reactions carried out under a 100% CO atmosphere. Dissolved CO concentrations are estimated to be in the range of 0.94 mM at 26 °C to 0.65 mM at 66 °C (using the α values for CO gas solubility listed in Ref. 38). For exchange with CO₂, dissolved CO₂ (0.74 mM at pH 7.2) was supplied by addition of 10 mM sodium bicarbonate from a 0.50 M stock solution. A methyl viologen redox buffer system was included for CO₂ exchange reactions and was kept reduced under H₂. All reactions were performed at pH 7.2 and contained 0.78 μM ACDS complex and 12 μM M. barkeri ferredoxin. Isotope exchange rates are expressed as \( v^*/[E_{\text{red}}] \), i.e. turnover number \((T,N)\). Error bars indicate standard errors of estimate for the initial exchange velocity values obtained from the reaction time course data fit as described under “Materials and Methods.”

ESR 900 flow cryostat with an ITC4 temperature controller. Spin quantification was carried out under nonsaturating conditions using 10 mM copper perchlorate as the standard (10 mM CuSO₄, 2 mM NaClO₄, 10 mM HCl).

RESULTS

Coupling of A and C Cluster Activities in the ACDS Complex—The ACDS complex catalyzes the overall reversible synthesis and cleavage of acetyl-CoA, whereby, in the direction of synthesis, individual steps lead to the formation of an A cluster acetyl species followed by transfer of the acetyl group to coenzyme A. In the reverse direction, cleavage of the acetyl C–C bond yields separate methyl and CO fragments bound to the A cluster. To measure the reversible fragmentation and reassembly of the A cluster species, the M. thermosthila ACDS complex, carbonyl exchange assays were performed using [1-¹⁴C]acetyl-CoA as substrate in the presence of either ¹²CO or ¹³CO₂. As shown in Fig. 1, isotope exchange rates were much higher in reactions with CO₂ as cosubstrate, as compared with reactions with CO. At 26 °C, the reaction was 350 times faster with CO₂ than with CO. Variation of the reaction temperature showed further differences in the kinetics, wherein the rate of exchange with CO₂ reached a maximum at around 50 °C and decreased at a higher temperature. By contrast, the activity with CO continued to rise with temperature up to the highest temperature tested, 66 °C. Eyring plots (see supplemental Fig. S1) of the CO₂ data were curved downward with a break beginning in the region around 40–50 °C; however, with CO, the plot of \( \ln(k/T) \) versus \( 1/T \) was linear throughout the temperature range examined. The free energy changes for activation, \( \Delta G^\circ \), for the CO and CO₂ exchange reactions were estimated to be 22.3 and 18.7 kcal/mol, respectively, at 25 °C (using only the three lowest temperature points for the CO₂ data, before the break). Activation enthalpy and entropy changes (\( \Delta H^\circ \) and \( \Delta S^\circ \)), which determine the different \( \Delta G^\circ \) values, were 17.6 kcal/mol and –16 cal/mol, respectively, for the CO exchange reaction, and 15.9 kcal/mol and –10 cal/mol for the reaction with CO₂. The error in the slope of the plots used to obtain \( \Delta H^\circ \) for the CO₂ exchange is much larger (18.5%) than for CO (1.5%), and therefore it is possible that the \( \Delta S^\circ \) values are similar and that variation in \( \Delta H^\circ \) is mainly responsible for the observed rate differences (supplemental Fig. S1). The different effects of temperature also indicate that CO and CO₂ exchange reactions are separate and distinct processes and that the free exchange of CO, released from the A cluster upon acetyl group fragmentation, is a minor reaction in comparison with the exchange of CO₂. These findings are consistent with a highly efficient channelling process in which CO produced at the A cluster remains well contained within the enzyme complex until it reaches the C cluster, where it is then oxidized to CO₂ and released, allowing for exchange with free unlabeled CO₂. The results demonstrate tight coupling of the A and C cluster activities in the ACDS complex, with minimal losses of CO derived from acetyl C–C bond cleavage.

Activity Differences between Bacterial ACS and the Archaeal ACDS β Subunit: A Direct Role of the ACS N-terminal Domain in Promoting Acetyl C–C Bond Fragmentation at the A Cluster—Because the results of carbonyl exchange assays indicated that CO produced by acetyl C–C bond cleavage at the A cluster in the ACDS complex is not readily released as free CO, we decided to compare various A cluster catalytic activities of the ACDS β subunit with full-length and N-terminally truncated forms of the bacterial ACS α-metalloprotein. The expressed β subunit protein, 44.6 kDa, is 42.9% identical over its entire length to a region containing the central and C-terminal domains of ACS from C. hydrogenoformans, corresponding to ACSCh residues 318–732. Therefore, in addition to the full-length 82.2-kDa ACSCh protein, an N-terminally truncated form, ACSChΔN, molecular mass of 46.8 kDa, which lacks the first 317 amino acids of ACSCh, was also expressed and purified. Anaerobic purification procedures were carried out, as described under “Materials and Methods,” in which extracts of anaeroically grown E. coli were subjected to anion exchange chromatography on Q-Sepharose as the initial capture step. Thereafter, different chromatographic procedures were used for the individual enzymes, which consisted of phenyl-Sepharose and Mono Q columns for the ACDS β subunit, HTP hydroxypatite, and Mono Q steps for ACSCh, and chromatography on a phenyl-Sepharose HP column for ACSChΔN. The resulting protein preparations were analyzed by SDS-PAGE, as shown in Fig. 2. All three proteins migrated according to their predicted molecular weights, and densitometric analyses indicated 94–95% purity for ACSCh and around 98% purity for both ACDS β and ACSChΔN.
Assays for acetyl-CoA synthesis using CO, CoA, and methylenecobinamide as the methyl donor substrate showed that all three proteins catalyzed rapid formation of acetyl-CoA. As shown in Fig. 3A, at 0.40 mM methylcobinamide and 0.48 mM CO, the ACSβ subunit was more active than ACSCh, whereas ACSChΔN showed the highest activity. By contrast, assays for [1-14C]acetyl-CoA/CO exchange activity revealed that only ACSCh was able to catalyze carbonyl group exchange at a significant rate (Fig. 3B). Thus, high activity of acetyl C–C bond fragmentation to yield a CO-bound A cluster species exchangeable with unlabeled CO was observed only with the full-length ACSCh.

