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Characterization of the *cdhD* and *cdhE* Genes Encoding Subunits of the Corrinoid/Iron-Sulfur Enzyme of the CO Dehydrogenase Complex from *Methanosarcina thermophila*

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The CO dehydrogenase enzyme complex from *Methanosarcina thermophila* contains a corrinoid/iron-sulfur enzyme composed of two subunits (δ and γ). The *cdhD* and *cdhE* genes, which encode the δ and γ subunits, respectively, were cloned and sequenced. The *cdhD* gene is upstream of and separated by 3 bp from *cdhE*. Both genes are preceded by apparent ribosome-binding sites. Northern (RNA) blot and primer extension analyses indicated that *cdhD* and *cdhE* are cotranscribed from a promoter located several kilobases upstream of *cdhD*. The putative CdhD and CdhE sequences are 37% identical to the sequences deduced from the genes encoding the β and α subunits of the corrinoid/iron-sulfur enzyme from *Clostridium thermoaceticum*. The CdhE sequence had a four-cysteine motif with the potential to bind a 4Fe-4S cluster previously identified in the corrinoid/iron-sulfur enzyme by electron paramagnetic resonance spectroscopy. A T7 RNA polymerase/promoter system was used to produce CdhD and CdhE independently in *Escherichia coli*. The purified CdhD protein was reconstituted with hydroxocobalamin in the base-on configuration. The purified CdhE protein exhibited an Fe-S center and base-off cobalamin binding in which the benzimidazole base nitrogen atom was no longer a lower axial ligand to the cobalt atom.

Acetate is a growth substrate for anaerobic microbes in the phylogenetically divergent *Bacteria* and *Archaea* domains. Most acetate-utilizing (acetotrophic) anaerobes from the *Bacteria* cleave acetyl-coenzyme A (CoA) and oxidize the methyl and carbonyl groups to CO_2 (38). Methanogenic *Archaea* also cleave acetyl-CoA but reduce the methyl group to CH_4 and oxidize the carbonyl group to CO_2 (10). Anaerobic acetogens (acetate producers) from the *Bacteria* obtain energy for growth by synthesizing acetyl-CoA from a methyl group, a carbonyl group, and CoA (23). Acetotrophic and acetogenic anaerobes have similar enzyme systems containing a CO dehydrogenase which either cleaves or synthesizes acetyl-CoA (9).

Two enzyme components can be resolved from the five-subunit CO dehydrogenase enzyme complex of *Methanosarcina thermophila*: a nickel/iron-sulfur (Ni/Fe-S) enzyme containing α and ε subunits and a corrinoid/iron-sulfur (C/Fe-S) enzyme containing γ and δ subunits (1). A fifth β subunit has not been characterized. The Ni/Fe-S enzyme is the proposed site of acetyl-CoA cleavage and carbonyl group (CO) oxidation (17). The C/Fe-S enzyme contains factor III {Coα-[α-(5-hydroxybenzimidazolyl)]-cobamide}, the cobalt atom of which is reduced to the Co^{1+} state with electrons donated directly by the Ni/Fe-S enzyme (1). The Co^{1+} supernucleophile displaces the methyl group bound to the Ni/Fe-S enzyme after acetyl-CoA cleavage. Electron paramagnetic resonance spectroscopy of the C/Fe-S enzyme (14) indicates that factor III is in the base-off configuration, in which the nitrogen atom of the 5-hydroxybenzimidazole base is no longer a lower axial ligand to the cobalt atom as is the case for the base-on configuration. The base-off configuration facilitates reduction to the methyl-accepting Co^{1+} redox state by changing the midpoint potential of the $Co^{2+/1+}$ couple to a value less negative than that with the base-on. The C/Fe-S enzyme from *M. thermophila* contains a single 4Fe-4S center with a midpoint potential of −502 mV, close to the −486 mV midpoint potential for the $Co^{2+/1+}$ couple (14); thus, the 4Fe-4S center presumably transfers electrons from the Ni/Fe-S enzyme to the cobalt atom of factor III. It has not been determined which subunits of the C/Fe-S enzyme contain factor III or the 4Fe-4S center.

The acetogenic anaerobe *Clostridium thermoaceticum* catalyzes the synthesis of acetyl-CoA by utilizing an enzyme system similar to the CO dehydrogenase complex from *M. thermophila* (23). The analogous clostridial C/Fe-S enzyme transfers a methyl group from methyl-tetrahydrofolate to a CO dehydrogenase which synthesizes acetyl-CoA from the methyl group, CO, and CoA. The clostridial C/Fe-S enzyme is an αβ dimer with subunit molecular weights of 55,000 and 34,000 (13). It contains cobalt in a corrinoid cofactor (5-methoxybenzimidazolylcobamide) and a single 4Fe-4S center, both of which are oxidized and reduced during catalysis (24). The 4Fe-4S center is located in the large subunit and the corrinoid in the smaller subunit (18).

