Nickel in Subunit β of the Acetyl-CoA Decarboxylase/Synthase Multienzyme Complex in Methanogens

CATALYTIC PROPERTIES AND EVIDENCE FOR A BINUCLEAR Ni-Ni SITE*

Received for publication, October 13, 2002, and in revised form, November 27, 2002
Published, JBC Papers in Press, December 2, 2002, DOI 10.1074/jbc.M210484200

Simonida Genic and David A. Grahame†
From the Department of Biochemistry and Molecular Biology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814-4799

The acetyl-CoA decarboxylase/synthase (ACDS) complex catalyzes the central reaction of acetyl C–C bond cleavage in methanogens growing on acetate and is also responsible for synthesis of acetyl units during growth on C-1 substrates. The ACDS β subunit contains nickel and an Fe/S center and reacts with acetyl-CoA forming an acetyl-enzyme intermediate presumably directly involved in acetyl C–C bond activation. To investigate the role of nickel in this process two forms of the Methanosarcina thermophila β subunit were overexpressed in anaerobically grown Escherichia coli. Both contained an Fe/S center but lacked nickel and were inactive in acetyl-enzyme formation in redox-dependent acetyltransferase assays. However, high activity developed during incubation with NiCl2. The native and nickel-reconstituted proteins both contained iron and nickel in a 2:1 ratio, with insignificant levels of other metals, including copper. Binding of nickel elicited marked changes in the UV-visible absorbance spectrum, with intact catalytic transfer bands indicating multiple thiolate ligation to nickel. The kinetics of nickel incorporation matched the time course for enzyme activation. Other divalent metal ions could not substitute for nickel in yielding catalytic activity. Acetyl-CoA was formed in reactions with CoA, CO, and methylcobalamin, directly demonstrating C–C activity. Acetyl-CoA was formed in reactions with CoA, as acetyl-enzyme formation in redox-dependent acetyltransferase assays. However, high activity developed during incubation with NiCl2. The native and nickel-reconstituted proteins both contained iron and nickel in a 2:1 ratio, with insignificant levels of other metals, including copper. Binding of nickel elicited marked changes in the UV-visible absorbance spectrum, with intact catalytic transfer bands indicating multiple thiolate ligation to nickel. The kinetics of nickel incorporation matched the time course for enzyme activation. Other divalent metal ions could not substitute for nickel in yielding catalytic activity. Acetyl-CoA was formed in reactions with CoA, CO, and methylcobalamin, directly demonstrating C–C bond activation by the β subunit in the absence of other ACDS subunits. Nickel was indispensable in this process too and was needed to form a characteristic EPR-detectable enzyme-carbonyl adduct in reactions with CO. In contrast to enzyme activation, EPR signal formation did not require addition of reducing agent, indicating indirect catalytic involvement of the paramagnetic species. Site-directed mutagenesis indicated that Cys-278 and Cys-280 coordinate nickel, with Cys-189 essential for Fe/S cluster formation. The results are consistent with an Ni₂[Fe₄S₄] arrangement at the active site. A mechanism for C–C bond activation is proposed that includes a specific role for the Fe₄S₄ center and accounts for the absolute requirement for nickel.

The methanogenic Archaea utilize a unique metabolic pathway for degradation of acetate under anaerobic conditions, and cleavage of acetate thereby accounts for a major proportion of the methane formed in the environment. The central reaction in this pathway is carried out by an unusual multienzyme complex, designated acetyl-CoA decarboxylase/synthase (ACDS), which contains five different polypeptide subunits and accounts for as much as 25% of the soluble protein in species such as Methanosarcina thermophila and Methanosarcina barkeri growing on acetate. The ACDS complex catalyzes cleavage of the acetyl C–C bond using the substrates acetyl-CoA and tetrahydroarasingartenin (H₂S₄Pt), a tetrahydrofolate analog which serves as methyl acceptor, and yields the products CoA, N⁵-methyltetrahydroarasingartenin, CO₂, and two reducing equivalents, as given in Reaction 1.

\[
\text{acetyl-CoA + H₂S₄Pt + H₂O} \rightleftharpoons \text{CoA} + \text{CH₃-H₂S₄Pt} + \text{CO₂} + 2\text{H}^+ + 2\text{e}^-
\]

**Reaction 1**

This overall reaction is made up of a series of partial reactions catalyzed by different protein subcomponents of the ACDS complex as shown in Scheme 1 (2). Acetyl-CoA binds to the β subunit, and under low redox potential conditions, as required for activity, transfers the acetyl group to a nuclophilic center on the enzyme forming an acetyl-enzyme species and releasing CoA (Scheme 1, *acetyl transfer*) (2, 3). The acetyl intermediate then undergoes C–C bond cleavage by a reaction that is presumed to involve metal-based decarboxylation and/or methyl group migration (Scheme 1, *cleavage*). The nascent methyl group is then transferred to a corrinoid cofactor present on the γδ subcomponent, which catalyzes subsequent methyl transfer to the substrate H₂S₄Pt (Scheme 1, *methyl transfer*) (2). The carbonyl group is oxidized to CO₂ by a process involving the αε CO dehydrogenase subcomponent, with regeneration of the reduced form of the β subunit. Previous studies on the β subunit have focused on a C-terminally truncated form of the protein purified from the native ACDS complex following partial proteolytic digestion (2–4).

