Genetic and Physiological Characterization of the *Rhodospirillum rubrum* Carbon Monoxide Dehydrogenase System

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A 3.7-kb DNA region encoding part of the *Rhodospirillum rubrum* CO oxidation (*coo*) system was identified by using oligonucleotide probes. Sequence analysis of the cloned region indicated four complete or partial open reading frames (ORFs) with acceptable codon usage. The complete ORFs, the 573-bp *cooF* and the 1,920-bp *cooS*, encode an Fe/S protein and the Ni-containing carbon monoxide dehydrogenase (CODH), respectively. The four 4-cysteine motifs encoded by *cooF* are typical of a class of proteins associated with other oxidoreductases, including formate dehydrogenase, nitrate reductase, dimethyl sulfoxide reductase, and hydrogenase activities. The *R. rubrum* CODH is 67% similar to the β subunit of the *Clostridium thermoaceticum* CODH and 47% similar to the α subunit of the *Methanothrix soehngenii* CODH; an alignment of these three peptides shows relatively limited overall conservation. Kanamycin cassette insertions into *cooF* and *cooS* resulted in *R. rubrum* strains devoid of CO-dependent H₂ production with little (*cooF::kan*) or no (*cooS::kan*) methyl viologen-linked CODH activity in vitro, but did not dramatically alter their photoheterotrophic growth on malate in the presence of CO. Upstream of *cooF* is a 567-bp partial ORF, designated *cooH*, that we ascribe to the CO-induced hydrogenase, based on sequence similarity with other hydrogenases and the elimination of CO-dependent H₂ production upon introduction of a cassette into this region. From mutant characterizations, we posit that *cooH* and *cooFS* are not cotranscribed. The second partial ORF starts 67 bp downstream of *cooS* and would be capable of encoding 35 amino acids with an ATP-binding site motif.

Carbon monoxide oxidation is a property of numerous bacterial genera with diverse physiological characteristics. The carbon monoxide dehydrogenases (CODHs) of aerobic organisms are O₂-stable three-subunit hydroxylases, often CO inducible, that catalyze the oxidation of CO to CO₂ (38, 61). The enzymes generally have high affinity for CO and couple its oxidation in vitro with the reduction of acceptors such as methylene blue; most do not reduce low-potential acceptors. The purified enzymes contain a molybdenum cofactor plus Fe and perhaps Se, Zn, and Cu (38). The structural genes from *Pseudomonas thermocarboxydovorans* have been cloned (5).

In anaerobic organisms, including acetogens, dissimilatory sulfate reducers, and methanogens, the CODHs are constitutively synthesized metalloproteins, usually O₂ labile, that catalyze the interconversion of single-carbon moieties with acetyl coenzyme A (acetyl-CoA); the readily assayed oxidation of CO to CO₂ (coupled with the reduction of low-potential electron acceptors) is but one catalytic function. The interconversion is a fundamental anaerobic metabolic process, and the physiology and biochemistry of these bacteria have been the subjects of several reviews (29, 33, 43, 54, 62). The purified 190-kDa *Methanothrix soehngenii* protein contains 2 Ni plus 18 Fe atoms per ($\alpha\beta$)₂ tetramer, the native form of this enzyme (28). The *Clostridium ther-*

moaceticum protein exists as a 440-kDa ($\alpha\beta$)₃ complex that contains 1.8 Ni, 11 Fe, and 1 Zn atom per $\alpha\beta$ dimer (34, 44). The DNA regions encoding the enzymes from *M. soehngenii* (18) and *C. thermoaceticum* (40, 46) have been cloned and sequenced.

The CODHs of phototrophic bacteria exhibit aspects of both the aerobic and anaerobic systems: the systems studied to date are CO inducible under anaerobic conditions, show a high $V_{\max}$ and low K_m for CO oxidation when coupled to the reduction of low-potential acceptors such as methyl viologen (MV), and are oxygen labile. CO oxidation is coordinated with the expression of a CO-tolerant hydrogenase and the CO-dependent synthesis of H₂ (10, 13, 56, 57).

The CODH from the phototroph *Rhodospirillum rubrum* has been thoroughly characterized biochemically. This enzyme is isolated as a 62-kDa monomer containing 1.2 Ni, 8.1 Fe, and 0.4 Zn atoms (11, 19, 22). In vitro, the purified protein catalyzes the oxidation of CO coupled to the reduction of MV, with a K_m for CO of 32 μ M (21) and a $V_{\max}$ of ca. 7,000 μ mol of CO oxidized min⁻¹ mg of protein⁻¹ (22). Unlike other Ni-CODHs, this protein is incapable of exchanging CO with the C-1 of acetyl-CoA (11).

Nickel is required for the synthesis of active holoenzyme (11), and cultivation of cells under inducing but Ni-limiting conditions results in the production of an inactive apo form. The apo-CODH is readily activated in vivo or in vitro upon addition of Ni (12, 20), and electron paramagnetic resonance signals associated with the Ni center have been determined by comparison of the holo and apo proteins (51). The nickel center is proposed to function as the binding site for CO and in the subsequent electron transfer to neighboring Fe/S centers in the protein (19).

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TABLE 1. Relevant characteristics of strains and plasmids

Strain or plasmid	Description	Source or reference
<i>E. coli</i>		
UQ324 (S17-1)	<i>pro thi recA</i> RP4 <i>tra</i> functions	50
UQ555 (XL1-Blue)	<i>recA1 endA1 lac</i> [F' <i>proAB lacI^qΔM15</i> , Tc ^r]	Stratagene
UQ917	UQ555(pLJC24)	16
UQ932	UQ555(pCO1)	This study
UQ991	UQ555(pCO3)	This study
UQ992	UQ555(pCO2R)	This study
<i>R. rubrum</i>		
UR1	Wild-type strain S1, naturally Nx ^r	P. W. Ludden
UR2	Spontaneous Sp ^r derivative of UR1	P. W. Ludden
UR279	UR2, Sp ^r Km ^r Nx ^r , <i>cooS1::kan</i>	This study
UR280	UR2, Sp ^r Km ^r Nx ^r , <i>cooS2::kan</i>	This study
UR281	UR2, Sp ^r Km ^r Nx ^r , <i>cooH3::kan</i>	This study
UR282	UR2, Sp ^r Km ^r Nx ^r , <i>cooF4::kan</i>	This study
UR284	UR2, Sp ^r Km ^r Nx ^r , <i>coo-6::kan</i>	This study
Plasmids		
pBSKS-	Ap ^r , <i>lacZ'</i>	Stratagene
pCO1	pBSKS- with 3.5-kb <i>HindIII</i> fragment containing <i>cooF</i> and most of <i>cooS</i> ; Ap ^r	This study
pCO2R	pBSKS- with 1.5-kb <i>ClaI</i> fragment containing 3' terminus of <i>cooS</i> and 5' terminus of ORF4; Ap ^r	This study
pCO3	pBSKS- with 0.2-kb <i>HindIII-ClaI</i> fragment subclone of pCO2R; Ap ^r	This study
pLJC24	pUC118 bearing 8.5-kb <i>EcoRI</i> fragment that hybridizes to entire pCO1 insert; Ap ^r	16
pSUP202	<i>mob⁺</i> , does not replicate in <i>R. rubrum</i> ; Ap ^r Cm ^r Tc ^r	32, 50

Under certain purification regimens, stoichiometric amounts of a 21-kDa peptide copurify with the 62-kDa CODH subunit. This peptide subunit contains four Fe atoms per monomer as the sole detected metal and is required in stoichiometric amounts in vitro for anchoring CODH to the membrane as well as for conveying the electrons to the CO-induced hydrogenase (22).