Although the genes encoding the two subunits of the Ni/Fe-S enzyme (CO dehydrogenase) from *Methanothrix soehngenii* have been cloned and sequenced (8), the sequence has not been reported for genes encoding subunits of the C/Fe-S enzyme from any methanogenic species. Here, we present the cloning, sequencing, and transcriptional analysis of the genes encoding the subunits of the C/Fe-S enzyme from *M. thermophila*. Amino acid sequence analysis and biochemical characterization of the proteins individually produced in *Escherichia coli* revealed putative subunit locations for factor III and the 4Fe-4S center.

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MATERIALS AND METHODS

Cloning the *cdhD* and *cdhE* genes encoding the δ and γ subunits of the C/Fe-S enzyme. The N-terminal sequence of the δ subunit was determined for use in the

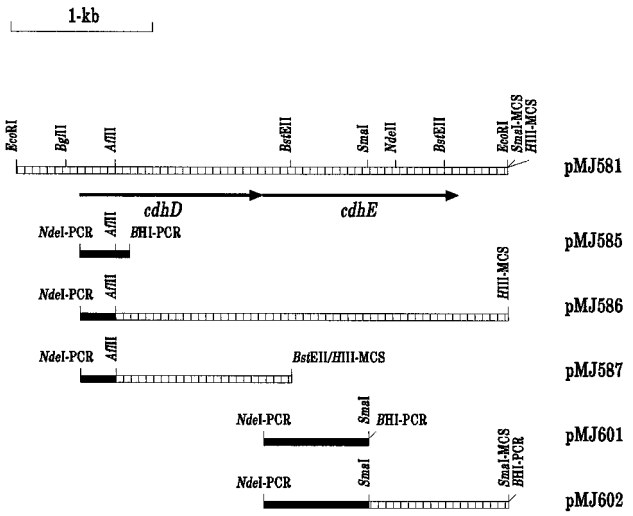

FIG. 1. Physical map of *M. thermophila* genomic DNAs in plasmids used in this study. The pUC19-based plasmid pMJ581 carries the 3.4-kb *Eco*RI fragment isolated from λMJ583. The remaining plasmids are derivatives of pMJ581. Nucleic acid fragments subcloned from pMJ581 are indicated by vertical hash marks. Fragments obtained after amplification by PCR are indicated by solid rectangles. The coding regions for *cdhD* and *cdhE* genes are represented by arrows. *B*HI, *Bam*HI; *H*III, *Hin*dIII; MCS, the multiple cloning region of plasmid pUC19; PCR, restriction endonuclease site synthesized by PCR.

synthesis of an oligonucleotide probe (National Biosciences, Plymouth, Minn.) on the basis of amino acids 1 to 13 (nucleotides +1 to +39 of the *cdhD* gene) (see Fig. 2). The CO dehydrogenase complex was purified as described elsewhere (37). Following sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of the purified complex, the subunits were electroblotted onto a polyvinylidene difluoride membrane (Millipore Corp., Bedford, Mass.) as described elsewhere (19). N-terminal sequences were determined on a model 470 gas phase peptide sequencer (Applied Biosystems, Inc., Foster City, Calif.) by the Virginia Polytechnic Institute and State University protein sequencing facility. The phenylthiohydantoin derivatives were identified with an on-line Applied Biosystems liquid chromatograph.

An *M. thermophila Sau*3A1 genomic library was prepared in λ phage vector GEM-11 (Promega, Madison, Wis.). The genes encoding the δ and γ subunits (*cdhD* and *cdhE*) were isolated from the library with an oligonucleotide probe which was labeled at the 3′ end with digoxigenin-11-ddUTP (Boehringer Mannheim Biochemica, Indianapolis, Ind.). Colony-plaque screen nylon membranes were used for plaque lifts (DuPont-New England Nuclear Research Products, Boston, Mass.). Hybridization, wash stringencies, and colorimetric detection with anti-DIG-alkaline phosphatase antibody were performed as recommended by the supplier (Boehringer Mannheim). Clones hybridizing specifically to the oligonucleotide probe were rescreened to ensure phage purity. Recombinant DNA (λMJ583) was isolated as described elsewhere (29) and then extracted with hexadecyltrimethylammonium bromide to remove polysaccharide (3). *M. thermophila* genomic and recombinant DNAs were compared for common restriction endonuclease fragments that hybridized with the oligonucleotide probe in Southern blots (31). These fragments were then isolated from recombinant DNA and ligated into vector pUC19. *E. coli* TB-1 [F^- *ara*Δ(*lac-proAB*) *rpsL* Δ*hsdR17* (r_K^-, m_K^+), Φ80d*lacZ*ΔM15] (Bethesda Research Laboratories, Gaithersburg, Md.) was grown in Luria-Bertani medium supplemented with ampicillin (100 ng/ml) (LB plus ampicillin) and was used for amplification of pUC19-based plasmids.

DNA sequence determination and analysis. A 3.4-kb *Eco*RI fragment (pMJ581) (Fig. 1) was isolated from a 16-kb *Sau*3A1 *M. thermophila* genomic fragment (λMJ583) which hybridized with the *cdhD*-specific probe. Plasmid pMJ581 and its exonuclease III deletion derivatives (12) were used for DNA sequence determination by the Sanger dideoxy method and Sequenase version 2.0 T7 DNA polymerase (U.S. Biochemical Corp., Cleveland, Ohio) (27). Synthetic oligonucleotides (Research Genetics, Huntsville, Ala.) based on the *cdhD* and *cdhE* sequence were used to complete the sequences of both DNA strands.