The genes encoding the five ACDS subunits are arranged together in an operon along with one additional open reading frame in all species of Methanosarcina and in certain other methanogens as well (5–7, 9). The operon structure is shown in Scheme 2, with the designated genes and corresponding subunit molecular masses indicated for *M. thermophila* TM-1. The additional open reading frame encodes an accessory protein thought to be involved in nickel insertion, and nickel is present in both the large CO dehydrogenase subunit α (CdhA) and in the β subunit (CdhC) containing the active site for...
Nickel in Acetyl C–C Bond Cleavage by the ACDS β Subunit

MATERIALS AND METHODS

Overexpression and Purification of Wild Type and Mutant Forms of the ACDS β Subunit—Previously we determined the complete sequence of the ACDS operon from M. thermophila strain TM-1 (GenBank accession number AF173830). The gene for the full-length ACDS β subunit (cdhC) and a truncated form of the gene coding for a protein lacking 75 amino acids at the C terminus (cdhC*) were overexpressed using a modified version of the pQE60 (Qiagen) vector from which the His tag was deleted, designated pQE60ΔHis. Modifications to remove the His tag were as described previously (14). PCR amplifications of cdhC and cdhC* were performed using genomic DNA as template isolated from M. thermophila strain TM-1 with forward primers incorporating an NcoI site and reverse primers containing a BamHI site. PCR products were initially subcloned into the plasmid pCRII-TOPO (Invitrogen). After digestion with NcoI and BamHI and purification by agarose gel electrophoresis, the eluted fragments were cloned into the pQE60ΔHis vector, previously cut with NcoI and BamHI. E. coli strain M15 [pREP4] was then transformed with the pQE60ΔHis expression constructs and used for overexpression as described below. Site-directed mutagenesis of cdhC* was performed using the QuikChange MultiSite-directed Mutagenesis kit, according to instructions provided by the manufacturer (Stratagene). The sequences of all genetic constructs and bordering regions were verified by sequencing of isolated plasmid DNAs using the method of dideoxynucleotide termination with the ABI PRISM Big-Dye Terminator cycle sequencing kit version 3.0 with AmpliTaq DNA polymerase FS (Applied Biosystems).

Overexpression of the wild type and all mutant forms of the ACDS β subunit was carried out at 31 °C under strictly anaerobic conditions in Luria-Bertani medium containing 1% glucose and 40 mM sodium fumarate as electron acceptor. Hydrogen gas evolved during growth was allowed to escape through a 22-gauge syringe needle fitted to one of two ports constructed in the stopper used to seal the culture vessels. A second port provided the means to remove aliquots for monitoring the growth and for required additions. Cultures were inoculated at 1:100 using an anaerobic starter culture grown overnight. About 3 h later, after reaching an OD of 0.7–0.9, the cells were induced by addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (Research Organics) and immediately supplemented with iron, sulfide, and nickel, 100 μM Fe(NH₄)₂(SO₄)₂, 200 μM Na₂S, and 5 μM NiCl₂ added from sterile anaerobic stock solutions. The cultures were further supplemented after 2 and 4 h following induction. These subsequent additions were needed to provide sufficient iron, as was evident from small amounts of black iron sulfide that remained in the final cell pellets under these conditions, but not when either lower levels or fewer additions of iron and sulfide were employed. Cells were harvested by centrifugation under anaerobic conditions 5 h after induction (at an OD of 2.5), and the cell paste was frozen in liquid nitrogen.

Crude buffer-soluble extracts were prepared by French press cell lysis at 20,000 pounds/square inch at 4 °C of 6.5 g of cell paste resuspended in 30 ml of 50 mM Tris·HCl, 25 mM Na₂SO₄, 10% glycerol, pH 7.5, and the lysate was centrifuged at 34,000 × g for 20 min at 4 °C. These steps as well as all others subsequently used for purification of various forms of the ACDS β subunit were performed under anaerobic conditions. The supernatant obtained following centrifugation was applied at ~1.6 ml/min to a 12 × 2.5 cm diameter column of Q-Sepharose
Fast Flow (Amersham Biosciences) equilibrated in buffer A (50 mM Tris-SO₄, 25 mM Na₂SO₄, pH 7.5) at room temperature. The column was then washed with ~270 ml of buffer A and eluted with 400 ml of linear gradient of 0–0.4 M Na₂SO₄ in buffer A. Fractions (8 ml) were collected and analyzed by SDS-PAGE and for UV-visible absorbance. Fractions with the maximum absorbance ratio A₄₀₀ nm/A₂₈₀ nm and displaying the highest purity on SDS gels were pooled, concentrated by ultrafiltration using an Amicon stirred cell, and dialyzed against Millipore water (2) and stored at −80 °C.

**Nickel Reconstitution**—Purified recombinant CdhC at a concentration of 7–35 μM that contained iron but lacked nickel was reconstituted with Ni⁷⁺ by incubation under anaerobic conditions in the presence of 10 μM NiCl₂ in 50 mM Hepes, pH 7.2, at room temperature. Aliquots were removed at different times, diluted with sufficient water to give 1.4 μM CdhC, and immediately assayed by transferring 10 μl of the diluted sample to 110 μl of acetyltransferase assay solution, and the reactions were completed as described below. Large scale reconstitution of the enzyme employed 80–80 μM CdhC and 180 μM NiCl₂ in reaction volumes of up to 12 ml. Sufficient time was allowed for metal incorporation (as determined in separate trials), and the reaction mixtures were thereafter concentrated to 1.3 ml by ultrafiltration on an Amicon YM30 ultrafiltration membrane, 44.5 mm diameter. Excess Ni⁷⁺ was removed by applying the concentrated enzyme to a 1.5-cm diameter, 10-ml bed volume column of Sephadex G-25 equilibrated in 40 mM sodium acetate buffer, pH 4.5, + 0.1 M NaCl. Ni⁷⁺ was monitored by absorbance at 280 nm (dotted line) and was monitored by absorbance at 280 nm (solid line) and 400 nm (dotted line). Fractions 49–52 were pooled (QAE pool). The inset shows SDS-PAGE analysis of total protein in E. coli extracts made prior to and following induction with isopropyl-β-D-thiogalactopyranoside (IPTG) and the purified protein obtained after chromatography.

UV-visible scans were recorded on a Hewlett-Packard 8452A spectrophotometer set up inside the anaerobic chamber. After addition of each aliquot of divalent metal solution, scans were recorded over time until no further changes were observed in the spectrum.