MATERIALS AND METHODS

General methods and buffers. Unless specified, the techniques, buffers, reagent solutions, and media used are those described by Sambrook et al. (47).

Strains and cultivation. Table 1 lists the strains and plasmids used in this work. *Escherichia coli* strains were grown in 2× LC (LC medium is identical to LB medium but with 5 g of NaCl per liter) or TB medium (47) at 31 or 37°C with vigorous agitation.

R. rubrum strains were cultivated at 30°C in SMN medium, which is malate-ammonium medium (24, 31) enriched with 0.3% casein enzyme hydrolysate (catalog no. C-0626; Sigma, St. Louis, Mo.) plus 0.3% yeast extract (Difco, Detroit, Mich.). This SMN medium was supplemented with 10 μM Ni, and cultures typically attained an optical density at 680 nm (OD₆₈₀) of 8 to 10 under phototrophic growth conditions (see Assays, below). For phototrophic growth, cells were cultivated in 100-ml serum vials (average volume, 121 ml; Wheaton, Millville, N.J.) containing 15 ml of N₂-sparged medium under an N₂ headspace. Vials were sealed with butyl rubber stoppers (Bellco, Vineland, N.J.) before autoclaving. The effect of headspace gas composition on growth entailed the addition of 32 ml of filter-sterilized CO or N₂ immediately after inoculation. The CODH system was induced for the H₂ accumulation experiments by the injection of 26.5 ml of filter-sterilized CO (or N₂) into the headspace of log-phase cultures (OD₆₈₀, 2.5 to 3.5). Vials were agitated (2.5-cm throw, 290 rpm) to ensure uniform exposure of the cultures to the headspace gases and the tungsten light illumination (intensity, ca. 825 microeinsteins

m⁻² s⁻¹). Aerobic growth in the dark was conducted in 500-ml flasks containing 50 ml of medium; the flasks were agitated at 270 rpm (2.5-cm throw).

Assays of CODH coupled to MV reduction (see below) entailed diluting log-phase cultures (OD₆₈₀, 3 to 4) into additional anaerobic SMN medium in sterile 25-ml stoppered vials to give 4 ml of culture at an initial OD₆₈₀ of 3. CODH was then induced by the injection of 5.2 ml of CO, and the vials were incubated phototrophically for 5 h.

Antibiotics were added as necessary at the following concentrations (in micrograms per milliliter): ampicillin, 100; chloramphenicol, 2.5 (*R. rubrum*) or 25 (*E. coli*); kanamycin sulfate, 10 (*R. rubrum*) or 25 (*E. coli*); nalidixic acid, 20; streptomycin, 100; and tetracycline, 10. *R. rubrum* inoculum cultures were grown under conditions identical to those in the experimental protocol except that kanamycin was added when appropriate. Antibiotics were not included in the medium used for growth, CODH activity, or H₂ accumulation experiments.

DNA isolation. Chromosomal DNA was isolated from *R. rubrum* UR1 by a modification of a standard method (47). Cell pellets were digested in 50 mM Tris-HCl (pH 8.0)–20 mM EDTA–100 μg of proteinase K per ml–5 mg of sodium dodecyl sulfate (SDS) per ml at 47°C for 5 h. The resulting viscous fluid was sequentially extracted with equal volumes of buffer-equilibrated phenol, phenol-chloroform (1:1), and then chloroform-isoamyl alcohol (24:1). The DNA in the recovered aqueous layer was precipitated overnight at –20°C by additions of NaCl to 0.2 M and ethanol to 68%, rinsed successively in 70 and 100% ethanol, dried, and dissolved in TE (Tris-EDTA) buffer. Plasmids were isolated from cells grown in 2× LC medium by an alkaline lysis procedure (47). Plasmids for DNA sequencing were isolated from TB-grown cultures by the ammonium acetate purification protocol (30). The use of high-purity ammonium acetate (catalog no. 24,019-2; Aldrich, Milwaukee, Wis.) facilitated the recovery of DNA suitable for restriction digests and sequencing.

Probes and hybridizations. Oligonucleotide probes were designed according to the amino acid sequences of fragments of CNBr-digested CODH that had been electrophoretically separated and transferred to a polyvinylidene difluoride membrane by the Probe-Design protocol (Promega, Madison, Wis.). These sequences contained multiple amino acids encoded by highly degenerate codons; therefore, the 17-mer probes COD10 and COD11 included one and two guesses, respectively, based on the codon frequency data of other *R. rubrum* genes, and were 64- and 32-fold degenerate, respectively. Probe SLP1, a 64-fold degenerate 17-mer containing one guess, was similarly fashioned according to the N-terminal sequence of the CODH-associated Fe/S protein (22). Probes were 5'-end labeled with [γ - 32 P]ATP by using T4 polynucleotide kinase (New England Biolabs, Beverly, Mass.), purified of unincorporated ATP by elution from a Sep-Pak C18 cartridge (Whatman, Millford, Mass.) in 50% methanol, and hybridized to digests of *R. rubrum* UR1 chromosomal DNA by the unblot protocol (39).

Cloning. The chromosomal *Hind*III fragment that hybridized to probes COD11 and SLP1 was recovered in a pool of 3.2- to 4-kb chromosomal DNA fragments by agarose gel electrophoresis and subsequent extraction by the GeneClean protocol (BIO 101, La Jolla, Calif.). Ligations into *Hind*III-digested pBSKS- (Stratagene, La Jolla, Calif.) and transformation of competent UQ555 cells were done by standard protocols (47). Colony hybridizations were performed with Colony/PlaqueScreen nylon hybridization membranes (Du Pont, Boston, Mass.) according to the manufacturer's directions except that the hybridization (omitting formamide) and wash duration and temperature were similar to those used in the unblot hybridizations (39). For oligonucleotide probe COD11, the overnight hybridization at 38°C was followed by 4-min stringent washes at 44, 50, and 53°C, and the membranes were exposed to X-OMAT AR film (Eastman Kodak, Rochester, N.Y.) overnight between each stringent wash. The plasmid inserts were sequenced with the COD11 primer pool (27) to verify cloning of the CODH region.