The GenBank, EMBL, and SwissProt databases were searched at the National Center for Biotechnology Information (Bethesda, Md.) by using the BLAST network server (2) at the University of Florida Interdisciplinary Center for Biotechnology Research Computing Facility in conjunction with the Genetics Computer Group program (Madison, Wis.) (7). BESTFIT (30) was used for protein sequence alignment.

Northern (RNA) hybridization and primer extension analyses. Total RNA was isolated (33) and further purified (22) from *M. thermophila* cells grown in marine medium supplemented with 100 mM acetate (32). Total RNA was fractionated by electrophoresis on formaldehyde-agarose gels and transferred to membranes as described elsewhere (4). A *cdhD*-specific 0.92-kb *Pst*I-to-*Hin*cII fragment spanning nucleotides +359 to +1279 of the *cdhD* gene (Fig. 2) and a *cdhE*-specific 1.1-kb *Bst*EII fragment spanning nucleotides +207 to +1315 of the *cdhE* gene (Fig. 2) were labeled with [α-^{32}P]dATP (DuPont-New England Nuclear Research Products) by using random hexanucleotides and the Klenow fragment of DNA polymerase (Boehringer Mannheim). Membranes were equilibrated in (final concentrations) 5× SSPE (3.6 M NaCl, 0.2 M $NaPO_4$, 0.02 M EDTA [pH 7.4]) containing 50% (vol/vol) formamide, 0.5% (wt/vol) SDS, and 5× Denhardt's solution (0.1% [wt/vol] Ficoll, 0.1% [wt/vol] polyvinylpyrrolidone, 1% [wt/vol] bovine serum albumin) for 2 h at 42°C. For independent hybridization, the *cdhD*- or *cdhE*-specific probe was added (4×10^5 cpm/ml) and the solution was equilibrated for 18 h. Membranes were washed at 68°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.01% (wt/vol) SDS. The X-AR film (Eastman Kodak, Rochester, N.Y.) was exposed to membranes for 24 h at −70°C.

The 5′ end of *cdhD*-specific mRNA was mapped by avian myeloblastosis virus reverse transcriptase primer extension analysis as recommended by the supplier (Promega) with the oligonucleotide 5′-GGGCTTCTACATCCATTCCCAC GAA-3′ complementary to nucleotides +40 to +64 of the *cdhD* gene (Fig. 2). The X-AR film (Eastman Kodak) was exposed to the vacuum-dried polyacrylamide sequencing gel for 48 h at 20°C.

Cloning *cdhD* and *cdhE* into expression vector pT7-7. PCR oligonucleotide-directed mutagenesis was used to generate restriction endonuclease cleavage sites for positioning the *cdhD* and *cdhE* genes downstream of the T7 RNA polymerase promoter in plasmid vector pT7-7 (36). Synthetic *Nde*I sites were incorporated at the translational start sites of the two coding regions to ensure proper 8-bp spacing from the ribosomal binding site in plasmid pT7-7. PCR amplification of large DNA fragments was avoided to minimize nucleotide misincorporation. The DNA encoding the N terminus of CdhD was amplified with two oligonucleotides, one with an adjoining *Nde*I-site (5′-CTGCCCATATG GCAAAGAAAATGA-3′) (RG-7) (Fig. 2) and one with a *Bam*HI site (5′-GGCTAGGATCCGCAACATCGAACTT-3′) (complementary to RG-8) (Fig. 2). The resulting 324-bp PCR fragment was digested with *Nde*I and *Bam*HI and ligated into pT7-7 (pMJ585) (Fig. 1). Plasmid pMJ586 (Fig. 1), which carries the complete *cdhD* and *cdhE* genes, was constructed by ligation of a 2.7-kb *Afl*II-to-*Hin*dIII fragment from pMJ581 into *Afl*II-*Hin*dIII-digested pMJ585. For CdhD expression, a 1.45-kb *Bst*EII-to-*Hin*dIII fragment was deleted from pMJ586. The remaining DNA fragment was filled in with the Klenow fragment of DNA polymerase and then ligated into pT7-7 to form pMJ587 (Fig. 1). A similar strategy was used to independently express the CdhE protein. Two oligonucleotides, one with an adjoining *Nde*I site (5′-CTGCCCATATGAAAATA AACAGCCC-3′) (RG-9) (Fig. 2) and one with a *Bam*HI site (5′-AGCTAG GATCCGCTTGGGTAGGTTC-3′) (complementary to RG-10) (Fig. 2), were used to amplify the coding region of the N terminus of CdhE. The resulting 751-bp fragment was digested with *Nde*I and *Bam*HI and then ligated into pT7-7 (pMJ601) (Fig. 1). Plasmid pMJ602 (Fig. 1), which carries the complete *cdhE* gene, was constructed by insertion of a 950-bp *Sma*I fragment from pMJ581 into the *Sma*I site of pMJ601.