To further characterize the differences found among the proteins, measurements of the kinetic parameters for acetyl-CoA synthesis were made and compared with CO exchange rates and with activities in acetyltransferase reactions. As shown in Table 1, under standard conditions with a CO concentration of 0.48 mM, the $K_m$ values for methylcobinamide were similar for ACSβ and ACSCh, around 0.5 mM. The acetyl-CoA synthesis activity of the ACSβ subunit was ~2-fold higher than ACSCh, as a result of the higher value of $V_{max}$. Truncation of the N-terminal region of ACSCh had a marked effect on both $K_m$ and $V_{max}$ values. In ACSChΔN, the $K_m$ value was decreased to about one-seventh of its value in the full-length protein, and the $V_{max}$ value was increased by a factor of around 4.4. Overall, the $V_{max}/K_m$ ratio increased by around 30-fold, indicating an apparent unmasking of the intrinsic catalytic efficiency for overall synthesis of acetyl-CoA. At the same time, CO exchange activity of ACSCh was drastically reduced in the N-terminally truncated protein, with around 3 orders of magnitude higher levels of CO exchange activity exhibited by the full-length protein. All three proteins catalyzed redox-dependent acetyl transfer reactions at rates that greatly exceeded those of CO exchange or overall acetyl-CoA synthesis (Table 1), as measured in reactions involving acetyl group transfer from acetyl-CoA to the reduced enzyme with release of CoA, followed by acetyl transfer from the enzyme to the CoA analog 3'-diphospho-CoA to yield 3'-diphospho-S-acetyl-CoA. Although transfer to CoA of nascent acetyl groups formed from CO and CH₃ condensation is therefore not rate-limiting for overall synthesis of acetyl-CoA, the ACSβ subunit was significantly

**FIGURE 2.** SDS-PAGE of *M. thermophila* ACSβ subunit and *C. hydrogenoformans* acetyl-CoA synthase, ACSCh, and its N-terminally truncated form ACSChΔN. Analysis was performed on a 12% acrylamide gel, stained with Coomassie Blue R-250 (40). Numbers above the lanes indicate relative amounts of protein loaded, with 100, 4, 2, and 1 corresponding to 6.0, 0.24, 0.12, and 0.06 μg of protein, respectively. Molecular weight markers (Bio-Rad) were loaded on lane M.

**FIGURE 3.** Direct role of the N-terminal domain of bacterial ACS in promoting acetyl C–C bond fragmentation at the A cluster. A, acetyl-CoA synthesis. Reactions carried out at 25 °C as described under “Materials and Methods” contained 0.40 mM methylcobinamide and 0.48 mM CO, the ACSβ subunit was more active than ACSCh, whereas ACSChΔN showed the highest activity. By contrast, assays for [1-14C]acetyl-CoA/CO exchange activity revealed that only ACSCh was able to catalyze carbonyl group exchange at a significant rate (Fig. 3B). Thus, high activity of acetyl C–C bond fragmentation to yield a CO-bound A cluster species exchangeable with unlabeled CO was observed only with the full-length ACSCh.

|          | ACSβ | ACSCh | ACSChΔN |
|----------|------|-------|---------|
| Turnover | 86.8 | 21.2  | 9.63    |

**TABLE 1.** Kinetic parameters for acetyl-CoA synthesis and CO exchange activities of bacterial ACS. Reaction conditions: 0.40 mM methylcobinamide, 0.48 mM CO, 34 μM Ti⁵⁺-NTA, 50% CO, 50% N₂ atmosphere, and 120 μM CoA. The concentrations of ACSβ, ACSCh, and ACSChΔN were 0.69, 0.84, and 0.19 μM, respectively. Initial rates and their standard errors of estimate were obtained from the time course data fit as described under “Materials and Methods” and are given as $v_0/(E_{total})$, turnover number (min⁻¹). B, exchange of [1-14C]acetyl-CoA with unlabeled CO. CO exchange reactions, 400 μl of total volume, contained 50 mM MES buffer, pH 6.5, 140 μM Ti⁵⁺-NTA, 25 μM [1-14C]acetyl-CoA, and were performed at 25 °C in sealed vials under 100% CO. Reactions were initiated by addition of [1-14C]acetyl-CoA, and 30-μl aliquots were removed at the indicated times and processed for scintillation counting as described under “Materials and Methods.” ACSβ, ACSCh, and ACSChΔN were 1.05, 0.89, and 1.34 μM, respectively.
more active in acetyltransferase assays than the bacterial enzyme, forming products 5–8 times more rapidly than \( \text{ACS}_{\text{Ch}} \Delta \text{N} \) and \( \text{ACS}_{\text{Ch}} \), respectively. Acetyltransferase activity of \( \text{ACS}_{\text{Ch}} \Delta \text{N} \) was about 150% that of \( \text{ACS}_{\text{Ch}} \), possibly related to the same underlying mechanism responsible for the more pronounced increase in acetyl-CoA synthesis activity. Because truncation of the ACSCh N-terminal domain virtually abolished the equivalent increase in acetyl-CoA synthesis activity. However, the data do show that the truncated enzyme, forming products 5–8 times more rapidly than \( \text{ACS}_{\text{Ch}} \Delta \text{N} \) and \( \text{ACS}_{\text{Ch}} \), respectively.