**RESULTS**

**Overexpression and Purification of the ACDS β Subunit**—The gene encoding the β subunit of the ACDS complex from *M. thermophila* was overexpressed in two separate forms in *E. coli*. One of these was the full-length 472-amino acid protein, CdhC, and the other was a 397-amino acid form truncated at the C terminus, CdhC*. CdhC* includes all major regions of conservation among β subunit homologs and is about 30 amino acids smaller than the estimated size of the native protein isolated in truncated form following disruption of the ACDS complex by partial proteolysis (2,3). SDS-gel electrophoresis showed that similar amounts of CdhC and CdhC* were produced over time after induction of *E. coli* cultures grown under anaerobic conditions, as described under “Materials and Methods.” However, CdhC was considerably less soluble than CdhC* as found by analysis of cell extracts and fractions from subsequent purification procedures. Yields of the purified proteins were around 3 mg per g of cell paste for CdhC versus 20–22 mg per g of cells for CdhC*. As shown in Fig. 1, Q-Sepharose anion exchange chromatography of the supernatant obtained from an extract of *E. coli* expressing CdhC* resulted in elution of a major peak of protein with absorbance at 280 and at 400 nm (due to Fe/S clusters), with fractions containing highly purified CdhC*.

**Activation of a Nickel-deficient Form of the Enzyme by Reconstitution with Nickel**—Samples of purified CdhC and CdhC* were assayed for acetyltransferase activity to determine their ability to react with acetyl-CoA under low redox potential conditions generating an acetyl-enzyme intermediate. As isolated, both recombinant proteins showed very low levels of acetyltransferase activity, less than 1% of the specific activity of the

---

**Nickel in Cysteine C Bond Cleavage by the ACDS β Subunit**

FIG. 1. Overexpression of the ACDS β subunit and purification by anion exchange chromatography. A buffer-soluble extract from 6.5 g of *E. coli* grown under anaerobic conditions and induced to express CdhC* was subjected to anion exchange chromatography on Q-Sepharose as described under “Materials and Methods.” Elution was carried out by application of a linear gradient of sodium sulfate (dashed line) and was monitored by absorbance at 280 nm (solid line) and 400 nm (dotted line). Fractions 49–52 were pooled (QAE pool). The inset shows SDS-PAGE analysis of total protein in *E. coli* extracts made prior to and following induction with isopropyl-β-D-thiogalactopyranoside (IPTG) and the purified protein obtained after chromatography.

UV-visible scans were recorded on a Hewlett-Packard 8452A spectrophotometer set up inside the anaerobic chamber. After addition of each aliquot of divalent metal solution, scans were recorded over time until no further changes were observed in the spectrum.
Nickel in Acetyl C-C Bond Cleavage by the ACDS β Subunit

Fig. 2. Nickel is required for redox-dependent acetyltransferase activity of recombinant ACDS β subunit. The time-dependent increase of acetyltransferase activity was observed in samples of purified CdhC during incubation with NiCl₂. Incubations were carried out as described for nickel reconstitution under "Materials and Methods," and aliquots were removed over time, diluted, and immediately assayed for acetyltransferase activity. HPLC was used to determine the extent of acetyl transfer from acetyl-CoA to the acceptor substrate 3'-dephospho-CoA (open circles) for calculation of acetyltransferase reaction rates. The nickel-reconstituted enzyme, obtained after 4 h of incubation with NiCl₂, showed no activity when reducing agent (TiCl₃) was omitted from the acetyltransferase assay mixture (solid square). The solid line is drawn for activation of the enzyme according to an apparent first order process, assuming that the concentration of free Ni²⁺ remains constant during reactivation because NiCl₂ is present in large excess over CdhC.

Native β subunit. However, high activity was found for both proteins, CdhC and CdhC*, after incubation with Ni²⁺. A progressive increase in activity was observed over time, reaching a maximum after several hours of incubation of CdhC, 7.1 μM, with NiCl₂, 100 μM, as shown in Fig. 2. Activation of the enzyme under these conditions in which [Ni²⁺] > [apoCdhC] followed pseudo-first order kinetics with an apparent half-time of about 32 min. Activity in the assay showed absolute dependence on low redox potential, as expected, because it is a characteristic property of the subunit isolated from the ACDS complex (3). Turnover rates for acetyl transfer were up to 1250 min⁻¹ for CdhC and 4500 min⁻¹ for CdhC*, as compared with the value of 3100 min⁻¹ (3) for the native β subunit isolated from the ACDS complex.

Plasma emission spectroscopic analyses on samples of the β subunit isolated from the ACDS complex and on samples from large-scale nickel reconstitution of CdhC* showed significant levels of iron and nickel but only trace amounts of other metals. In particular, levels of copper were extremely low, as shown in Fig. 3. Notably, the measured iron/nickel ratio was 1.9 for nickel-reconstituted CdhC*, and a similar value of 2.2 was observed for the isolated β subunit. These results indicate that binding of nickel is required for activation of the enzyme and that the enzyme contains iron and nickel in a ratio of ~2:1. Therefore, direct spectrophotometric methods were developed to examine further the interaction of nickel with the β subunit.

Binding of Nickel to the β Subunit Results in Characteristic UV-visible Changes and Proceeds with Defined Stoichiometry and Kinetics—Preparations of the brown, iron-containing, nickel-deficient β subunit (CdhC and CdhC*) exhibited absorbance at around 400 nm typical of a simple Fe/S protein with minimum values for the ratio A₂₈₀nm/A₄₀₀nm of around 5.1. Upon addition of NiCl₂ a marked, time-dependent change was observed in the UV-visible spectrum of the recombinant protein resulting in a final spectrum closely resembling that of the subunit isolated from the ACDS complex. Difference spectra obtained by subtracting the spectrum of the apoenzyme from that of the nickel-reconstituted protein showed a sharp peak of absorbance increase centered around 332–336 nm and a broader peak at around 550 nm, as shown in Fig. 4A (middle panel). A third peak with the highest intensity was found at 262 nm (not shown). These features are attributed to ligand-to-metal charge transfer absorption formed when nickel binds to the enzyme, with multiple S ligation indicated by the high values of molar absorptivity. In addition, similar features were observed for the enzyme after incubation with other divalent first row transition metals including Co²⁺ and Cu²⁺, Fig. 4A (top and bottom panels), with lower intensity d-d transitions at 680 and 720 nm found in the spectrum of the Co²⁺-substituted protein.