Purification of heterologously produced CdhD and CdhE proteins. The *cdhD* (pMJ587) and *cdhE* (pMJ602) genes were independently expressed in *E. coli* BL21(DE3) with the bacteriophage T7 RNA polymerase/promoter system (34). Approximately 900 colonies of freshly transformed cells were pooled, inoculated into 500 ml of LB plus ampicillin medium, and incubated for 3 h at 37°C and 300 rpm. This culture was used to inoculate a 20-liter fermentor maintained at 30°C. Cells were grown aerobically in LB plus ampicillin medium containing 50 μM $FeSO_4$, 1 μM hydroxocobalamin (Sigma, St. Louis, Mo.), and 0.05% Antifoam C (Sigma). Once cells reached an A_{600} of about 0.6 to 1.0, T7 RNA polymerase-dependent transcription was induced with 0.4 mM isopropyl-γ-D-thiogalactopyranoside (Sigma) for 2 h. The cells were concentrated to 1 liter by a tangential flow and were centrifuged at 5,000 × *g* for 30 min at 4°C. The cell pellets were frozen overnight at −70°C and then stored in liquid nitrogen. All remaining steps in the purification were performed at 20 to 25°C (unless otherwise indicated) in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) containing N_2:H_2 (19:1). All solutions were made anoxic as previously described (37). Approximately 5 g (wet weight) of cells was thawed in 3 volumes (wt/vol) of Tris buffer (50 mM Tris-HCl [pH 7.6]) and disrupted by passage through a French pressure cell at 20,000 lb/in^2. The lysate was centrifuged at 10,000 × *g* for 30 min (4°C). The pellet, containing inclusion bodies, was washed twice in an equal volume of Tris buffer and then resuspended in 6 ml (final volume) of the same buffer. By a modification of a previously described procedure (18), protein aggregates were solubilized in 15 ml of 50 mM Tris-HCl (pH 7.6) containing 6 M urea, 3 μM $FeCl_3$, 3 μM Na_2S, and 3 μM hydroxocobalamin. The sample was centrifuged at 10,000 × *g* for 20 min at 13°C to remove insoluble protein and then diluted 20-fold in Tris buffer containing 3 μM hydroxocobalamin. This solution was incubated for 3.5 h at 13°C and then centrifuged at 10,000 × *g* for 20 min at 13°C. Dithiothreitol was added (2 mM final concentration) to the supernatant solution, which was then concentrated to 20 to 40 mg of protein per ml with an ultrafiltration unit fitted with a YM30 membrane (Amicon, Beverly, Mass.). The proteins were further purified and unbound hydroxocobalamin was removed by gel filtration chromatography. The protein samples (1% column volume) were