In addition to the substantial changes observed in the \( V_{\text{max}} \) and \( K_{\text{m}} \) values for methylocobinamide in acetyl-CoA synthesis, removal of the N-terminal domain from the bacterial protein also resulted in marked changes in the effects of CO concentration on the kinetics, as shown in Fig. 4. For the full-length \( \text{ACS}_{\text{Ch}} \) enzyme, acetyl-CoA synthesis activity was maximal at low concentrations of CO and declined as levels of CO were increased, a situation indicative of substrate inhibition. Although CO inhibition of acetyl-CoA synthesis has been shown previously for the native CODH/ACS \( \alpha_2 \beta_2 \) heterodimeric enzyme from \( \text{M. thermoacetica} \) (34, 42, 43), such effects were not exhibited by the recombinant \( \text{ACS}_{\text{Ch}} \alpha \Delta \) subunit in those studies in the absence of contacts with the CODH \( \beta \) subunits (34, 43). In contrast, our results on \( \text{ACS}_{\text{Ch}} \), indicate that strong substrate inhibition does occur even in the absence of interactions with CODH subunits. The \( K_{\text{m}} \) value for CO could not be determined under the experimental conditions used in Fig. 4 because significant consumption of total available CO was encountered at the lowest CO level tested, 0.0095 mM; however, the data do show that the \( K_{\text{m}} \text{value must be significantly lower than that value. Analysis of the truncated enzyme, ACSCh} \Delta \text{N, revealed a distinctly different kinetic pattern, wherein activity showed the typical hyperbolic dependence on substrate concentration, with a} K_{\text{m}} \text{for CO of 0.36 ± 0.05 mM and a} V_{\text{max}} \text{of 159 ± 13 min}^{-1} \text{ (Fig. 4). Because the level of methylocobinamide (0.40 mM) used in these assays is nearly saturating for this form of the enzyme, the observed} V_{\text{max}} \text{value also provides an estimate of} k_{\text{cat}} \text{for acetyl-CoA synthesis. Analysis of the effect of CO concentration on the ACDS \( \beta \) subunit (supplemental Fig. S2) also showed standard hyperbolic kinetics, and yielded values of} K_{\text{m}} \text{for CO of 0.087 ± 0.028 mM and} V_{\text{max}} \text{of 20.8 ± 3.6 min}^{-1} \text{. In this case, the 0.40 mM level of methylocobinamide is slightly less than half-saturating. However, the} V_{\text{max}} \text{of 43.7 min}^{-1} \text{ (Table 1) provides a reasonable estimate of} k_{\text{cat}} \text{for the ACDS} \beta \text{ subunit, because the level of 0.48 mM CO used in those assays is near saturation for that enzyme. Overall, the kinetic changes from low} K_{\text{m}} \text{values for CO and strong substrate inhibition in the full-length ACSCh \( \beta \) to relatively high} K_{\text{m}} \text{values and little or no CO inhibition in the truncated ACSCh} \Delta \text{N and ACDS} \beta \text{ subunit are consistent with

### TABLE 1

| Catalystic properties of ACDS \( \beta \) subunit and bacterial ACS protein forms | Acetyl-CoA synthase activity$^a$ | Acetyl-CoA synthase activity $^a$ | [\(^{1-14}\text{C}\)]Acetyl-CoA/CO exchange activity T.N.$^b$ | Acetyltransferase activity T.N.$^b$ |
|-----------------------------------------------|------------------------------------------|------------------------------------------|---------------------------------|---------------------------------|
| | \( V_{\text{max}} \text{ min}^{-1} \) | \( K_{\text{m}} \text{ mM} \) | \( k_{\text{cat}} \text{ min}^{-1} \) | \( k_{\text{cat}} \text{ min}^{-1} \) |
| ACDS \( \beta \) | 43.7 ± 2.4 | 0.53 ± 0.06 | ≤0.012 | 10,800 ± 1,500 |
| ACSCh | 21.3 ± 4.2 | 0.46 ± 0.09 | 8.5 ± 0.3 | 1,350 ± 230 |
| \( \text{ACS}_{\text{Ch}} \Delta \text{N} \) | 93.2 ± 5.1 | 0.066 ± 0.016 | ≤0.007 | 2,050 ± 200 |

$^a$ Standard conditions for acetyl-CoA synthase employed a fixed [CO] of 0.48 mM (50% CO, 50% N\(_2\) atmosphere) with varied concentrations of CH\(_3\)-cobinamide. Values of \( K_{\text{m}} \) and \( V_{\text{max}} \) are therefore apparent at this level of CO. Standard errors were obtained from the direct fits of the velocity versus concentration data to the Michaelis-Menten equation for acetyltransferase assays, means ± S.E. are for two determinations on each enzyme form. Error analyses methods were as described in Ref. 41.

$^b$ T.N. means turnover number, \( v / [E_{\text{tot}}] \).

$^c$ Data are from Ref. 16.

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*FIGURE 4. Changes in the kinetics of acetyl-CoA synthase possibly due to differences in CO accessibility to the A cluster in different forms of the enzyme.* Acetyl-CoA synthase reactions were carried out at a fixed concentration of methylocobinamide (0.40 mM) with varied concentrations of CO at 25 °C. Gas mixtures in which CO levels were varied from 1 to 48% with a balance of N\(_2\) were used to provide substrate levels of dissolved CO ranging from 0.0095 to 0.456 mM. Preincubation under the various CO gas atmospheres, initiation of the reactions by addition of CoA, and analysis of acetyl-CoA formation over time were carried out as described under “Materials and Methods.” Error bars are indicated for the estimates of the initial velocities obtained from fits of reaction time course data, except for the single ACSCh point at the lowest CO concentration. For this reaction, all of the available CO had been consumed within the first 30 s after addition of CoA, prior to removal of the first aliquot. The plotted rate value corresponds to 0.0095 mM CO consumed in 30 s and is a substantial underestimate of the correct initial rate. The apparent \( K_{\text{m}} \) value for CO for ACSD3 \( \beta \) could not be determined but must be less than 0.0095 mM. The data for ACSCh \( \Delta \text{N} \) were analyzed by a direct fit to the Michaelis-Menten equation and yielded a \( K_{\text{m}} \) for CO of 0.36 ± 0.05 mM and \( V_{\text{max}} \) of 159 ± 13 min\(^{-1} \). The line for ACSCh \( \Delta \text{N} \) is drawn from the equation for competitive substrate inhibition in a Ping Pong Bi Bi system (39), in which substrate CO would combine with the free enzyme to form an enzyme-CO dead-end complex, with \( V_{\text{max}} \) values assumed to be similar to that of ACSCh \( \Delta \text{N} \). The ascending portion of the curve lies very close to the y axis and is essentially indistinguishable in the figure as drawn.