Fig. 3. Metal content of fractions containing the native ACDS β subunit and nickel-reconstituted CdhC* exhibit an iron/nickel ratio close to 2:1 with insignificant levels of copper. Plasma emission spectroscopic analysis (inductively coupled plasma-atomic emission spectroscopy) of metal content was performed on fractions obtained by ion exchange chromatographic separation of the β subunit from the ACDS complex (A), and on fractions obtained from gel filtration following large scale nickel reconstitution of CdhC* (B). Nickel reconstitution, involving gel filtration on Sephadex G-25, was performed as described under "Materials and Methods." Isolation of the β subunit from the ACDS complex employed chromatography on a Mono Q HR 10/10 column following partial proteolytic digestion of the ACDS complex with chymotrypsin and was carried out by methods described previously (3). Fractions 65–85 correspond to a region containing 0.58–0.83 mM Na₂SO₄ in the linear salt gradient used for elution.

The kinetics of nickel binding to the β subunit were monitored spectrophotometrically by following the increase in absorbance over time after a single addition of excess Ni²⁺ to CdhC*. Under conditions in which the concentration of CdhC* was significant relative to [Ni²⁺], the reaction followed second order kinetics in which the rate was dependent upon the concentration of both apoenzyme and [Ni²⁺], with an apparent second order rate constant of ~3 × 10⁻⁴ M⁻¹ min⁻¹, as shown...
in Fig. 5. These results agree with the time course for activation of the enzyme in Fig. 2 in which $[\text{Ni}^{2+}/\text{H}]$ corresponds to pseudo-first order conditions, indicating that activation is limited by the rate of nickel binding. Nickel reconstitution at pH 7.2 (Fig. 5) was much faster than at pH 6.5 but markedly slower than the reaction at pH 8.0. The magnitude of these differences was consistent with deprotonation of more than one cysteine thiol group in metal ion coordination and is in accordance with results described later from site-directed mutagenesis.

The ACDS Subunit Catalyzes Nickel-dependent Acetyl C–C Bond Activation in the Absence of All Other Protein Subunits—Methods were developed to assay for net synthesis of acetyl-CoA from one-carbon precursors using the purified recombinant subunit as the only protein component. Reactions contained nickel-reconstituted subunit protein and CoA under an atmosphere of CO, serving as the precursor of the carbonyl group of acetyl-CoA, in the presence of methylcobalamin as a methyl group donor and Ti$_3$ citrate as a reducing agent. The results of HPLC analyses of samples removed over time showed that acetyl-CoA was indeed formed in the absence of all other ACDS subunit proteins (Fig. 6). Besides methylcobalamin, methyl iodide was also tested, but no evidence was found for activity of methyl iodide as a methyl group donor. The

Fig. 4 A, spectrophotometric changes occurring upon binding of Ni$^{2+}$ and other divalent metal ions to the ACDS $\beta$ subunit. Difference spectra were obtained by subtraction of the spectrum of CdhC* taken before addition of metal ions from that recorded after metal ion addition corrected for dilution. Molar absorptivity was calculated on the basis of absorbance change per mol of metal ion. B, stoichiometry of metal binding to the $\beta$ subunit. Spectrophotometric measurements were made on samples of CdhC*, 16.4 nmol, determined on the basis of one-fourth of the measured iron content of the protein. Titrations were carried out with the indicated metal ions (6 nmol per addition, except for the last three additions of NiCl$_2$ which contained 12 nmol each), as described under “Materials and Methods.” The dashed lines are drawn from fits of the absorbance values to the quadratic equation for binding at a single site or class of sites. The solid lines show intersection at the apparent equivalence points: Co$^{2+}$ 1.4, Ni$^{2+}$ 1.9, and Cu$^{2+}$ 1.8 eq/mol CdhC*.

Fig. 5 Apparent second order kinetics of Ni$^{2+}$ binding to the $\beta$ subunit in vitro. Spectrophotometric traces were recorded during nickel incorporation into samples of CdhC*, 12 $\mu$M (24 $\mu$M total nickel sites), initiated by addition of 49.5 $\mu$M NiCl$_2$ (open circles) or 98 $\mu$M NiCl$_2$ (open squares). The solid lines are drawn to the best fits of the data to the second order rate equation describing the progress of the reaction shown in the inset with $k = 3.3 \times 10^{-4}$ and $2.8 \times 10^{-4}$ $\mu$M$^{-1}$ min$^{-1}$ for the reactions with 49.5 and 98 $\mu$M NiCl$_2$, respectively.
rate of acetyl-CoA synthesis (Fig. 6) was only about 1/3000th of the rate catalyzed by the native ACDS complex. However, because the $\beta$ subunit protein was expressed in E. coli, which lacks ACDS and other CO dehydrogenase proteins, these levels of activity cannot be due to contamination from other ACDS proteins. Thus, the results reliably demonstrate that the active site for acetyl-CoA cleavage and C–C bond activation resides on the $\beta$ subunit. Furthermore, as shown in Fig. 6, formation of acetyl-CoA was observed only with nickel-reconstituted samples of the protein and was undetectable with samples that had not been preincubated with $\text{Ni}^{2+}$, which demonstrates that nickel is essential for net acetyl-CoA synthesis activity.

*Formation of a Characteristic A-cluster EPR Spectrum by Reaction of the ACDS $\beta$ Subunit with CO Requires $\text{Ni}^{2+}$ and Occurs in the Absence of Reducing Agents*—Preparations of the isolated $\beta$ subunit were tested both in the absence of nickel and after nickel reconstitution for the formation of an EPR-detectable adduct in the presence of CO. As shown in Fig. 7, reaction of the nickel-reconstituted $\beta$ subunit with CO generated a strong signal with $g$ values and power saturation characteristics of an $S = 1/2$ system similar to those found for the A-cluster NiFeC species observed in the native methanogen ACDS complex (17, 18) and clostridial CO dehydrogenase/acetyl-CoA synthase (19, 20). The signal was observed only in samples of the nickel-reconstituted protein and was undetectable in preparations of the nickel-deficient enzyme. Notably, exposure of the nickel-deficient enzyme to CO resulted in rapid bleaching of the Fe/S center absorbance, which was not found for the holoenzyme. This finding indicates that CO reacts with the Fe/S center in the apoenzyme causing its destruction in the absence of nickel and suggests that nickel plays a structural role in addition to its catalytic function, modulating the properties of the Fe/S center and preventing permanent alteration by strong ligands such as CO.