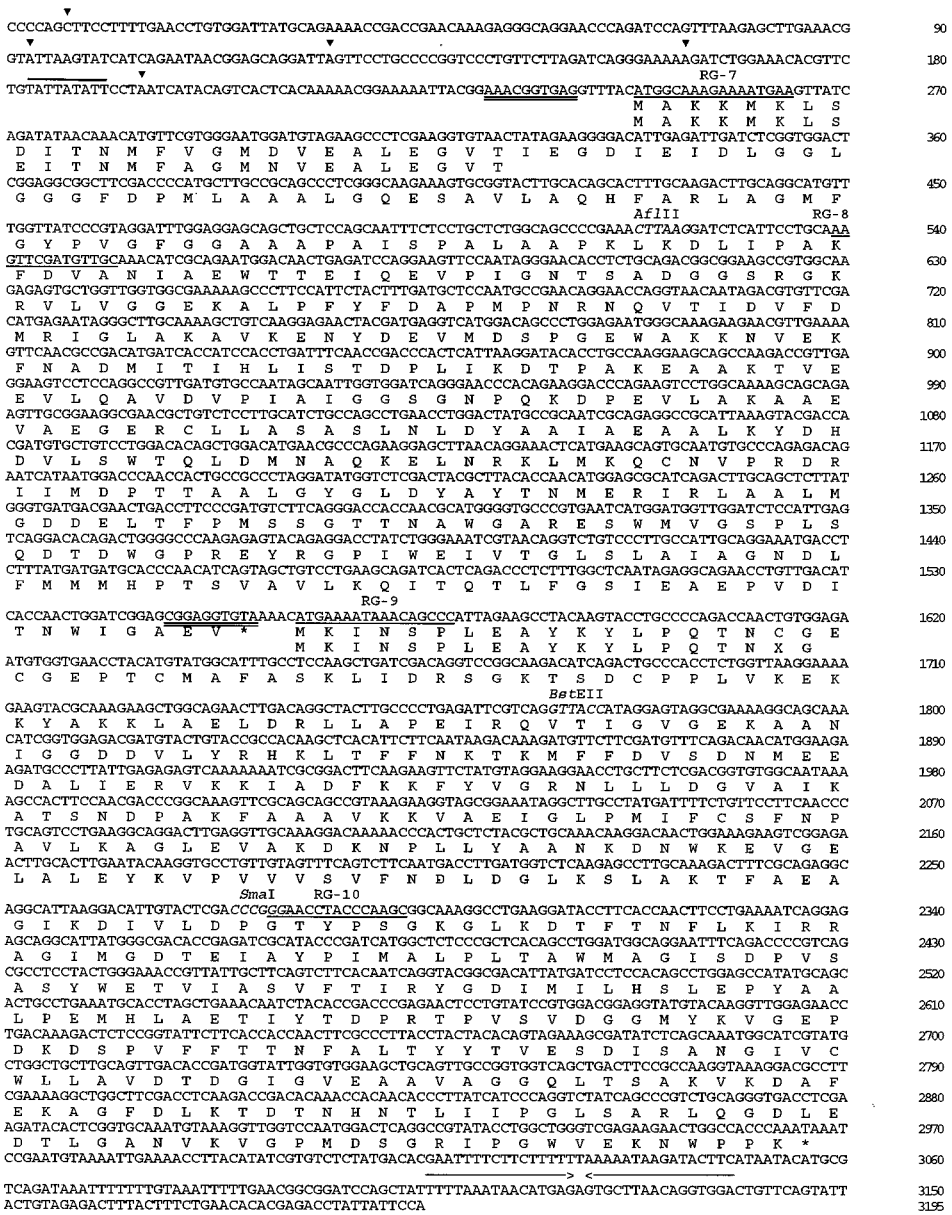

FIG. 2. Nucleic acid sequences and predicted amino acid sequences of *cdhD* and *cdhE* genes encoding the δ and γ subunits of the C/Fe-S enzyme from the CO dehydrogenase complex of *M. thermophila*. A 3,195-bp region of the 3.4-kb *Eco*RI fragment (see text) is shown. The *cdhD* gene is located upstream of *cdhE*. The predicted amino acid sequences are shown directly below the DNA sequences in single-letter code beginning at the first base of each codon. The N-terminal protein sequences determined for the δ and γ subunits of the C/Fe-S enzyme are represented below the sequences predicted for CdhD and CdhE, where X is an uncertain assignment. The bases complementary to the 5′ ends of the primer extension products (see Fig. 4) are indicated with triangles. Nucleotide sequences used for primers in PCR amplification are underlined and labeled RG-7, RG-8, RG-9, and RG-10 (see Materials and Methods). The indicated restriction endonuclease sites were used for cloning *M. thermophila* DNA into expression vector pT7-7. The sequence closest to the consensus archaeal *boxA* promoter sequence is overlined. Double underlining, putative ribosome-binding sites; asterisks, translation stop codons; inverted arrows, a potential stem-loop structure.

applied to a Bio-Gel P6 (Bio-Rad, Richmond, Calif.) column (70 by 1.5 cm) equilibrated with 50 mM Tris-HCl (pH 7.6) containing 150 mM KCl and eluted with the same buffer. Protein concentrations were estimated by the dye-binding assay (5) (Bio-Rad). The purified proteins were stored at −70°C.

Biochemical analysis of CdhD and CdhE. SDS-PAGE was performed as described elsewhere (15) with 10% gels. The amounts of acid-labile sulfide (28) and Fe (11) were determined as described. Protein-bound corrinoid was quantified by dicyano derivatization of the cofactor as described elsewhere (37). Protein concentrations were estimated by the Biuret (16) assay. All calculations were based on CdhD and CdhE molecular masses of 47 and 51 kDa, which were deduced from the respective genes.

Nucleotide sequence accession number. A 3,195-bp region (Fig. 2) of the 3.4-kb *Eco*RI fragment which contains the *cdhD* and *cdhE* genes has been assigned GenBank accession number U30484.

RESULTS AND DISCUSSION

Cloning, sequencing, and transcriptional analysis of *cdhD* and *cdhE* encoding the δ and γ subunits of the C/Fe-S enzyme. A degenerate oligonucleotide, which was specific for the N-terminal sequence of the δ subunit of the C/Fe-S enzyme (Materials and Methods), was used to probe an *M. thermophila* genomic library in λGEM-11. Of the approximately 1,800 plaques analyzed, two clones hybridized specifically with the oligonucleotide. DNA sequence analysis of a 3.4-kb *Eco*RI genomic fragment revealed two open reading frames with deduced N-terminal amino acid sequences which identified them

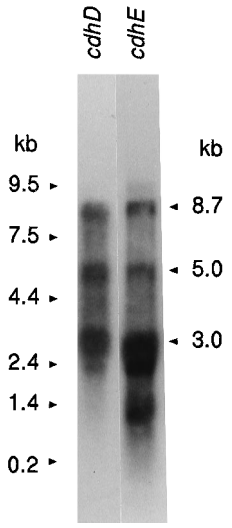

FIG. 3. Northern analysis of *cdhD*- and *cdhE*-specific transcripts. Total RNA isolated from acetate-grown *M. thermophila* was probed with *cdhD*- and *cdhE*-specific DNA fragments (see Materials and Methods). RNA molecular size markers (U.S. Biochemicals) (left) and transcript sizes (right) are as indicated.