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Fig. 5, the EPR spectra of ACDS proteins, "frozen, and analyzed by EPR spectroscopy. As shown in Fig. 5 for comparison, and it exhibits a slightly higher average spin value than bacterial CODH/ACS. Very similar spin values have been reported for ACDS signal I of 2.089, 2.074, and 2.028 from M. thermophila (48) and 2.089, 2.076, and 2.028 for the enzyme complex from M. barkeri (49). Interestingly, the A cluster activities of the proteins correlated with the type of signal observed, in that only samples that displayed type I signals (ACSCh and the ACDS complex) were able to carry out significant carbonyl exchange activity. Recent crystallographic analyses on an N-terminally truncated form of ACS from M. thermoacetica indicated that a substantial reorientation of the relative positions of the central and C-terminal domains had taken place, as compared with either the closed or open forms of the protein where some degree of contact with the N-terminal region is always maintained (50). Thus, the change in the EPR signal from type I to type II is seen to take place in parallel with the conformational changes that result from loss of interactions of the N-terminal domain with the central and C-terminal domains in ACS or in the case of the ACDS complex with the loss of contact of the β subunit with other protein components in the complex such as the αε2 CO dehydrogenase subcomponent.

**DISCUSSION**

The efficient catalysis of acetyl C–C bond cleavage by nickel- and iron-containing A cluster enzymes is essential for decomposition of acetate by anaerobic acetate-oxidizing bacteria and Archaea and is also critical for disproportionation of large amounts of acetic acid by methanogens. By contrast, proficient coupling of CO and CH₃ groups at the A cluster is required for large scale production of acetate by acetogenic bacteria (51). Thus, at the outset it seems surprising that exchange of the carbonyl group of acetyl-CoA with CO, a process that requires acetyl C–C bond cleavage (to form CO at the A cluster that can be released and exchanged), is much more active in M. thermoacetica CODH/ACS involved in acetate synthesis than in ACDS from methanogenic species engaged in acetate cleavage. It was reported early on that the kcat for CO exchange was an order of magnitude lower for the ACDS complex from M. thermophila than for clostridial CODH/ACS from M. thermoacetica (29). In addition, exceedingly low CO exchange rates were noted using cell extracts of M. barkeri, but rapid exchange was observed when CO₂ was employed (30). The results in Fig. 1 agree with these previous studies and indicate that the ACDS complex catalyzes carbonyl exchange up to several hundred

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5 An error was found in the g values reported in Fig. 7 of Ref. 11, and the numbers on the abscissa of that plot are incorrect. Recalculation from the original data gives values of peak maxima and minima at g 2.055 and 2.020, respectively, much closer to the results found here.
times faster with CO₂ than with CO. If, in fact, exchange takes place only with CO₂, then the low rates observed with CO actually might have been due to the presence of a small amount of CO₂ formed from CO oxidation by highly active CO dehydrogenase. However, because the two reactions show distinct temperature dependences, it is likely that the observed loss of label from [1-¹⁴C]acetyl-CoA in the presence of CO is not due to reaction with small amounts of CO₂ produced by CO oxidation and that direct exchange with CO actually does take place. Differences in activation enthalpy and entropy changes both appear to contribute favorably to the increased rate of exchange with CO₂ relative to CO, although it is possible, because of experimental limitations, that the major effects are mainly due to differences in ΔH°.

The low rates of exchange of CO versus CO₂ by ACDS indicate that CO binding and release at the A cluster are tightly coupled to acetyl-CoA synthesis, perhaps even more so than in bacterial CODH/ACS, where CO exchange rates around 46.5 min⁻¹ (300 nmol/min/mg) at 55 °C have been observed (32). One possibility would be that transfer of CO from the ACDS β subunit to the α₁ε₂ CO dehydrogenase might be more efficient (therefore not exchanged with free CO) because of a tight association of subunits at the interface between the α₁ε₂ and the β subunit. Because the N-terminal domain of the bacterial ACS, which intervenes between the A cluster and subunit interface with CODH, is lacking in the ACDS β subunit, highly effective transfer of CO to the ACDS complex might be the result of a direct contact of the A cluster with the α₁ε₂ CODH protein. If interaction with the α₁ε₂ protein prevents the escape of CO by a mechanism involving physical containment, similar to that proposed for the tunnel in the CODH/ACS (14, 15, 35), then, in the absence of the α₁ε₂ protein, the free β subunit should be able to exchange CO derived from acetyl C–C bond cleavage with bulk CO more rapidly than when it is associated with the α₁ε₂ protein. However, as shown in Fig. 3B, the free ACDS β subunit does not catalyze CO exchange at an increased rate, with the observed activity being only slightly above the level of detection, even lower than found for the ACDS complex. As shown in a previous study, loss of carbonyl exchange activity of ACDS also takes place upon disruption of the complex by partial proteolysis (10).