In contrast to previous studies (11, 12), these results show that addition of a reducing agent was not required to form the NiFeC species. Samples identical to those employed in Fig. 7 were also prepared except that 1 mM Ti$^{3+}$-citrate was included as a reducing agent during the reaction with CO. Under these conditions the NiFeC signal intensity was equivalent to that observed in the absence of reducing agent. These results indicate that reduction of the enzyme is not required for reaction with CO to generate the A center NiFeC signal and that the paramagnetic enzyme-CO adduct contains nickel in the Ni$^{2+}$ oxidation state.

*Site-directed Mutagenesis Indicates That Amino Acid Residues Cys-278 and Cys-280 Function as Ligands to Nickel, and Cys-189 Is Required for Fe/S Cluster Formation*—A comparison of available DNA sequence data indicated that there are 6 cysteine, 3 histidine, and 2 tryptophan residues conserved in the $\beta$ subunit, with a number of conserved proline residues found at positions in close proximity, as shown in Scheme 3. To obtain information on which of the histidine or cysteine residues may function as ligands to nickel and/or iron, several single site-directed mutants of CdhC* were prepared, including C189S, C278S, C280S, and H394N, and overexpressed and purified as described for the wild type CdhC*. As shown in Fig. 8, substitution of asparagine for histidine at position 394, an invariant residue near the end of the last region of conservation among all $\beta$ subunit homologs, had little or no effect on the UV-visible spectrum of the protein or on the process of nickel binding as judged from the difference spectrum obtained following incubation with Ni$^{2+}$. In contrast, replacement of cysteine residues at either position 278 or 280 with serine resulted in a pronounced alteration of the difference spectrum with a complete lack of the peak at 550 nm and marked attenuation of the absorbance around 330–340 nm (Fig. 8, right). This demonstrates that both Cys-278 and Cys-280 are essential for proper binding of nickel and indicates that coordination of nickel involves thiolate ligation from both cysteine residues.

**Table 3.** Conserved cysteine, histidine, proline, and tryptophan residues in the ACDS $\beta$ subunit.

| Residue | Function |
|---------|----------|
| Cys-189 | Required for Fe/S cluster formation |
| Cys-278 | Function as ligands to nickel |
| Cys-280 | Function as ligands to nickel |
| His-394 | Invariant residue near the end of the last region of conservation among all $\beta$ subunit homologs |

---

**Fig. 6.** The ACDS $\beta$ subunit catalyzes acetyl C–C bond formation in the absence of all other ACDS subunit proteins. Acetyl-CoA formation was quantified by HPLC analysis of samples removed as a function of time from reaction mixtures set up as indicated under “Materials and Methods.” Results using recombinant protein as isolated (−Ni) are compared with reactions employing the same amount of nickel-deficient enzyme (−Ni). The level of CdhC used was 3.9 μM, indicating less than a single turnover; however, reactions carried out for longer time periods showed levels of product corresponding to more than a single turnover.

**Fig. 7.** EPR spectra of the recombinant ACDS $\beta$ subunit prior to and following nickel reconstitution. Reduction of Ni$^{2+}$ at the active site is not required to form the NiFeC signal at the A center. Samples of the ACDS $\beta$ subunit (CdhC*+, 240 μg) as isolated in the apoenzyme form (−Ni) or after nickel reconstitution (+Ni) were incubated in 40 mM HEPES buffer at pH 7.2 for 30 min under an atmosphere of 100% CO, transferred to quartz EPR tubes, and frozen in liquid N$_2$ prior to EPR analysis. Note that no reagents capable of potentially reducing Ni$^{2+}$ to the Ni$^{+}$ oxidation state such as Ti$^{3+}$–citrate or dithionite were added. Spectra were recorded at 90 K on a Varian E 9 X-band EPR spectrometer with 10-milliwatt microwave power at 9.120 GHz, with 8 gauss field modulation at 100 kHz and a receiver gain of 1600 (+Ni) or 2000 (−Ni).

**Scheme 3.** Conserved cysteine, histidine, proline, and tryptophan residues in the ACDS $\beta$ subunit. All conserved Cys, His, and Trp residues are shown, but only proximal conserved proline residues are indicated.
Regardless of the major effects on the formation of the nickel site, the UV-visible spectra of the C278S and C280S mutants in the absence of nickel were virtually identical to that of the nickel-deficient wild type protein (Fig. 8, left), indicating that Cys-189 is required for proper coordination of iron in the assembly of the Fe/S cluster. Difference spectra recorded after addition of NiCl$_2$ were unique, showing that nickel was bound to the C189S mutant with substantial thiolate ligation but in an altered form compared with the wild type protein. This demonstrates that the presence of the Fe/S center has a significant influence on the coordination environment of the nickel-binding site.

Acetyltransferase activity of the mutants was measured after incubation with Ni$^{2+}$ and corresponded to the results from UV-visible spectroscopic analyses. No significant level of activity was found with the mutants C278S or C280S, both lacking a thiol group needed for coordination to nickel (Table I). The mutant C189S in which the Fe/S center is absent was also inactive. However, high levels of activity were found with the mutant H394N (Table I) which exhibited native Fe/S and nickel binding characteristics (Fig. 8), despite the fact that His-394 is highly conserved.

![Fig. 8. Single site-directed cysteine-to-serine mutations implicate Cys-278 and Cys-280 as direct ligands to nickel and demonstrate that Cys-189 is required for assembly of the Fe/S center.](http://www.jbc.org/)

**DISCUSSION**

To investigate the role of the ACDS $\beta$ subunit in activation of the acetyl C–C bond, two forms of the protein were overexpressed in *E. coli* under anaerobic growth conditions and purified and characterized. One of these was the 472-amino acid full-length ACDS $\beta$ subunit CdhC, and the other was a truncated form of the protein lacking 75 amino acid residues at the C terminus designated CdhC*, designed to mimic the form of the $\beta$ subunit isolated previously by partial proteolytic digestion of the ACDS complex (2). CdhC* was obtained in higher yields than CdhC and was considerably more soluble as indicated by the finding that significant amounts of CdhC but not CdhC* were found in the pellet obtained after centrifugation of *E. coli* extracts and were present in cloudy fractions that did not bind to the Q-Sepharose column used for purification. The increase in solubility resulting from truncation of the C-terminal region is consistent with previous findings that proteolytic removal of the C terminus takes place at an early stage during dissociation of the ACDS complex by limited proteolytic digestion (2). Loss of the C terminus also correlates closely with a marked decline in the ability of the complex to catalyze net synthesis of acetyl-CoA (see Fig. 4 in Ref. 2). Thus, it is suggested that the C-terminal region may function as a docking domain in the ACDS complex needed for protein-protein interactions and proper communication between subunits (while promoting the non-physiological aggregation of the full-length protein when expressed unaccompanied by the other ACDS subunits).