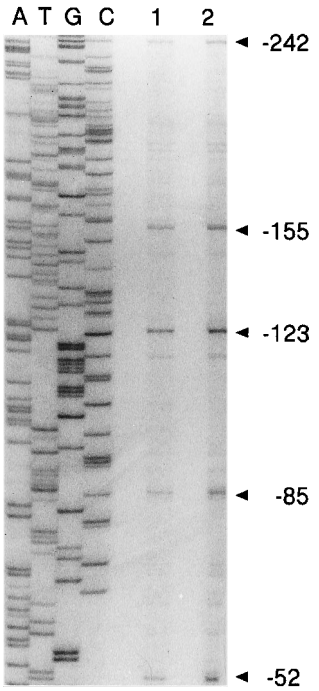

FIG. 4. Mapping of the 5′ end of *cdhD*-specific mRNA. Primer extension products with total RNA from acetate-grown *M. thermophila* cells (lanes 1 and 2) and DNA sequencing reactions (lanes A, T, G, and C) in which the same oligonucleotide (see Materials and Methods) was used. The numbers at right indicate the bases relative to the putative translational start site (+1) of the *cdhD* gene (Fig. 2) that were complementary to the 5′ ends of the primer extension products.

as the genes (designated *cdhD* and *cdhE*) encoding the δ and γ subunits of the C/Fe-S enzyme (Fig. 2). The *cdhD* gene was located upstream of and separated by 3 bp from *cdhE*. Each gene was preceded by an apparent ribosome-binding site (25). Northern analysis revealed 8.7-, 5.0-, and 3.0-kb mRNA species which hybridized with both *cdhD*- and *cdhE*-specific probes (Fig. 3), indicating coexpression of the genes. An additional 1.4-kb mRNA species hybridized with only the *cdhE*-specific probe.

Typically, transcription of genes from the methanogenic *Archaea* initiates 22 to 27 bp downstream of a box A sequence [consensus sequence, 5′-TTTA(T/A)ATA-3′] at a purine-pyrimidine dinucleotide, ideally in a box B sequence (consensus sequence, 5′-ATGC-3′) (25). Only one potential box A sequence was present upstream of *cdhD* (Fig. 2); however, the corresponding downstream box B sequence was absent. Primer extension analysis yielded multiple signals complementary to nucleotides upstream of *cdhD* (Fig. 4), none of which was at the expected 20 to 27 bp downstream of the only potential box A sequence (Fig. 2). The *cdhE* gene was followed by an inverted repeat and poly(dT) stretches (Fig. 2) which may serve in transcription termination (25). Northern blotting, primer extension, and gene organization suggest that *cdhD* and *cdhE* are cotranscribed from a promoter located several kilobases upstream of *cdhD* and that the 3.0-kb mRNA species derives from processing of the larger mRNA transcript. The source of the 1.4-kb *cdhE*-specific mRNA is unknown; however, processing of the 3.0-kb mRNA is a possibility.

Sequence analysis of CdhD and CdhE. The *cdhD* and *cdhE* genes encode putative 436- and 468-amino-acid proteins (CdhD and CdhE) (Fig. 2) with calculated anhydrous molecular masses of 46,953 and 51,035 Da, respectively. Substantial identity (37%) and similarity (59%) were observed between CdhE and the 55-kDa α subunit (AcsC) of the C/Fe-S enzyme from *C. thermoaceticum* (18) (Fig. 5A). A single 4Fe-4S center, which was previously identified by electron paramagnetic resonance spectroscopy (14), is present in the C/Fe-S enzyme from *M. thermophila*. The sequence of CdhD contained only two remotely spaced cysteines (Fig. 2), whereas the N-terminal sequence of CdhE contained a four-cysteine motif ($CX_2CX_4CX_{16}CP$) with a spacing identical to that of a four-cysteine cluster in the N-terminal sequence of the clostridial AcsC (Fig. 5B). It is not known if the four-cysteine motif coordinates the low-potential 4Fe-4S center in the clostridial AcsC (18); however, only one other cysteine is present in AcsC, and only two other cysteines were identified in the *M. thermophila* CdhE (Fig. 2), none of which is conserved between the two proteins (Fig. 5A), suggesting that they are less likely to coordinate 4Fe-4S centers. The 4Fe-4S centers in the clostridial and *M. thermophila* C/Fe-S enzymes have similar redox potentials (<-500 mV) (14, 24), suggesting similar amino acid environments. All of the results described above are consistent with the $CX_2CX_4CX_{16}CP$ motif coordinating the low-potential 4Fe-4S centers present in the C/Fe-S enzymes from *M. thermophila* and *C. thermoaceticum*. The cysteine spacing in this novel motif is notably different from that of $CX_2CX_2CX_3CP$, which coordinates low-potential 4Fe-4S centers of diverse proteins (6); thus, additional experiments will be important to show that the cysteines of the $CX_2CX_4CX_{16}CP$ motif coordinate the 4Fe-4S center. A similar cysteine motif (Fig. 5B) is present in the sequence deduced from the gene encoding a putative low-potential Fe-S protein from *Rhodobacter capsulatus* (26), suggesting that this novel motif is present in other low-potential 4Fe-4S proteins.