DNA sequencing. Sets of nested deletions (26) were introduced into the cloned region encoding CODH with the Erase-a-Base kit (Promega) with the modification that ammonium acetate-purified DNA was used (see above) and the desired bands of exonuclease-treated DNA were purified by the GeneClean method prior to ligation. Sequencing of plasmid DNA with the reverse (catalog no. 1201; New England Biolabs), forward (Promega Q5391), or custom (Department of Biochemistry Oligonucleotide Synthesis Facility, University of Wisconsin-Madison) primers was performed with the Sequenase 2.0 kit (United States Biochemical, Cleveland, Ohio) according to the manufacturer's protocol (version 5). The dGTP labeling reactions were modified by including single-stranded binding protein (United States Biochemical) and increasing the termination temperature to 42°C to minimize pausing. In addition, nearly all of the sequenced regions were run with duplicate dITP labeling reactions to remedy gel compression artifacts. Linear 0.4-mm sequencing gels containing 5% acrylamide (Bio-Rad, Richmond, Calif.) and 8.3 M urea were run with an electrolytic gradient (48). The complementary DNA strands were sequenced as independent projects and matched without conflict in the final comparison. Sequence analyses were performed with the University of Wisconsin Genetics Computer Group package (17). Protein identity and similarity comparisons were made by using the GAP program with default parameters.

Mutagenesis. The Km^r cassette from pUC-4K (58) was

excised with *Hinc*II and ligated into the cloned CODH region of pCO1 (Table 1) linearized by partial digestion with *Hinc*II or *Eag*I (ends made flush with Klenow polymerase). These cassette-containing inserts were excised by digestion with *Pvu*II, ligated into the *Eco*RV site of the suicide vector pSUP202 (50), transformed into UQ324, and mated into *R. rubrum* (32). The resulting Km^r *R. rubrum* mutants were screened for Cm^s to identify double-reciprocal recombinants, and the introduction of the cassette into the CODH-encoding region of chromosomal DNA was verified by Southern hybridization (data not shown).

Assays. CODH activity was measured by diluting 0.25 ml of culture into 1 ml of anaerobic lysis buffer at room temperature in a sealed 9-ml vial. The lysis buffer, reduced by the injection of 10 μ l of 10 mM sodium dithionite, contained 100 mM MOPS (morpholinepropanesulfonic acid, pH 7.5), 2 mM EDTA, 0.25 mM MV, and SDS (500 μ g/ml). Five minutes after a gentle mixing, 10 to 50 μ l of the lysis mixture was assayed at room temperature in a buffer containing 100 mM MOPS (pH 7.5), 2 mM EDTA, and 10 mM MV as described previously (19). The CODH activity of *R. rubrum* is stable in buffers containing SDS (11), and the activity decreased <20% during a 90-min room temperature incubation in the lysis solution.

H₂ was assayed with a Shimadzu GC8A gas chromatograph equipped with a thermal conductivity detector and a 1-m molecular sieve 5A column operated with N₂ carrier gas. Samples (0.25 ml) were obtained with a 1-ml glass syringe equipped with a Mininert syringe valve (Supelco, Bellefonte, Pa.).

A Beckman DU-6 spectrophotometer was used for growth measurements of SMN-diluted cultures against an SMN blank. An OD₆₈₀ of 1 (1-cm path) corresponds to 394 mg of cell (dry weight) per liter.

Nucleotide sequence accession number. The nucleotide sequence data reported in this article will appear in the EMBL, GenBank, and DDBJ nucleotide sequence data bases under accession number M90421.

RESULTS

Cloning the CODH-encoding region. Probes COD11 and SLP1, which were based on the amino acid sequences of CODH and the functionally associated Fe/S protein (22), respectively, showed overlapping patterns of hybridization to endonuclease-digested *R. rubrum* DNA (Fig. 1). We attribute the presence of the bands unique to the SLP1 hybridization to the recognition by one of the probes in this 64-fold degenerate pool of another region of DNA, conceivably encoding a similar Fe/S protein motif. Probe pool COD10, while hybridizing faintly to the same bands as COD11, also showed considerable hybridization to numerous other DNA fragments (data not shown), a curious result since sequence data ultimately indicated that this probe was properly designed.

Fragments of *Hind*III-digested chromosomal DNA in the 3.5-kb size range were extracted from an agarose gel, ligated into pBSKS-, and transformed into *E. coli* UQ555. Of approximately 400 insert-bearing transformants, 3 hybridized strongly to COD11 in colony hybridizations. Plasmids from the three clones were sequenced with COD11 as a primer and in each case revealed a coding region appropriate to the remainder of the CNBr peptide fragment sequence from which COD11 was designed, confirming the cloning of a portion of the CODH-encoding region. One such plasmid, designated pCO1, was used in all subsequent studies. A

FIG. 1. *R. rubrum* chromosomal DNA bands detected by unblot hybridizations (39) with oligonucleotide probes based on CODH (COD11) and the associated Fe/S protein (SLP1). Probes COD11 and SLP1 were 32- and 64-fold degenerate 17-mers, respectively.

schematic representation of the pCO1 insert is presented in Fig. 2.

DNA sequencing and analysis. Sets of nested deletions were produced from both ends of the cloned region in pCO1 and used both to map the hybridization sites of probes SLP1 and COD11 and in subsequent sequencing procedures. Probe SLP1 hybridized to a region at approximately position 1000 and probe COD11 hybridized to a region at approximately position 3000 of the insert (data not shown). When the sequence analysis indicated that pCO1 lacked the 3' terminus of the gene encoding CODH, the adjacent *HindIII*-*ClaI* fragment was subcloned into pBSKS⁻ to generate plasmid pCO3 (Fig. 2). This subcloning started with pLJC24, a plasmid containing the 8.5-kb *EcoRI* fragment (Fig. 1) identified from an *R. rubrum* EMBL4 lambda library (24) with COD11 as a probe (16). A 1.5-kb *ClaI* fragment was excised from pLJC24 and ligated into *ClaI*-digested pBSKS⁻ to generate pCO2R (Fig. 2), and then excision of the *ClaI*-*HindIII* segment already present in pCO1 resulted in pCO3 (Fig. 2). The location of the pCO2R *ClaI* insert was verified by hybridization (16) and sequence analyses (data not shown).

The insert sequence of pCO1, combined with that of

pCO3, contained all or portions of four open reading frames (ORFs) with the expected G+C content and codon usage (data not shown), based on the 65% G+C content of *R. rubrum* (55) and a compilation of 4,136 *R. rubrum* codons extant in the GenBank data base. This sequence is presented in Fig. 3, with expected translation initiation sites and ORFs (*cooH*, *cooF*, *cooS*, and ORF4) indicated both in Fig. 3 and schematically in Fig. 2. In addition to the proposed ORFs, there is a 450-bp "A+T-rich" interval extending from positions 568 to 1017 with a G+C content of 52%.