Consistent with the behavior of the free ACDS β subunit, the bacterial ACS₇ΔN protein, which mimics the ACDS β subunit by being composed solely of the homologous central and C-terminal regions, is also nearly inactive in catalyzing [1-¹⁴C]acetyl-CoA/CO exchange. Removal of the N-terminal domain of ACS₇ results not only in the nearly complete loss of CO exchange activity but also causes a marked stimulation of acetyl-CoA synthesis in which the Kₘ value for methylcobinamide is decreased and the Vₘₐₓ value is increased overall (Table 1). The results indicate that the N-terminal domain, although not required for acetyl-CoA synthesis, is essential to catalyze the reaction in the reverse direction, i.e. acetyl C–C bond fragmentation, as judged by CO exchange. Although the CO exchange reaction has been known since the time it was first recognized as a proof that bacterial CODH/ACS catalyzes the synthesis and cleavage of the C–C and C–S bonds of acetyl-CoA (26), relatively few experiments have been performed subsequently to examine the C–C fragmentation step directly, and the essential involvement of the ACS N-terminal domain had not been recognized.

**Mechanism of Acetyl C–C Bond Fragmentation**

**Energetic States of Intermediates**—To explain how the N-terminal domain is involved in altering the relative rates of catalysis of the forward and reverse reactions, the energetics of acetyl C–C bond fragmentation need to be taken into account. Following reaction of the enzyme with acetyl-CoA to form an A cluster acetyl-Niₚ²⁺ intermediate, energy input would be required to cleave the acetyl C–C bond to generate separate CO and CH₃ ligands bound to Niₚ. Density functional theory calculations have estimated that +6.2 kcal/mol would be required to cleave the C–C bond of an acetyl moiety bound to trigonal-planar Niₚ²⁺ in a model of the A cluster, in proceeding to a square-planar Niₚ²⁺ (CO)(CH₃) fragmented species (52). Here, the coordination number of Niₚ increases from 3 to 4 upon fragmentation, and an increase in coordination number also has been proposed (albeit from 4 to 5) in the mechanism deduced from quantum chemical studies, whereby four-coordinate square-planar Niₚ²⁺-acetyl is converted to the higher energy state, five-coordinate, distorted square-pyramidal Niₚ²⁺(CO)(CH₃) (53). Our results suggest that the energy required for the coordination geometry changes needed to facilitate C–C bond fragmentation is derived from conformational changes involving interaction of the N-terminal domain with the central and C-terminal domains. One possibility for such an interaction would be thermally driven conformational changes, transmitted through the interdomain contacts, that result in alteration of the distances or angles of approach of the three bridging sulfur ligands to Niₚ such that a higher energy and higher coordination number state could be achieved. The average distance between the three sulfur ligands (bound to Niₚ) is significantly larger in the square-planar arrangement at Niₚ in the open conformation of ACSₗ₅₆₄ as compared with tetrahedral configuration in the closed form (Scheme 2) (15), suggestive of a situation in which conformational changes in the enzyme may be coupled to ligand repositioning.

Stabilization or destabilization of the Niₚ²⁺(-acetyl intermediate in different conformational states of the protein would have different effects on the individual partial reactions catalyzed by the A cluster, as indicated in Scheme 3. The ACS mechanism thereby can be viewed as an “ensemble” of conformations that support the catalytic network of chemical steps, using theory and terminology from studies on other enzymes in which conformational effects on catalysis are well established (54). The minimal reaction coordinate landscape that includes open-like and closed-like ACS conformations qualitatively illustrates the relative differences in stabilities of the chemical intermediates in the two different conformations. For the methylated intermediate, the closed-like form cannot be arrived at directly by methylation of the reduced enzyme in the closed conformation, because of inaccessibility of the large corrinoid substrate. Thus, the lower Kₘ value for methylcobinamide and the higher Vₘₐₓ value observed following removal of the N-terminal domain would be due to greater accessibility of the large corrinoid substrate to the A cluster in an open-like
Conformational Changes Coupled to Catalysis at the A Cluster

SCHEME 3. Reaction coordinate diagram for acetyl-CoA synthase with two conformations of the enzyme having different abilities to stabilize/destabilize the Ni\(_{p}^{2+}\)-acetyl intermediate. The reaction coordinate covers a portion (steps II–IV) of the overall process of acetyl-CoA synthesis starting from the methylated enzyme and ending in the formation of acetyl-CoA. Step I, methylation of the reduced enzyme is not shown. Two conformational states are considered as follows: State 1, an open-like form, and State 2, which is closed-like. The methylated form of the closed-like state is positioned at higher energy because methylation of the closed-like conformation is improbable due to inaccessibility of the large corrinoid substrate. To achieve the methylated, closed-like state, a conformational change is employed to convert the open methylated state to the closed form, arrow a (State 1 \(\rightarrow\) 2). Similarly, reaction of acetyl-CoA with the open-like form (step IV, in reverse) leads to a relatively stable Ni\(_{p}^{2+}\)-acetyl intermediate, with a subsequent conformational change that affords the closed-like acetylated form, arrow b (State 1 \(\rightarrow\) 2). In the closed form, the Ni\(_{p}^{2+}\)-acetyl species is destabilized as a result of interdomain contacts involving the N-terminal domain of ACS (or by intersubunit interactions of the β subunit with the α\(_{2}\)ε\(_{2}\) protein within the ACDS complex). Although the Ni\(_{p}^{2+}\)-acetyl intermediate is stable in the open-like arrangement in the truncated enzyme, access to the closed-like state is disallowed due to the absence of interdomain interactions. The exchange of CO is precluded in the truncated enzyme because of the inability to utilize the change in conformational state to surmount the barrier to step III in reverse. Potential energy differences are not to scale. A comprehensive description of the steps is given in the text.