All three proteins CdhC, CdhC*, and the native $\beta$ subunit isolated from the ACDS complex had similar Fe/S cluster contents, as judged by comparisons of their UV-visible spectra. Metal analyses showed that CdhC and CdhC* lacked nickel under the conditions used for their expression and purification, and both proteins were inactive in catalysis of acetyl group transfer at low redox potential. Nevertheless, high enzymatic activity was observed following incubation with Ni$^{2+}$ (Fig. 2) demonstrating that the nickel-reconstituted recombinant enzyme is able to form an acetyl-enzyme intermediate in reaction with acetyl-CoA and possesses redox properties comparable with those of the native protein. Although NiCl$_2$ was added during expression, one explanation for why nickel was not incorporated into the protein in *E. coli* might be that levels of the metal ion were limited due to competition for insertion into other nickel-containing proteins such as hydrogenases needed for anaerobic growth. However, such competition should be overcome at least in part by the large amounts of $\beta$ subunit protein formed (Fig. 1), and the finding of less than 1% active protein with an undetectably low level of nickel suggests that other factors, lacking in *E. coli*, are needed for specific incorporation of nickel into the methanogen $\beta$ subunit. Indeed, the strong dependence on Ni$^{2+}$ concentration in the apparent second order kinetics (Fig. 5) indicates that the rate of nickel insertion would be too low to support formation of the active

![Fig. 8](http://www.jbc.org/)

**TABLE I**

| $\beta$ mutant | Fe/S center formation | Nickel binding$^a$ | Acetyltransferase-specific activity$^b$ |
|----------------|-----------------------|-------------------|--------------------------------------|
| WT             | +                     | +                 | 100                                  |
| H3894N         | +                     | +                 | 73                                   |
| C280S          | +                     | –                 | 0.07                                 |
| C278S          | +                     | –                 | 0.04                                 |
| C189S          | –                     | Altered           | 0.08                                 |

$^a$ Strong charge transfer band intensity indicating multiple thiolate ligation.

$^b$ Wild type (WT) activity was 108.3 μmol/min/mg.
Nickel in Acetyl C–C Bond Cleavage by the ACDS β Subunit

Acetyl C–C Bond Activation—Direct evidence that the ACDS β subunit contains the site for activation of the acetyl C–C bond was obtained from the finding that significant levels of acetyl-CoA were formed in reactions of the β subunit with CoA, CO, and methylcobalamin in the absence of all other ACDS protein components. As shown in Fig. 6, at low redox potential acetyl-CoA was generated over time from CoA and the one-carbon precursors CO and methylcobalamin. Previously, it was shown unequivocally that the ACDS β subunit is responsible for activation of the C–S bond of acetyl-CoA (2, 3); however, only indirect evidence for activation of the C–C bond was available (2, 4). The results presented here demonstrate for the first time that the β subunit active site indeed has the intrinsic chemical properties needed for C–C bond activation. These results are in agreement with recent findings on the homologous subunit from the clostridial CODH/ACS enzyme (12) and further show that the β subunit is capable of interacting with the free methyl-B12 cofactor, not bound to a corrinoid protein. This indicates that the β subunit forms a complex with the corrinoid cofactor, to a significant degree, even in the absence of corrinoid protein. Binding of methylated corrinoid would thereby increase the effective concentration of methyl groups at the active site; thus, the failure to form acetyl-CoA in reactions using methyl iodide as methyl donor may result from an inability of methyl iodide to bind to the enzyme. If methylation of the enzyme is rate-limiting to begin with, then inefficient binding of a methyl donor would be especially detrimental.

Absolute Requirement for Nickel—Binding of nickel to the β subunit was a conditio sine qua non for each of the four major characteristics investigated as follows: acetyltransferase activity involving formation of an acetyl-enzyme intermediate (Fig. 2); the ability to carry out C–C bond activation as indicated by net synthesis of acetyl-CoA (Fig. 6); formation of an EPR-detectable enzyme-CO adduct (Fig. 7); and the ability to generate a UV-visible spectrum characteristic of the native enzyme (Fig. 4A and Fig. 8). The binding of nickel occurs with a stoichiometry of 2 eq/mol protein, as shown by values of the iron/nickel ratio close to 2:1 by inductively coupled plasma-atomic emission spectroscopy metal analyses of the native and recombinant proteins (Fig. 3) and by direct titration of the apoenzyme (Fig. 4B). Divalent metal ions other than Ni²⁺ exhibited comparable stoichiometries in titrations and generated UV-visible spectral changes related to those produced by Ni²⁺. However, none of the other metal ions tested (Mn²⁺, Fe²⁺, Co²⁺, and Cu²⁺) was able to substitute for nickel in formation of a catalytically active enzyme. This all-or-none quality indicates that remarkably specific electronic structure and reactivity are demanded for the metal ion to participate successfully in the catalytic process, properties possessed only by nickel.