***cooH*.** Presumably, this ORF begins outside of the cloned region in pCO1 and extends to position 567. The sequence has a 64% G+C content. This ORF encodes 188 amino acids that are 37% identical (59% similar) to approximately the last third of the *E. coli hycE* gene product, the formate hydrogenlyase hydrogenase 3 isozyme (8). The inferred peptide is also similar to other oxidoreductases, in particular the bovine mitochondrial NADH-ubiquinone oxidoreductase, to which it is 35% identical (57% similar) (23). Given the presence in *Desulfovibrio baculatus* of a TGA-encoded selenocysteine at the 3' end of the [NiFeSe] hydrogenase (59), there is a formal possibility that the TGA codon at position 565 of *cooH* encodes selenocysteine. Translation would then continue for an additional 24 amino acids until the next in-frame stop, also TGA, was reached. However, in this interval, 5 of the 24 codons are rare in *R. rubrum*, compared with only 6 of the preceding 188, diminishing the likelihood of continued translation through the TGA at 565. We have provisionally designated this partial ORF *cooH* (for CO oxidation, hydrogenase).

***cooF*.** This ORF begins at position 1018 and extends to position 1590; it is preceded by an acceptable ribosome-binding-like sequence (Fig. 3) and has a G+C content of 63%. The N-terminal amino acids of the purified protein and those inferred from the DNA sequence match exactly (Fig. 4). There is a possibility that translation starts 45 nucleotides upstream of the ATG start codon designated here and that the nascent polypeptide contains a 15-amino-acid N-terminal signal sequence. This is not likely because (i) the putative sequence includes several rarely used codons, (ii) it shows atypical signal sequence structure, assuming that extrapolation

FIG. 2. Organization of the *coo* region. The figure presents the 3.7-kb cloned and sequenced region, indicating the *HindIII* and *ClaI* restriction sites used in cloning pCO1, pCO2R, and pCO3. It also shows the locations of the cassette insertions (and the *R. rubrum* strain bearing the particular insert) and designates the putative transcripts.

cooB (3' terminus)

1 K L E P T L R D E I I P L Y Q T N P S I V D R T R G I G V L
 1 AAGCTTGAACCAACGCTGCGGACGAGATCATCCCGCTTTATCAGACCAACCCGTCGATCGTTGATCGCACCCGGGGCATTTGGCGTTCTG
HindIII

31 S A A D C V D Y G L M G P V A R G S G H A Y D V R K Q A P Y
 91 AGCGGCGCGATTGCGTGGACTATGGCTGATGGGGCCGGTGGCGCGCGCTCGGGCCATGCCATGACGTGCGCAAGCAGGCGCCCTAT

61 A V Y D R L D F E M A L G E H G D V W S R A M V R W Q E A L
 181 GCGGTTTATGATCGTCTGGACTTCGAGATGGCCCTGGGCGAGCACGGCGATGTGTGGTCGCGCGCCATGGTGGCTGGCAAGAGGCCCTG

91 T S I G L I R Q C L R D M P D G P T K A G P V P P I P A G E
 271 ACCTCGATCGGGCTGATCCGCCAATGCCGCGGACATGCCCCAGCGTCCGACGAAGGCGGGCCGGTGGCGCCCATTTCCCGCTGGCGAG

121 A V A K T E A P R G E L I Y Y L K T N G T D R P E R L K W R
 361 GCGGTGCGCAAGACCGAGGCGCGCGCGGAGTTGATCTATTACCTGAAGACCAACGGCACCGACCGGCGCGAAGCGCTGAATGGCGC

151 V P T Y M N W D A L N V M M A G A R I S D I P L I V N S I D
 451 GTTCCACCTATATGAAGTGGGATGCGCTCAATGTGATGATGGCGGGGCGGGATTTCGATATCCCGTGATCGTCAACAGCATCGAT
HincII

181 P C I S C T E R *
 541 CCCTGCATCTCCTGTACGGAACGCTGAACGCCCCAGACAGAGAAGCGTTCTCATCTTCTAGTTCTGCTGGTTTTTCTTGATAAATCCCCG

631 CGATGGCGATGAACGCTTCGTGGGATTTTGCCGAGTCTTCGCGCTCTTTTCATACCCCATAAAACTCTGGATAACTGTCTATCTGGC

721 CGACAGACGGGGCGGGCTTTTGTGCGTTACTCGGCGCCAGGGGCAAAGGCCCGCGCGGTCTGAAGGGCGACGAAGCACCCGCGGGC
EagI

811 CGTGGTTTTTGATCGGCGAAGTCTTTGTTTCGTCTCGTTTTTAAGGAAACCCGAACAGGGGTGGCGTTAATTCACGAGATGACTTTGT

901 AAATTACGCTCTGAGAACAGCGTTTCGCTCCAGGGAATGATTGTTGCAGACAGTGCAGTCTAAAAAGGCAATGCGTTGTTTTGACGTG

cooF
 M P P I K E N V I I Y A N P D H C L S C H

991 GTAATCAATGCGTGGAGGCGAGTACGAATGCGCGCAATCAAAGAAATGTAATCATCTACGCCAATCCCGATCATGTCTGTCTGCCAT

22 S C E L A C A V A H S G G H D M I E A I A A N L P L H A R N
 1081 TCATGCGAGTTGGCTTGGCGCTGCGCCATAGTGGCGGTACGACATGATCGAGGCGATTGCGGCCAATTGCGCGTGCATGCGCGCAAC

52 K V V S V D G T A M P M Q C R Q C E D A P C T F A C P T G A
 1171 AAGGTGGTCAGCGTCGATGGCACGGCGATGCCATGCACTGCGCGCAGTGGCAGGATGCGCCCTGCACCTTCGCGTGGCGCGCGC

82 C R Q A D G Q V Q I V E Q H C I G C K L C V M V C P F G A I
 1261 TGTCGTGAGCGCGATGGTCAGGTTGAGATCGTGCAGCAGCTGATCGGTTGCAAGCTCTGCGTGATGGTCTGCCCCCTCGGGGCGATC

112 T V R S E T V V E Q G A C T N R G V A K K C D L C V D W R A
 1351 ACCGTGCGTCCGAAACGGTCGTGAGCAGGGCGCTGACCAACCGGGGCGTGGCAAGAAATGCGATCTGTGTGCGACTGGCGCGCC
HincII

142 S T G K T A P A C V E A C P T K A I R M V D L D A Y R I A L
 1441 AGCACGGCAAGAGGCTCCGGCTGTGTGAGGCTTGCCCGACCAAGGCCATCGCATGGTGCATCTCGACGCTATCGGATCGGCTTG

cooS
 M T H H

172 R E A R A R E I A K S H R H M R V Q F *
 1531 CGCGAGGCGCGGGCGCGGAGATCGCCAAGTCGACCGCCATATGCGCGTCCAATTCTAAGCACCAGGAATCGCATGATGACCCATCA