In the structure of ACS\(_{\text{mt}}\) (15, 35), a strictly conserved phenylalanine residue (Phe-512 in ACS\(_{\text{mt}}\), that corresponds to Phe-195 in the ACDS β subunit) is present with its side chain positioned within 4 Å above Ni\(_{p}\) and at a similar distance in ACS\(_{\text{Ch}}\) (24), where it shields axial approach to Ni\(_{p}\) in the open form. In the closed form, which is where the tunnel is continuous between the A and C clusters, rotation of the phenylalanine side chain displaces it by as much as 7 Å, which then allows access of CO from the tunnel to the proximal metal at the axial position. So, in the native enzyme, following methylation of the open form, a change of conformation to the closed state (Scheme 3, arrow a, State 1 \(\rightarrow\) 2) would provide for ready access of CO to the axial position of Ni\(_{p}\). The rate-determining step in this case no longer corresponds to CO addition to the methylated enzyme but rather to the intrinsic open-to-closed transition rate of process a, and in the full-length ACS\(_{\text{Ch}}\), with unrestricted access of CO, the rate of acetyl-CoA synthesis is high even at low CO concentrations and does not increase at higher levels of CO. Instead, the full-length enzyme becomes inhibited at high concentrations of CO, possibly due to CO binding to the unmethylated enzyme in the closed state (discussed below). In step III, CO and CH\(_{3}\) groups combine by the process of carbonyl insertion or methyl group migration to form the Ni\(_{p}^{2+}\)-acetyl intermediate, which, as predicted by density functional theory calculations (52), is substantially more stable than the fragmented form. Relative to State 1, the Ni\(_{p}^{2+}\)-acetyl intermediate in State 2 is destabilized as a result of interdomain interactions with the N-terminal domain of ACS (or by intersubunit interactions of the β subunit with the α\(_{2}\)ε\(_{2}\) protein within the ACDS complex). In step IV, acetyl group transfer to CoA forms acetyl-CoA. Interaction of acetyl-CoA with the closed conformation is not depicted because acetyl-CoA and CoA would bind predominantly to the open form.

Exchange of the carbonyl group of acetyl-CoA with CO involves steps II–IV, starting with the reverse of step IV, acetyl-CoA binding to the open-like State 1 and formation of the Ni\(_{p}^{2+}\)-acetyl intermediate in an open-like conformation. Thereafter, the conformational change indicated by Scheme 3, arrow b (State 1 \(\rightarrow\) 2), would act to destabilize the acetyl intermediate, resulting in C–C bond fragmentation (reverse of step III), followed by CO release (step II in reverse). Rebinding of unlabeled CO, and retracing of steps II–IV in the forward direction completes one cycle of CO exchange. The open-like arrangement in the truncated enzyme would provide greater stability to the Ni\(_{p}^{2+}\)-acetyl intermediate; however, without access to the closed-like state, CO exchange is precluded because of the high barrier to reverse step III. In order for efficient CO exchange to take place, a shift from State 1 to State 2 (Scheme 3, arrow b) would be required to increase the energy of the acetyl intermediate, thereby overcoming the barrier to fragmentation. The closed-like form State 2 is characterized by interdomain interactions involving the N-terminal domain in ACS or intersubunit interactions of the β subunit with the α\(_{2}\)ε\(_{2}\) protein in the ACDS complex, and in this conformation the enzyme is able to promote chemical cleavage of the acetyl C–C bond. Thus, the overall exchange reaction is obligately coupled to conformational changes. In summary, the relative differences in energy states of the intermediates depicted in Scheme

state. In the truncated enzyme, which exists only in the open form, carbon monoxide addition to the methylated enzyme, step II, would be rate-limiting for acetyl-CoA synthesis, and therefore the rate increases as CO concentration is raised to high levels (Fig. 4).

In the structure of ACS\(_{\text{mt}}\) (15, 35), a strictly conserved phenylalanine residue (Phe-512 in ACS\(_{\text{mt}}\), that corresponds to Phe-195 in the ACDS β subunit) is present with its side chain positioned within 4 Å above Ni\(_{p}\) and at a similar distance in ACS\(_{\text{Ch}}\) (24), where it shields axial approach to Ni\(_{p}\) in the open form. In the closed form, in which the tunnel is continuous between the A and C clusters, rotation of the phenylalanyl side chain displaces it by as much as 7 Å, which then allows access of CO from the tunnel to the proximal metal at the axial position. So, in the native enzyme, following methylation of the open form, a change of conformation to the closed state (Scheme 3, arrow a, State 1 \(\rightarrow\) 2) would provide for ready access of CO to the axial position of Ni\(_{p}\). The rate-determining step in this case no longer corresponds to CO addition to the methylated enzyme but rather to the intrinsic open-to-closed transition rate of process a, and in the full-length ACS\(_{\text{Ch}}\), with unrestricted access of CO,
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SCHEME 4. Chemical steps and conformational changes in the mechanism of acetyl-CoA synthase. Intermediates formed in the closed-like conformation are indicated in red, and species in the open-like state are in black and are designated with subscripts c and o, respectively. Vertical arrows denote conformational changes. Steps i–iv and conformational changes a and b correspond with Scheme 3. The red arrows, including conformational changes a and b, trace the major pathway used by the native enzyme, whereas only the lower set of reactions (arrows between open-like species only) is available for catalysis by the truncated ACSCh ΔN enzyme or ACDS β in the absence of other protein contacts. No reaction is indicated for direct methylation of the closed conformation because the active site would be inaccessible in that form. Similarly, reaction with CoA is assumed to occur primarily with the open form, and the closed state containing an acetyl-intermediate is shown to require a conformational change (indicated as b) prior to reaction with CoA.