Residues 111 to 420 of CdhD had strong identity (37%) and similarity (62%) to the entire 34-kDa β subunit (AcsD) of the C/Fe-S enzyme from *C. thermoaceticum* (Fig. 6), which is postulated to bind the corrinoid cofactor (18). It was not possible to identify conserved sequences with the potential to bind corrinoids, because no consensus corrinoid-binding primary sequence motif is available.

FIG. 5. (A) Alignment of *M. thermophila* CdhE (γ) with *C. thermoaceticum* AcsC (α); (B) Alignment of the four-cysteine motif common to *M. thermophila* CdhE and *C. thermoaceticum* AcsC with a similar motif deduced from the gene (ORFU3) encoding a putative Fe-S protein from *R. capsulatus*. Dashes, gaps introduced for alignment; cola and periods, identical and functionally similar residues, respectively. *Mt*, *M. thermophila* (this report); *Ct*, *C. thermoaceticum* (22); *Rc*, *R. capsulatus* (31).

Alignment of the CdhE and CdhD sequences identified 25.5% identical and 50% similar amino acids (data not shown). The identity between CdhD and CdhE is in agreement with the cross-reactive nature of polyclonal antibodies raised against the δ and γ subunits isolated from the C/Fe-S enzyme (unpublished results). Likewise, the clostridial AcsC and AcsD proteins contained 23.5 identical and 48.5% functionally similar amino acids; thus, the *cdhD*, *cdhE*, *acsC*, and *acsD* genes may have evolved from an ancestral gene which duplicated.

The sequence identities of *M. thermophila* CdhE with *C. thermoaceticum* AcsC and of *M. thermophila* CdhD with *C. thermoaceticum* AcsD are surprising considering that these microbes are at phylogenetic extremes (20). These results suggest either horizontal gene transfer or a common ancestor for the

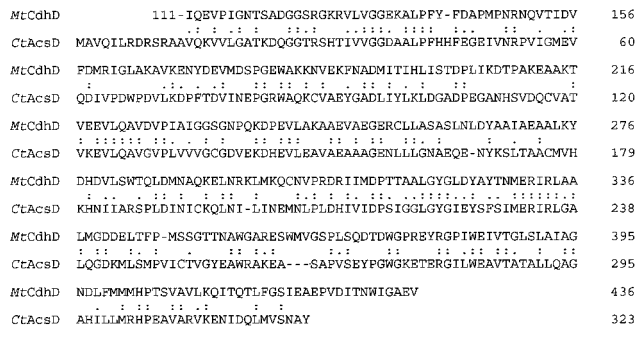

FIG. 6. Alignment of *M. thermophila* CdhD (δ) with *C. thermoaceticum* AcsD (β). Residues 111 to 420 of CdhD are aligned with the entire sequence of AcsD. Dashes, gaps introduced for alignment; cola and periods, identical and functionally similar residues, respectively. *Mt*, *M. thermophila* (this report); *Ct*, *C. thermoaceticum* (22).

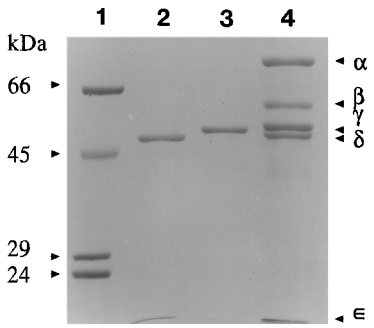

FIG. 7. SDS-PAGE of the *M. thermophila* CO dehydrogenase enzyme complex and the heterologously produced CdhD (δ) and CdhE (γ). Lanes: 1, marker proteins (bovine plasma albumin, 66 kDa; ovalbumin, 45 kDa; bovine carbonic anhydrase, 29 kDa; and bovine trypsinogen, 24 kDa) (Sigma); 2, 1 μg of CdhD protein; 3, 1 μg of CdhE protein; 4, 5 μg of *M. thermophila* CO dehydrogenase enzyme complex. Subunit designations are shown on the far right. Proteins were stained with Coomassie blue R-250.

M. thermophila and *C. thermoaceticum* genes which cannot be distinguished at this juncture.

Characterization of heterologously produced CdhD and CdhE. The CdhD and CdhE proteins were produced independently in *E. coli* with a T7 RNA polymerase/promoter system. Both proteins formed inclusion bodies that were isolated by centrifugation as a first step in purification. The isolated inclusion bodies containing CdhE were dark brown, whereas inclusion bodies which contained only CdhD were white. The brown of CdhE was retained after urea solubilization of the inclusion bodies and disappeared when the protein was acidified, a result consistent with an Fe-S center that is stable in 6 M urea. There was no spectroscopic evidence for bound corrinoid after urea solubilization of either CdhD or CdhE; therefore, the proteins were renatured in the presence of ferric iron, sulfide, and hydroxocobalamin to optimize the reconstitution of Fe-S centers and to incorporate the corrinoid cofactor. The molecular masses of the purified CdhD (δ) and CdhE (γ) proteins produced in *E. coli* (52 and 54 kDa) were slightly less than the masses of the authentic proteins isolated from *M. thermophila* (Fig. 7); the reason for this discrepancy is unknown.