5 D C A H C S S D A C A T E M L N L A E A N S I E T A W H R Y
 1621 CGACTGCGCCACTGTTCTCCGACGCTGCGCGACCGAAATGTAATTGGCCGAGGCCAACAGCATCGAAACCGCTTGGCATCGCTA

35 E K Q Q P Q C G F G S A G L C C R I C L K G P C R I D P F G
 1711 CGAAAAGCAGCAGCCCCAATGCGGCTTCGGCAGCGCGCGCTGTGTGCCGATCTGCCTGAAGGGCCCTGTCCGATCGATCCCTTTGG

65 E G P K Y G V C G A D R D T I V A R H L V R M I A A G T A A
 1801 CGAAGGCCCAAGTACGGCGTCTGCGGCGCGACCGGACCATCGTCGCGCGCCATCTGGTGCGCATGATGCGCGCGGACCGCGCGC

95 H S E H G R H I A L A M Q H I S Q G E L H D Y S I R D E A K
 1891 CCATTCCGAGCAGGCGCGCATCGCCCTGGCCATGCAGCATATTTCACAAGGCGAGCTTACGATTATTCGATCCCGGATGAGGCCAA

125 L Y A I A K T L G V A T E G R G L L A I V G D L A A I T L G
 1981 GCTCTATGCCATCGCAAGACCTGGGCGTGGCCACCGAGGGCGGTGTTCTGTGGCGATCGTGGGCGATTGGGGGCGATCACCTGGG

155 D F Q N Q D Y D K P C A W L A A S L T P R R V K R L G D L G
 2071 TGACTTCCAGAACGAGATTACGACAACTTGGCTTGGCTTGGCGGTGCGTGACGCGCGTGGGTGAAGCGCTTGGTGATCTGGG

185 L L P H N I D A S V A Q T M S R T H V G C D A D P T N L I L
 2161 ACTGTGCCCCATAATATCGATGCTTGGTGGCGCAGACGATGTGCGCACCCATGTGGGTGCGATGCGGATCCCAACATCTGATCCT

215 G G L R V A M A D L D G S M L A T E L S D A L F G T P Q P V
 2251 GGGGCGGTTGGCGCTGCCATGGCGGATCTTGACGGTTCGATGTGCGGACCGAGCTGTCCGATGCCCTGTTGGCACGCCCCAGCGGT

FIG. 3. Sequence of the *coo* region of *R. rubrum*. The DNA sequence and proposed gene products are indicated. The putative ribosome-binding sites are double underlined (2). Restriction sites used in cloning and cassette mutagenesis are also marked. Residues of the putative ATP-binding motif (GKGG-N₁-GK-N₁₄-G-N₈-DP, where N_x indicates a stretch of the indicated number of any nucleotides [49]) in the ORF4 gene product are overlined.

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245  V S A A N L G V M K R G A V N I A V N G H N P M L S D I I C
2341  GGTCAGTCCGCCCAATCTGGGCGTCATGAAGCGGGGCGGTCAATATCGCCGTCACACGGCCATAACCCGATGCTCAGCGACATCATTTG
                                     HincII
275  D V A A D L R D E A I A A G A A E G I N I I G I C C T G H E
2431  CGATGTGGCGCGGATCTGCCGACGAGGCGATCGCCGCGGCGCGGCGGAGGCATCAACATCATCGGCATCTGCTGTACCGGCCATGA

305  V M M R H G V P L A T N Y L S Q E L P I L T G A L E A M V V
2521  GGTGATGATGCCACGGCGTGGCGCTGGCCACTAACTACCTGTGCGAGGAATTGCCGATCCTGACCGCGCCCTGGAGGCGATGGTCTG

335  D V Q C I M P S L P R I A E C F H T Q I I T T D K H N K I S
2611  CGATGTCCAGTGCATCATGCCGTGCTGCCGCGCATCGCCGAATGCTTCCACACCCAGATCATCACCACCGACAACACAACAAGATCAG

365  G A T H V P F D E H K A V E T A K T I I R M A I A A F G R R
2701  TGGCGCCACCATGTGCCCTTCGACGAGCACAAGGCGGTGGAGACCGCAAGACGATTATCCGATGGCGATCGCCGCTTTGGCCGGC

395  D P N R V A I P A F K Q K S I V G F S A E A V V A A L A K V
2791  CGATCCCAACCGCGTCGCCATTCCCGCTTCAAGCAGAAGTCGATCGTCGGCTTCAGCGCCGAAGCCGTCGTCGCCGCTGGCCAAAGGT

425  N A D D P L K P L V D N V V N G N I Q G I V L F V G C N T T
2881  CAATGCGGACGATCCGCTGAAGCGCTGGTGCATAATGTGTCACCGCAATATCCAGGGTATCGTGTGTTGCTGGGTGCAACACGAC
                                     HincII
455  K V Q Q D S A Y V D L A K S L A K R N V L V L A T G C A A G
2971  CAAGTTTACGCAAGACAGCGCTATGTCGATCTCGCCAAATCCCTGGCCAAGCGCAATGTGCTGGTGTGCGACGGGCTGCGCCGCCG

485  A F A K A G L M T S E A T T Q Y A G E G L K G V L S A I G T
3061  CGCCTTCGCCAAGGCGCGATTGATGACCTCCGAGGCGACCAACCAATACGCTGGCGAGGGTTGAAGGGGGTGTGAGCGCCATCGGCAC

515  A A G L G G P L P L V M H M G S C V D N S R A V A L A T A L
3151  CGCCGCCGCTGGGGGGGCGCTGCCCTTGGTCATGCATATGGGCTCTTGCCTGCATAACAGCCGGGCGGTGGCTTTGGCCACGGCTT

545  A N K L G V D L S D L P L V A S A P E C M S E K A L A I G S
3241  GGCCAATAAGCTGGGCGTCGATCTCTCGGATCTGCCGCTGGTTCGCCAGCGCGCGGAATGCATGAGCGAGAAGGCCCTGGCCATCGGCTC

575  W A V T I G L P T H V G S V P P V I G S Q I V T K L V T E T
3331  GTGGGCGGTGACCATCGGTCTGCCGACCCATGTCCGCTCGGTGCCTCCGGTCATCGGTTGCGAGATCGTGACCAAGCTGGTGACCGAAAC

605  A K D L V G G Y F I V D T D P K S A G D K L Y A A I Q E R R
3421  GGCCAAGGACTTGGTGGGCGGTTATTTTCATCGTTGATACCGACCCCAAGCGCGGGGACAGCTTTACGCCGCCATTCAAGAGCGCTCG
                                     HindIII
635  A G L G L *
3511  CGCCGGGCTTGGCCTGTAAGACCTTAACGACCCCGGGCGGGGAAGTTGATCCTCGCCCTCTCTTTCTTATCGGTGAGGTTGCCATGA
                                     ORF4
                                     M K