3 reflect the following observations. 1) Acetyltransferase activity is much higher than either acetyl-CoA synthesis or CO exchange but is not faster than the overall thermodynamically favorable process of acetyl-CoA synthesis. 3) The acetyl-enzyme intermediate is, at a lower energy state, relative to the fragmented Ni₄(CO)(CH₃) species, as indicated by density functional theory results (52). 4) The presence of the N-terminal domain is required to generate a conformational change to allow CO exchange to take place. 5) The decreased $K_m$ value for methylcobinamide in ACSCh ΔN versus full-length ACSCh is due to greater accessibility of substrate in the open-like form. 6) Acetyl-CoA synthesis activity increases as CO concentrations are raised to high levels only with the truncated open-like forms, wherein methylation is readily achieved but CO addition faces a barrier due to restricted access.

Inhibitory Effects of CO—The kinetic differences in CO utilization in acetyl-CoA synthesis, i.e. low $K_m$ values for CO and strong CO inhibition in the full-length ACSCh, but high $K_m$ values for CO and the absence of significant inhibition in the truncated ACSCh ΔN and ACDS β subunit, can be interpreted in view of differences in CO accessibility that would exist in different conformational states available to different forms of the enzyme. CO inhibition is pronounced for the native enzyme where, in the closed state, CO would be freely accessible to the axial position of Ni₄. However, little or no inhibition is seen for permanently open forms of the enzyme, ACSCh ΔN, and the isolated ACDS β subunit, in which access of CO would be restricted by the shielding phenylalanine residue. In general, the nucleophilicity of a reduced metal center is decreased upon binding of CO as a result of back-π donation that lowers the electron density on the metal. Therefore, if CO arrives at Ni₄ before CH₃, then lower reactivity for methylation would result, and the overall rate of acetyl-CoA synthesis would be expected to be lower than in the case where CO adds to a Ni₄(CH₃) species already formed. It has been shown that the presence of a CO ligand in the Ni(0) compound (bis(2-diphenylphosphinoethyl)phenylphosphine)NiCO prevents methylation by methylcobaloxime (55). And, in recent studies on a binuclear Ni(II)-Ni(II) complex bearing methyl and thiol ligands, it was found that thioester formation, initiated by addition of CO, takes place as a single turnover process because of the concomitant formation of an inactive Ni(0)-carbonyl complex that does not react further with methylcobaloxime (56). This was interpreted to indicate that CO addition to Ni₄ in ACS must be strictly regulated to prevent the formation of such inactive Ni(0)-CO species. Thus, the effect of decreased nucleophilicity could provide the basis for inhibition by CO binding to the unmethylated enzyme.

In the absence of CO, increased levels of the methylated enzyme open form $E_o-CH₃$ would be produced as the concentration of methyl donor is increased according to an equilibrium process, as indicated in Scheme 4. As small amounts of CO are added, reaction with the methylated enzyme would take place most effectively in the closed conformation where CO has unrestricted access to Ni₄. The rate of reaction would therefore be dependent on the concentration of the $E_o-CH₃$ species capable of undergoing the rate-determining conformational change, indicated as a, to generate the closed form with which CO would react rapidly. As the level of CO is increased, the concentration of the methylated intermediate would decline because of substrate inhibition, with CO competing for the unmethylated form of the enzyme, binding most efficiently to the closed form to produce an $E_c-CO$ species that is essentially a dead-end complex. A simple two-equilibrium situation in which the equilibrium constant for CO binding to free enzyme is about 14 times higher than the effective equilibrium for methylation (at the 0.4 mM level of methylcobinamide employed) also provides a close approximation of the descending portion of the curve in Fig. 4. As CO levels are raised, activity declines in proportion to the decreasing concentration of the $E_c-CH₃$ species. A cooperative model of CO binding, that was suggested to explain CO inhibition of the $\alpha_2\beta_2$ CODH/ACS enzyme from M. thermoacetica (42) was not required.

In enzyme forms that are unable to undergo a conformational change to the closed state, such as ACSCh ΔN and the isolated ACDS β subunit, acetyl-CoA synthesis would proceed according to the lower set of reaction steps given in Scheme 4. Here, methylation occurs readily because of the ease of approach of methylcobinamide, but access of CO to the axial
position of Niₚ would be hindered. However, at sufficiently high levels, CO gains entry into the active site and reacts with the Eₒ-C₃H₇ species, the predominant form of the enzyme. Following transient formation of the fragmented Eₒ(CO)(CH₃)₃ species, the Niₚ-acetyl intermediate is formed and rapidly captured by CoA to generate acetyl-CoA and the free enzyme as products. Because of the hindered access to CO, remethylation of Niₚ is sterically crowded by residues from the N-terminal domain that impinge on the A cluster in the closed ACS conformation. As mentioned above, in the open form a nearby chain of Ile-146 would be in steric conflict with any equatorial ligand to Niₚ equal to or larger in size than a methyl group (15). Thus, conformationally driven steric effects on the group(s) bound to Niₚ (Scheme 5) can be proposed as an alternative mechanism for the input of energy required to cleave the acetyl C–C bond. In this mechanism, steric force from the bacterial N-terminal domain, or from an analogous region in the ACDS complex, would distort the square-planar geometry of the A cluster Niₚ⁺⁺-acetyl intermediate to initiate methyl group migration and C–C bond cleavage in adopting a five-coordinate, fragmented Niₚ⁺⁺(CO)(CH₃)₃ form that could then undergo release of CO. In that form, the conserved Phe-195 would also be displaced, as needed to accommodate a nascent axial ligand and permit CO to be released/exchanged. Studies using site-directed mutagenesis to examine the roles of specific residues would help to establish their involvement in such a mechanism.