Approximately 0.2 mol of hydroxocobalamin per mol of purified CdhD was detected. Incubation of CdhD with the strong reducing agent Ti(III)citrate increased the A_{394} (Fig. 8A) that is characteristic of the Co^{1+} redox state of corrinoids (24). The 394-nm peak decreased with the addition of the artificial methyl donor CH_3I (Fig. 8A), which indicated formation of the methyl-cobalt bond. Fe and acid-labile sulfur in CdhD were undetectable, and no absorbance attributable to Fe-S centers was discernible (Fig. 8A).

CdhE contained approximately 4.5 mol of Fe, 2.7 mol of acid-labile sulfur, and 0.3 mol of hydroxocobalamin per mol of purified CdhE. The spectrum of the as-isolated CdhE showed broad absorbance centered at 410 nm (Fig. 8B), which is suggestive of oxidized Fe-S centers. Addition of Ti(III)citrate decreased the broad absorbance, indicating reduction of the Fe-S center, and increased the A_{394}, indicating reduction of the cobalt atom of the corrinoid to Co^{1+}. Although electron paramagnetic resonance experiments are necessary to determine if the center is of the 4Fe-4S type, these results further support the idea that the γ subunit (CdhE) contains the single 4Fe-4S center in the C/Fe-S enzyme from *M. thermophila*. Addition of CH_3I to the Ti(III)citrate-reduced CdhE decreased the A_{394}, which indicated methylation of the corrinoid. Addition of CH_3I also produced a broad absorbance for CdhE centered at

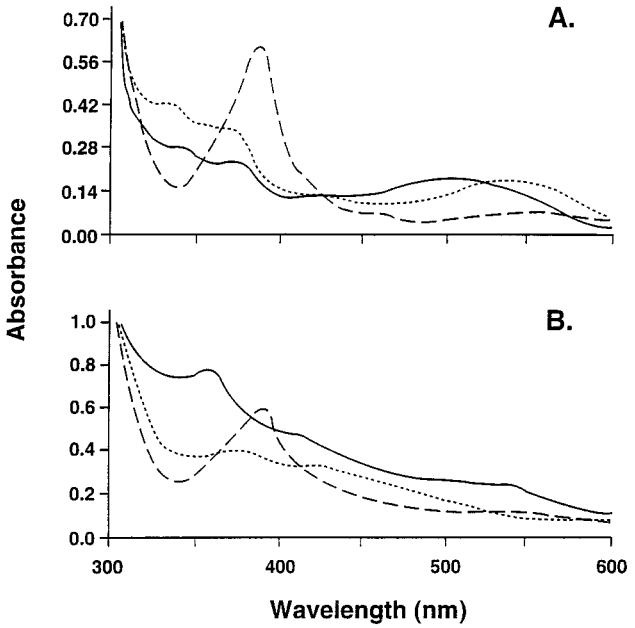

FIG. 8. UV light-visible spectroscopy of heterologously produced CdhD (δ) and CdhE (γ). Concentrations of 35 μM CdhD (A) and CdhE (B) were used. Lines, as-isolated proteins; dashed lines, proteins reduced with 1 mM (final concentration) Ti(III)citrate; dotted lines, reduced proteins treated with 93 μM (final concentration) CH_3I. Proteins were at 23°C in serum-stoppered cuvettes which contained 101 kPa of N_2. Spectra were obtained with a Hewlett-Packard 8452D diode array spectrophotometer.

450 nm (Fig. 8B). This A_{450} is indicative of methylated corrinoid in the base-off form. The C/Fe-S enzyme purified from *M. thermophila* contains one molecule of factor III in the base-off form which facilitates reduction of the cobalt atom to the Co^{1+} redox state, which is a requirement for methylation (1, 14). Factor III contains a 5-hydroxybenzimidazole base; however, the hydroxocobalamin used in the experiments reported here contains a 5,6-dimethylbenzimidazole base (21). The ability to bind hydroxocobalamin in the base-off configuration suggests that the amino acid environment is more essential for this configuration than is the corrinoid structure. Indeed, vitamin B_{12} (containing 5,6-dimethylbenzimidazole) is a functional substitute for factor III in *Methanobacterium thermoautotrophicum* (35). The spectrum of reduced and methylated CdhD indicated no significant A_{450} (Fig. 8A), suggesting that the corrinoid was in the base-on configuration, which is contrary to the configuration of factor III in the C/Fe-S enzyme purified from *M. thermophila*. Either the corrinoid in CdhD was not incorporated correctly or corrinoid bound to CdhD nonspecifically. Although the ability to bind corrinoid in the base-off form favors localization of factor III to the γ subunit (CdhE) of the C/Fe-S enzyme, the substoichiometric amounts of corrinoid detected in CdhE and CdhD preclude a firm assignment. The reason for the low amount of corrinoid is unknown; however, it is possible that an accessory protein which is not present in *E. coli* is required for efficient insertion of factor III in *M. thermophila*, and this may prove interesting for future research.

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