3    I A V T G K G G V G K S T I V G M L A R A L S D E G W R V M
3601  AGATCGCGGTGACGGAAAGGGCGGGTTGGCAAGTCGACCATCGTTGGCATGCTGGCCCGCGCCTGTGTCGACGAGGGGTGGAGGGTGA

33   A I D
3691  TGGCCATCGAT
                                     ClaI

```

FIG. 3—Continued.

tion from data for *E. coli* is valid (42), and (iii) the upstream ATG is not preceded by a recognizable ribosome-binding sequence.

The amino acid composition of the encoded 190-amino-acid (20.6-kDa) peptide is very similar to that determined experimentally for the purified Fe/S protein (data not shown). The protein contains 16 cysteines arranged in four

4-cysteine motifs, and data base searches identified seven proteins, all associated with oxidoreductases, which each contained four similar cysteine groupings (Fig. 5). We have designated this ORF *cooF* (for Fe/S protein).

***cooS*.** The third ORF starts at position 1610, 19 nucleotides downstream from the end of *cooF*, extends to position 3529, has a G+C content of 64%, and is preceded by a potential ribosome-binding site (Fig. 3). The N-terminal amino acid sequence inferred from the DNA sequence matches that determined for purified CODH (Fig. 4), and the DNA-based and experimental amino acid compositions are very similar (11) (data not shown). In addition, the amino acid sequences determined from two other CNBr fragments of CODH also matched those of portions of the predicted peptide (data not shown).

The predicted peptide is 639 residues long, has a size of 66.9 kDa, and has a pI of 6.5, which are similar to previously published values of 626 amino acids and 62 kDa for the purified protein (11). Data base sequence comparisons indicated 46% identity with the *C. thermoaceticum* CODH β subunit (67% similarity) (40) and 23% identity with the *M. soehngenii* CODH α subunit (47% similarity) (18). We have designated this ORF *cooS*.

A. Fe/S protein (CooF)

Protein Analysis	.PPIKENV(q,i)IYANPDHwLSI
Sequence Prediction	MPPIKENV..I..IYANPDHCLSC

B. CODH (CooS)

Protein Analysis	.THx(d,k)xAHxS(y,s)DAxxtemlnlaeans
Sequence Prediction	MTHH..D..CAHCS..S..DACATEMLNLAeANS

FIG. 4. Experimental and sequence-predicted N-terminal alignments of the Fe/S (CooF) and the CODH (CooS) proteins. Lower-case letters for the protein analysis residues indicate marginal determinations, with parentheses including residue alternatives for a single position and an x indicating an unknown amino acid. The periods are for alignment purposes only.

Sequence	Position	
		* * * *
Group 1		
<i>Ec</i> FdnH	35	DVSTCIGCKACQVACSEWN
<i>Ec</i> Orf2	8	EPLWCTGCTCLAACSDVH
<i>Ec</i> HycB	8	DSLTCIGCHTCEAACSETH
<i>Rr</i> Coof	13	NPDHCLSCHSCELACAVAH
<i>Ec</i> NarY	12	NLDKCI GCHTCSVTCKNVW
<i>Ec</i> NarH	12	NLDKCI GCHTCSVTCKNVW
<i>Ec</i> DmsB	9	DSSRCTGCKTCELACKDYK
<i>Ws</i> FdhB	12	DEARCIDCHGCDVACKEAH
Consensus		---CigCh-C--aC---
		* * * *
Group 2		
<i>Ec</i> FdnH	96	RKDGCI DCEDPGKACPSAG
<i>Ec</i> Orf2	47	APVVCCHCEAPCLQVCPVNA
<i>Ec</i> HycB	47	APQLCHCEDAPCAVCPVNA
<i>Rr</i> Coof	61	MPMQCRQCEDAPCTFACPTGA
<i>Ec</i> NarY	179	LPRLCEHCLNPSCVATCPSGA
<i>Ec</i> NarH	180	LPRLCEHCLNPACVATCPSGA
<i>Ec</i> DmsB	62	LSISCMHCEDPACTKVCPSGA
<i>Ws</i> FdhB	55	LSIACMHCSAPCAQVCPVDC
Consensus		-p--C-hCed--C---CP--a
		* * * *
Group 3		
<i>Ec</i> FdnH	129	QSENCIGCGYCIAGCPFNI
<i>Ec</i> Orf2	78	NESLCIGCKLCAVCPFGA
<i>Ec</i> HycB	78	NESLCVSKLCLGACPFGA
<i>Rr</i> Coof	92	VEQHCIGCKLCLVMVCPFGA
<i>Ec</i> NarY	212	DQDKCRGWRICISGCPYKK
<i>Ec</i> NarH	213	DQDKCRGWRICITGCPYKK
<i>Ec</i> DmsB	94	DEDEVICGRYCHMACPYGA
<i>Ws</i> FdhB	87	DKEKICIGCGYCLYACPFGA
Consensus		---CigCh-C---CPFGa
		* * * *
Group 4		
<i>Ec</i> FdnH	156	RVYKCTLCVD--7-PACVKCTPTGA
<i>Ec</i> Orf2	142	VAVKCDLCSF--5-PACVRACPNQA
<i>Ec</i> HycB	139	IAVKCDLCSF--5-PACARMCPPTKA
<i>Rr</i> Coof	129	VAKKCDLCSF--9-PACVEACPPTKA
<i>Ec</i> NarY	239	KSEKICFCYP--7-TVCSETCVGRI
<i>Ec</i> NarH	240	KSEKICFCYP--7-TVCSETCVGRI
<i>Ec</i> DmsB	121	HMTKCDGCDYD--6-PTCVESCPPLRA
<i>Ws</i> FdhB	119	PMDKCTFCAG-24-PVCAAMCSTKA
Consensus		---KC--C-----p-C---Cp--a

FIG. 5. Comparison of cysteine clusters of *cooF* with those of other Fe/S proteins. The sequences of *E. coli* nitrate-inducible formate dehydrogenase subunit (*Ec* FdnH [3]), *E. coli* bacterioferri-ritin-associated protein (*Ec* Orf2 [1]), *E. coli* hydrogenase subunit (*Ec* HycB [8]), *R. rubrum* Fe/S protein (*Rr* Coof; this work), *E. coli* second nitrate reductase subunit (*Ec* NarY [7]), *E. coli* nitrate reductase subunit (*Ec* NarH [6]), *E. coli* dimethyl sulfoxide reductase subunit (*Ec* DmsB [4]), and *Wolinella succinogenes* formate dehydrogenase subunit (*Ws* FdhB [9]) are shown. Asterisks indicate cysteines (except for tryptophan in the third cluster of the *nar* gene products), and nominal proline positions are designated by an arrow. Consensus amino acids are capitalized if present in all positions, lowercase if present in at least five sequences. The position in the protein of the first indicated residue is given.

ORF4. Analysis of the sequence data indicated a fourth ORF with suitable codon usage starting at position 3597, 67 nucleotides downstream from the 3' terminus of *cooS*, and extending to the end of the pCO3 insert (Fig. 2 and 3). The 35 amino acids encoded by this partial ORF are very similar to the putative ATP-binding motif found, for example, in the *nifH*-encoded nitrogenase reductase (49); this ATP-binding motif is marked in Fig. 3. While determination of the function of the ORF4 product awaits further sequencing and mutant analyses, recent Northern (RNA blot) hybridizations indicate that its expression is CO inducible (data not shown).