NiFeC EPR Signals I and II

The results of EPR analysis indicate that the NiFeC type I signal, with a maximum in the gₓ,y region at 2.08–2.09, is found in systems in which interdomain (ACS) or intersubunit (ACDS) contacts are present. A previous study showed that removal of the N-terminal domain from ACSₘᵢ also resulted in loss of the type I signal, with low levels 0.05 spin/mol of type II signal observed instead; however, that protein was reported to be inactive (58). The requirement for intersubunit interactions to produce signal I is also supported by an earlier finding that treatment of the ACDS complex with a cationic detergent inactivated CO exchange activity and caused the loss of signal I and the formation of signal II (10). After removal of the detergent by ultrafiltration, signal I was found to return, and much of the exchange activity was restored as well. Our findings agree with the conclusion from that study that interactions between the subunits harboring ACS and CODH components are important for carbonyl exchange; however, they do not indicate involvement of a “covert” electron transfer to explain why CO exchange activity is lost. Signal II has sometimes been referred to as the “pseudo” NiFeC species; however, it is important to emphasize that signal II is not an artifact but instead reports on the electronic state of the A cluster Niₚ⁺⁺-CO species when it is exposed to solvent, in an open-like conformational state of the protein in which interdomain/intersubunit interactions are minimal or absent. Thus, the variable levels of signal II reported for some preparations of ACDS and CODH/ACS are best explained by variable losses of protein-protein interactions with the A cluster-containing domain, a result that could be due to damage during purification and handling or to instability intrinsic to the interdomain/intersubunit contacts, or both. It is also noteworthy, that the major difference between types I and II spectra in ACDS and CODH/ACS are in the gₓ,y region, which...
indicates similar changes in the local environment of nickel. Such changes are likely in the equatorial region of Ni₄, which is in contact with hydrophobic residues (Ile-146 and Val-149) in the closed conformation but exposed to polar solvent in the open state.

**Coupling of A and C Clusters in Bacteria and Archaea**

Bacterial CODH/ACS and archaenal ACDS systems both catalyze carbonyl exchange of the acetyl group of acetyl-CoA with CO and CO₂; however, in ACDS, the rate of the reaction with CO₂ greatly exceeds that of CO. Studies on acetogenic CODH/ACS indicate that little or no incorporation of free CO takes place during exchange of 15CO₂ with acetyl-CoA to yield [1-15C]acetyl-CoA (32) and that the reduction of CO₂ is greatly stimulated under conditions in which the product CO is being consumed in the synthesis of acetyl-CoA (33). Structural studies show how CO₂ reduction at the C cluster can be coupled to the synthesis of acetyl-CoA without exchange with bulk CO, by means of a 70-Å-long intraprotein tunnel that connects the A and C clusters (14, 15, 31). In the open conformation of ACS₅₄₋, in which the A cluster is exposed to solvent, α-helix 7 in the ACS N-terminal domain occupies a position that blocks the tunnel ~20 Å from the A cluster, which prevents escape of CO (15, 35). Closure of this tunnel gate would thereby prevent leakage of CO to the solvent, block the reduction of additional molecules of CO₂, and halt the further expenditure of reducing equivalents. The proposed gating mechanism prevents loss of CO that potentially could engage in unwanted binding to other metal centers in the cell or could result in wasteful loss of substrate and reducing potential (35). Furthermore, in acetogens, the loss of CO by leakage from the tunnel would amount to uncoupling of the highly unfavorable step of the reduction of CO₂ to the remaining steps in the overall favorable pathway of acetate synthesis, a physiologically detrimental situation. In addition to the aforementioned means of preventing escape of CO from the tunnel, the integrity of the interface between CODH and ACS also would be essential for acetyl genesis. However, this is not a requirement in *C. hydrogenoformans* growing autotrophically in the presence of high levels of CO, conditions under which most of the ACS exists as a monomeric protein free from CODH (24). Release of CO, as measured by isotopic exchange, takes place readily in both CODH/ACS and in ACS in the absence of contact with CODH. In both cases, the ACS N-terminal domain is available to interact with the A cluster to promote acetyl C–C bond cleavage, without which C–C bond fragmentation is severely compromised, as is evident from the catalytic properties of ACS₅₄₋ΔN. In ACDS, the escape of CO would be harmful to cells carrying out methanogenesis by disproportionation of acetate, due to the loss of low potential electrons needed to drive methyl group reduction to methane coupled with energy conservation. In the process of acetate cleavage, CO loss is prevented because the A cluster-acetyl species, formed upon reaction with acetyl-CoA, remains stable and unfragmented in the absence of a productive contact with the α₁,ε₂ CO₂–CDH protein. The requirement for the appropriate contact with the α₁,ε₂ protein to promote acetyl C–C bond scission ensures that CO is released only under conditions in which it can be efficiently transferred to the C cluster for subsequent rapid oxidation to CO₂. Because of the very high efficiency of CO transfer in ACDS, the predominant carbonyl exchange activity is observed with CO₂ rather than CO. The decline in CO₂, but not CO, exchange activity that takes place at a higher temperature, e.g. above around 50 °C, might be due to selective loss of C cluster function, or, alternatively, could result from conformational changes that lead to disruption of the β-α interface. Interdomain contacts within ACS are used to promote C–C bond fragmentation in bacteria, which has the potential to result in loss of CO regardless of whether or not a gating mechanism is active, particularly if the intersubunit interface between CODH and ACS becomes compromised, with such contacts not directly connected to acetyl C–C bond cleavage. With ACDS, transfer of CO across the intersubunit interface between acetyl-CoA synthase and CO dehydrogenase subunits takes place as well, but here these same intersubunit contacts, rather than interdomain interactions in ACS, also mediate acetyl C–C bond fragmentation. Thus, the structural arrangement in ACDS appears to be optimized for the transfer of CO to the C cluster to coincide with CO release from cluster A.

In conclusion, this work highlights the importance of protein conformational changes directly involved in specific steps of catalysis at the A cluster. Although conformational changes previously have been strongly suspected to be involved during catalysis by ACDS and CODH/ACS, the role of intersubunit/interdomain contacts in promoting cleavage of the acetyl C–C bond was unanticipated. Further advancements in understanding the mechanism of this step and in identification and characterization of other conformationally coupled steps in catalysis at the A cluster will be a focus of future investigations.

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