Ni-Fe/S Center—The recombinant β subunit expressed in E. coli contained all of the ligands needed for nickel binding to generate a functionally active Ni-Fe/S site, because no additions other than NiCl₂ were required to reconstitute activity. Multiple thiolate ligation to each nickel was indicated by the high values of molar absorbptivity of the charge transfer bands formed upon nickel reconstitution. Characteristic UV-visible difference spectra obtained for binding of three different metals (Co²⁺, Ni²⁺, and Cu²⁺) also implied multiple thiolate ligation, consistent with findings from extended x-ray absorption fine structure (EXAFS) studies on the related clostridial CODH/ACS α metallosubunit of nickel coordination by more than one sulfur ligand (21, 22). The strong effect of pH on the rate of nickel incorporation was also consistent with deprotonation of more than one thiol group in the process of nickel binding, and site-directed mutagenesis (Fig. 8 and Table I) identified Cys-278 and Cys-280 as ligands potentially involved in direct coordination to nickel. In addition, the results may be viewed in relation to the recently published crystallographic structure of the A center of clostridial CODH/ACS (13), in which an Fe₃S₇ cluster is bridged to a binuclear metal site with two cysteine residues in shared coordination to both metal ions, corresponding to Cys-278 and Cys-280 in the methanogen β subunit. The binuclear center of CODH/ACS contained nickel, but the metal ion proximal to the Fe/S center was suggested to be copper. In contrast, our results indicate that copper is not a component of the Ni-Fe/S center in the β subunit, because only trace levels of copper are detectable (Fig. 3). Rather, the data presented here are consistent with a structure for the Ni-Fe/S center similar to that of the clostridial CODH/ACS, but containing a binuclear metal site with nickel residing at both positions in an Ni₄[Fe₃S₇] arrangement.

In some respects the Fe₃S₇ and nickel-nickel subsites appear to act relatively independently, e.g. altered nickel binding in the C278S and C280S mutants had little effect on the spectrum of the Fe/S center itself (Fig. 8). However, a notable exception was revealed in reactions with CO, which provided evidence for interaction of the nickel and Fe/S sites. We found that CO caused a rapid bleaching of the nickel-deficient enzyme, not observed in reactions with the nickel-reconstituted protein, suggesting decomposition of the Fe/S center in the absence of
nickel by reaction of the strong ligand CO with iron in the Fe/S center. This provides the first evidence to date that nickel modulates the reactivity of the Fe/S center and would be consistent with bridging of the Fe/S center to nickel at the proximal site, by analogy to the A center structure in CODH/ACS (13).

Site-directed mutagenesis experiments also provided information on groups involved at the Fe/S center. The complete absence of an Fe/S center in the C189S mutant (Fig. 8) implied that Cys-189 is directly coordinated to one of the iron atoms in the Fe/S center. Although mutations that strongly affected the binding of nickel had marginal effects on the UV-visible spectrum of the Fe/S center, the absence of an Fe/S center in C189S strongly influenced the nickel-binding characteristics as observed by the pronounced alteration of the difference spectrum formed upon addition of Ni2+ (Fig. 8). These changes may result from local conformational rearrangements and displacement of groups normally involved in coordination of nickel. Alternatively, the altered nickel binding properties in the C189S mutant may reflect the loss of interaction between the Fe/S cluster and the nickel-nickel site needed for proper coordination of the binuclear nickel center and formation of its characteristic UV-visible spectrum. The close proximity of Cys-189 to Cys-192 indicates that Cys-192 also may be coordinated to the Fe/S cluster, and these residues correspond to two of the four cysteines that are coordinated to the Fe/S center in the structure of the A center in CODH/ACS (13). Because an Fe/S cluster is not formed even partially in the C189S mutant, Cys-189 along with another group such as Cys-192 may provide the site of nucleation for the initial step in Fe/S cluster insertion.

Mechanism of C–C Bond Activation—The mechanism of acetyl C–C bond activation by the β subunit can be interpreted on the basis of the present results together with previous findings on the β subunit isolated from the methanogen ACDS complex and information available from studies on the homologous subunit from clostridial CODH/ACS. It has been established that activation of the enzyme by one-electron reduction is required for acetyltransferase activity, involving reaction with acetyl-CoA to form an acetyl intermediate. The enzyme nucleophile needed to generate this intermediate is depicted as Ni(I) in Scheme 4. The Ni(I) species would be formed by reduction of one of the nickel ions at the binuclear site in the as-isolated, all Ni(II)-enzyme. Formation of the acetyl-enzyme intermediate would occur by attack of Ni(I) on the carbonyl group of acetyl-CoA. In the case of the copper-substituted enzyme, reduction to the Cu(I) level with its d10 closed shell configuration may be readily accomplished; however, subsequent action of Cu(I) as a nucleophile would be highly unfavorable. Therefore, it is not surprising that the copper-enzyme is completely inactive. Reduction of the Co(II)-substituted enzyme would be substantially more difficult, but if a Co(I) species was formed, then the ensuing nucleophilic attack would yield an acetyl-Co(III)-enzyme potentially too stable to undergo rapidly the reverse reaction, i.e. attack by CoA (or homolog 3′-dephospho-CoA) as required for the overall exchange. Thus, Ni(I) provides sufficient nucleophilicity without generating unreactive products, and the indicated Ni(III) intermediates should be relatively high energy species. Accordingly, we propose that the role of the Fe/S center is to stabilize the nascent Ni(III) adducts formed in reactions a and b (Scheme 4) by converting them to the more stable Ni(II) forms. This would also serve to maintain electron density on nickel, as required for interaction with the α-acid acceptor CO in the step of carbonation/decarbonylation involving migration of the methyl group, reaction c. In addition to migratory steps that may occur within the coordination sphere of a single nickel, the presence of a binuclear nickel-nickel center opens the possibility for migration of species between the two nickel ions distinguished by different coordination environments. The overall cleavage of acetyl-CoA would require the reverse of reaction a, i.e. transfer of the methyl group from the β subunit to the corrinoid protein by nucleophilic attack of the Co(I) corrinoid on the bound methyl group. Similarly, the reverse of reaction b is needed for acetyltransferase activity through nucleophilic attack of CoA on the β subunit acetyl group. The Fe/S center in its oxidized form would facilitate both processes, generating the corresponding reactive Ni(III) species by accepting an electron in the equilibrium internal electron transfer steps indicated in Scheme 4.