Mutant generation and characterization. The genetic techniques developed for *R. rubrum* allowed us to construct site-specific insertions in the cloned regions and replace the wild-type chromosomal *coo* region with these mutagenized DNA fragments by homologous recombination. Km^r cassettes from pUC-4K (58) were introduced into *Hinc*II sites in pCO1 at the 3' terminus of *cooH* (position 528, strain UR281) and *cooF* (position 1428, UR282) and in two locations in

cooS (position 2395 [UR280] and position 2923 [UR279]). In addition, a cassette was placed into the *Eag*I site at position 808, near the center of the interval between *cooH* and *cooF* (UR284; see Fig. 2 and 3). As detailed in Materials and Methods, phenotypic characterization of the mutants and parental strains was done with enriched malate-containing (30 mM) medium (SMN) supplemented with 10 μ M Ni. Cultures were agitated to ensure adequate gas-liquid mixing.

Under aerobic conditions, the cassette insertion mutants (UR279 through UR282) and the control strain (UR2) grew similarly (average doubling time [T_d], 3.3 h; final OD₆₈₀, 5.2; the growth of UR284 was not assayed). Likewise, the growth of the insertion mutants and the control strain (UR2) under anaerobic photoheterotrophic conditions in the absence of CO was similar (T_d , 3.5 h; final OD₆₈₀, 7.7). The addition of CO (ca. 30%) to the photoheterotrophic growth condition resulted in only slight growth rate changes: the most significant alterations were the increased growth rate of strain UR2 (T_d , 3.0 h) and the decreased growth rate of strain UR281 (*cooH*; T_d , 4.0 h). In addition, UR281 cultures developed an unusual orange color as growth proceeded, unlike the normal deep red color present in the control without CO. The similar photoheterotrophic growth of these strains regardless of the presence or absence of CO permitted subsequent comparisons of their physiological characteristics, including CODH activity and CO-dependent H₂ production.

As indicated in Table 2, insertion mutations *cooS1* (UR279) and *cooS2* (UR280) eliminated detectable MV-linked CODH activity (which requires only a functional *CooS*). In contrast, strains UR281 (*cooH*) and UR284 (*coo-6::kan*) exhibited CODH activity levels similar to that obtained with the UR2 control. Finally, in a peculiar result, three different experiments indicated that strain UR282 (*cooF*) retained 5% of the control CODH activity level. As this activity appears when CO is present, it might reflect poor transcription through the cassette or the presence of a regulated but weak transcription start immediately upstream of *cooS*. We favor an alternative hypothesis, in which gratuitous transcription out of the cassette into *cooS* is translated into a product(s) that is stable only in the presence of CO or other CO-induced factors. This question awaits further transcriptional studies.

CO-dependent H₂ synthesis, a measure of the overall integrity of the synthesis and function of a system in which, at minimum, CO oxidation (by *CooS*) is coupled to H₂ production (presumably by *CooH*) via an Fe/S protein (*CooF*), was absent in all insertion mutants except UR284 (Fig. 6A). This culture accumulated H₂ at a rate identical to that of the UR2 control culture, and in these cultures, the molar ratio of H₂ accumulated to CO present initially was 0.75. All strains accumulated small amounts of H₂ upon the addition of N₂ rather than CO (Fig. 6B). In cultures of mutant strains UR279 through UR282, this basal H₂ accumulation was inhibited by CO, and the final amount of headspace H₂ (average, 43 μ mol) was similar to the amount present at the time of CO addition (average, 40 μ mol).

DISCUSSION

The sequence, mutational, and physiological analyses presented here suggest that CODH and its functionally associated Fe/S protein are integral and unique parts of an elaborate CO oxidation system. The evidence for the identity of *cooF* and *cooS* is manifold and includes both hybridization and sequence analysis results and the phenotypic

TABLE 2. MV-linked CODH activities in *R. rubrum* strains

Strain	CODH induced ^a	OD ₆₈₀ ^b	CODH sp act ^c
UR2	—	8.6	0.0 (0)
	+	8.7	4.3 (100)
UR279 (<i>cooS1</i>)	—	9.1	0.0 (0)
	+	8.4	0.0 (0)
UR280 (<i>cooS2</i>)	—	8.6	0.0 (0)
	+	8.6	0.0 (0)
UR281 (<i>cooH3</i>)	—	9.3	0.0 (0)
	+	8.3	5.0 (116)
UR282 (<i>cooF4</i>)	—	10.5	0.0 (0)
	+	9.8	0.2 (5)
UR284 (<i>coo-6</i>)	—	8.9	0.1 (2)
	+	9.3	3.3 (78)

^a Cultures were induced for CODH activity at an initial OD₆₈₀ of 3 with a 25% CO headspace for 5 h prior to the assay.

^b OD₆₈₀ of the culture at time of assay.

^c Micromoles of CO oxidized per minute per OD₆₈₀. Numbers in parentheses indicate the percentage of activity in the CO-induced control (UR2).

characteristics of mutants. In addition, the sequence analysis, the nearly wild-type levels of MV-linked CODH activities of strains UR281 (*cooH::kan*) and UR284 (*coo-6::kan*), and the similar CO-dependent H₂ accumulation by strains UR2 and UR284 suggest that at least two transcriptional units are involved, one ending with *cooH* and one starting with *cooF*. The full extent of these transcripts is unknown at present.

The identification of *cooH* is less certain than the assignments of *cooF* and *cooS*. Besides overall similarity with the C-terminal third of the *E. coli hycE* gene product, this partial ORF encodes two regions, in the expected order, that are highly conserved in Ni/Fe hydrogenases (starting with amino acid 126, EAPRGxL; starting with amino acid 178, SxD PCIXC, where x is any amino acid [37, 45]). Furthermore, insertion of a cassette immediately upstream from the *cooH*-encoded C-terminal conserved region generated a mutant