The findings from EPR analyses of reactions of the β subunit with CO are relevant for understanding the role of the paramagnetic NiFeC species in the mechanism of C–C bond activation. In Scheme 4, reaction d, CO is indicated to react with the enzyme at the Ni(II) level, forming an EPR active species not directly involved in the catalytic cycle. This is based on the finding that the active enzyme obtained following reconstitution with NiCl2 contains nickel in the Ni(II) form and that addition of a reducing agent was not needed to generate the NiFeC EPR signal (Fig. 7). This finding contrasts with the apparent requirement for reducing agent reported for NiFeC signal formation in the isolated α metallosubunit from clostridial CODH/ACS (11, 12). The fact that reduction is not required to form the EPR signal with the ACDS β subunit raises questions about the relevance of proposed species such as Aα–CO and Ni3+.CO. Theoretically, one-electron reduction of the paramagnetic enzyme-CO adduct could convert it directly to an EPR silent form involved in the catalytic cycle. However, we found that the EPR signal intensity was unchanged by the presence of a strong reductant (1 mM Ti3+-citrate). This indicates that disappearance of the NiFeC signal upon reaction with an appropriate methyl donor or during catalytic turnover (8, 18, 23) takes place by the reversal of reaction d, with the shift in equilibrium becoming favorable only in the presence of substrates.

In conclusion, the present work highlights the importance of the β subunit in the overall process of acetyl-CoA cleavage by the methanogen ACDS complex. The focus on characterization of the spectroscopic and catalytic properties of nickel as part of an unusual Ni-Fe/S cluster at the active center of the β subunit emphasizes its role in activation of the acetyl C–C bond.

REFERENCES
1. Grahame, D. A. (1991) J. Biol. Chem. 266, 22227–22233
2. Grahame, D. A., and DeMoll, E. (1996) J. Biol. Chem. 271, 6352–6358
3. Bhakar, R., DeMoll, E., and Grahame, D. A. (1998) Biochemistry 37, 14491–14499
4. Murakami, E., and Ragsdale, S. W. (2000) J. Biol. Chem. 275, 4699–4707
5. Maupin-Purlow, J. A., and Ferry, J. G. (1996) J. Bacteriol. 178, 6849–6856
6. Galagian, J. E., Nusbaum, C., Roy, A., Endrizzi, M. G., Macdonald, P., FitzHugh, W., Calvo, S., Engels, R., Smursov, S., Atinour, D., Brown, A., Albers, N., Nayler, J., Stange-Thomann, N., Detrellano, K., Johnson, R., Linton, J., McEwan, P., McKernan, K., Talasans, J., Turrell, A., Ye, W., Zimmer, A., Barber, R. D., Cai, I., Graham, D. E., Graham, D. A., Guss, A. M., Heidrich, R., Ingram-Smith, C., Kuettner, H. C., Kryczy, J. A., Leal, J. A., Li, W., Liu, J., Mukhopadhyay, B., Reeve, J. N., Smith, K., Springer, T. A., Umayam, L. A., White, O., White, R. H., Conway de Macario, E., Ferry, J. G., Jarrell, K. F., Jing, H., Macario, A. J. L., Paulsen, I., Pritchett, M., Sowers, K. R., Swanson, R. V., Zinder, S. H., Lander, E., Metcalfe, W. F., and Birren, B. (2002) Genome Res. 12, 532–542
7. Deppenmeier, U., Johann, A., Hartsch, T., Merkl, R., Martinez-Arias, R., Henne, A., Wieder, A., Baumgarten, M., I. Lienard, T., Christmann, A., Ilonmeko, M., Steckel, S., Bhattacharyya, A., Lykidis, A., Overbeck, R., Klen, H. P., Gunsalu, R. P., Fritz, H. J., and Gottschalk, G. (2002) J. Mol. Microbiol. Biotechnol. 4, 453–461
8. Servellati, J., Kumar, M., and Ragsdale, S. W. (2002) Biochemistry 41, 1807–1819
9. Smith, D. B., Doucette-Stamm, L. A., Deleugher, C., Lee, H., Dubois, J., Aldredge, T., Bashirzadeh, R., Blakeley, D., Cook, G., Gilbert, K., Harrison, D., Hoang, L., Reagle, P., Lumm, W., Pothier, B., Qiu, D., Spadafora, R., Vicare, R., Wang, Y., Wierzbowski, J., Gibson, R., Jiwani, N., Caruso, A.
Nickel in Acetyl C–C Bond Cleavage by the ACDS β Subunit

10. Xia, J., and Lindahl, P. A. (1995) Biochemistry 34, 6037–6042
11. Xia, J., and Lindahl, P. A. (1996) J. Am. Chem. Soc. 118, 483–484
12. Loke, H.-K., Tan, X., and Lindahl, P. A. (2002) J. Am. Chem. Soc. 124, 8667–8672
13. Doukov, T. I., Iverson, T. M., Seravalli, J., Ragsdale, S. W., and Drennan, C. L. (2002) Science 298, 567–572
14. Gencic, S., LeClerc, G. M., Gorlatova, N., Peariso, K., Penner-Hahn, J. E., and Grahame, D. A. (2001) Biochemistry 40, 13068–13078
15. Perkins, S. J. (1986) Eur. J. Biochem. 157, 169–180
16. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
17. Terlesky, K. C., Barber, M. J., Aceti, D. J., and Ferry, J. G. (1987) J. Biol. Chem. 262, 15392–15395
18. Grahame, D. A., Khangulov, S., and DeMoll, E. (1996) Biochemistry 35, 593–600
19. Ragsdale, S. W., Wood, H. G., and Antholine, W. E. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 6811–6814
20. Lindahl, P. A., Munck, E., and Ragsdale, S. W. (1990) J. Biol. Chem. 265, 3873–3879
21. Xia, J., Dong, J., Wang, S., Scott, R. A., and Lindahl, P. A. (1998) J. Am. Chem. Soc. 120, 7502–7510
22. Russel, W. K., Stalhandske, C. M. V., Xia, J., Scott, R. A., and Lindahl, P. A. (1998) J. Am. Chem. Soc. 120, 7502–7510
23. Barondeau, D. P., and Lindahl, P. A. (1997) J. Am. Chem. Soc. 119, 3959–3970
Nickel in Subunit $\beta$ of the Acetyl-CoA Decarboxylase/Synthase Multienzyme Complex in Methanogens: CATALYTIC PROPERTIES AND EVIDENCE FOR A BINUCLEAR Ni-Ni SITE
Simonida Gencic and David A. Grahame

J. Biol. Chem. 2003, 278:6101-6110.
doi: 10.1074/jbc.M210484200 originally published online December 2, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M210484200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 23 references, 10 of which can be accessed free at http://www.jbc.org/content/278/8/6101.full.html#ref-list-1