that contained active CODH yet failed to produce H₂ in the presence of CO, again consistent with the identification of *cooH*. However, comparing the *R. rubrum* CO-induced hydrogenase with other Ni hydrogenases may not be appropriate, as the presence of Ni in this enzyme is not proved (though its function depends on Ni in the medium [12]), and the effect of the insertion is consistent with *cooH* encoding another protein involved in electron transfer from CODH to the hydrogenase. Notably, *CooH* shows considerable similarity to an NADH-ubiquinone oxidoreductase (23).

The analysis of *CooF* and seven other cysteine-rich oxidoreductase-associated proteins indicates four potential Fe/S motifs in each with highly conserved organization and placement. One motif (group 3, Fig. 5) is a typical [4Fe-4S] center arrangement (14), except for the nitrate reductase-associated NarY and NarH proteins, and a second group (group 1, Fig. 5) is similar but lacks the expected proline following the fourth cysteine. Atypical but conserved cysteine spacings are evident in groups 2 and 4, and extensive conservation of neighboring residues is clear (Fig. 5). In contrast to other ferredoxins (for example, the 55-amino-acid *R. rubrum* ferredoxin I [36] contains two [4Fe-4S] motifs and has a calculated pI of 3.5), these *CooF*-like proteins are larger (190 to 514 residues) and have a more basic predicted pI (5.2 to 7.5). These striking similarities suggest an evolutionary relatedness and conserved function and mechanism in electron transfer. The role of *CooF* in electron transfer from CODH to the CO-induced hydrogenase has been clearly demonstrated in reconstitution experiments with purified CODH, *CooF*, and membranes containing the hydrogenase (22). Curiously, however, metal analysis of the purified *CooF* indicated the presence of only four Fe atoms per peptide (22), suggesting that Fe may be absent from some of the cysteine clusters or lost during purification of the protein, which was nevertheless catalytically competent. It would be interesting to compare *CooF* with the subunits of the *C. thermoaceticum* CODH-associated corrinoid Fe/S protein (46) when their coding sequences become available.

Though the physiological role of the *R. rubrum* CODH appears to differ from that of the acetyl-CoA synthase/CODH enzymes (see the introduction), *CooS*, the *C. thermoaceticum* β subunit, and the *M. soehngenii* α subunit have certain biochemical and DNA sequence similarities. Comparisons of the peptide sequences deduced from the DNA sequences indicates 67% similarity of the *R. rubrum* *CooS* subunit with the *C. thermoaceticum* β subunit (40) and 47% similarity of *CooS* with the *M. soehngenii* α subunit (18). An alignment of these proteins, presented in Fig. 7, indicates a limited number of conserved residues and even fewer clusters of highly conserved amino acids, and thus the sequences from positions 602 to 607 and 655 to 658 are notable. In addition, these proteins contain several cysteine residues with conserved position and local environment, as well as an HxxHxxH sequence beginning at position 121 (where x is not histidine). Similar histidine motifs are also present in Ni-containing hydrogenases (37, 45) and urease (41), but not in an Fe hydrogenase (60). A data base search showed, however, that this pattern was not unique to known Ni enzymes. Finally, the two eubacterial sequences differ from the *M. soehngenii* sequence by their lack of two cysteine motifs characteristic of [4Fe-4S] clusters (Fig. 7).

The sequence-based similarities of *CooS* to the *C. thermoaceticum* β and the *M. soehngenii* α subunits are not preserved in comparisons of other subunits of these enzymes. The *M. soehngenii* β subunit (187 residues) is similar

FIG. 6. H₂ accumulation in phototrophically grown *R. rubrum* cultures. Injections of 26.5 ml of CO (A) or N₂ (B) were made at the time indicated by the arrows to the ca. 106-ml headspace of log-phase (OD₆₈₀, 2.5 to 3.5) cultures of UR2 (○), UR279 (▲), UR280 (△), UR281 (■), UR282 (□), and UR284 (●). The line in panel B delineates the average headspace hydrogen content for all cultures.

<i>Rr</i> CooS	MT	HHDCAHCSDD	ACATEMLNLA	EA.....NS	50	<i>Rr</i> CooS	RDPNR.VAI.	.PAFKQKS..	IVGF	SAEAVVAALA	500	
<i>Ct</i> CmbB	MPRFRDLSDN	CRPSEAPRVM	EPKNCRTVD	PAVLEMLVKS	DK	<i>Ct</i> CmbB	KESNRPPVYI	.PQIKNRV..	VAGM	SLEALTKLLA		
<i>Ms</i> CdhA						<i>Ms</i> CdhA	EQYKKESLFT	EQQFKDTLAT	CTECNQCAFV	CPPHIRISEM	ISEALKGNLE	
Consensus				E		Consensus		K	A A A A	EA L		
<i>Rr</i> CooS	IETAMHRYEK	QQPQCGF..G	SAGLCRIEL	KGPEDIDPFG	EGPKYGVCGA	100	<i>Rr</i> CooS	KVNA.....				550
<i>Ct</i> CmbB	VITAFDRFVA	QQPQCKI..G	YEGICCRFCF	AGPCRIKATD	GPGRSREICGA	<i>Ct</i> CmbB	TQNA.....					
<i>Ms</i> CdhA	VATLRDNDKF	ICNRYKIMYS	PADDTCTCLT	YGPCDL....	TGNKGACGI	<i>Ms</i> CdhA	PFSSTYEVCV	GCQRCEQTCP	QEIPILKLYE	YANREYIRNQ	KFKMRAGRGP	
Consensus	T		C C	GPC	G CG	Consensus		A A A A				
<i>Rr</i> CooS	DRDTIVARHL	VRMIAAGTAA	HSEHGRHIAL	ANQHISQDEL	HOYSIRDEAK	150	<i>Rr</i> CooS	..DDPLKPLV	DNVVGNIQGG	IVLVGCEHTT	KVQDSAYVD	LAKSLAKRNV
<i>Ct</i> CmbB	SAWTIVARNV	GLMILTGAAG	HCEHGNHIAH	ALVENAEGKA	PDYSVKDEAK	<i>Ct</i> CmbB	..QNPPIRVLN	QAILDGLAG	VALICGCHNL	KGFQNSHLT	VMKELLKNNV	
<i>Ms</i> CdhA	DMAAACGKIV	LVAVLMGTCA	HTAHRH....	LYHMLDKF	GDMPF.....	<i>Ms</i> CdhA	VLDTIRKVG	APLVLGQIPG	VIALVGCNSY	PNGTKECY.D	IAKEFVDRGY	
Consensus		G A H H G H		D		Consensus		G G	GC		K	
<i>Rr</i> CooS	LYAIAKTLGV	ATEGRGLLAI	VGDLAAITLG	DFQNDYDKP	CAWLAASLTP	200	<i>Rr</i> CooS	LVLATGCAAG	AFAKAGLMTS	EATTQYAGEG	LKGVLSAIGT	AAGLGGPLPL
<i>Ct</i> CmbB	LKEVCRVRGI	EVEGKSVLEL	AQEVGEKALE	DFRRLKGEGE	ATWMLTIME	<i>Ct</i> CmbB	FVVATGCSAQ	AAGKLGLLDP	ANVETCYGSG	LKGFLLRLGE	GANIEGLPP	
<i>Ms</i> CdhA			DMGSEILV	D.....	APLYRTITLG	<i>Ms</i> CdhA	IVVATGCM.A		MDMSLYKDED	GKTINEQYEG	A....FDGRN	
Consensus			L D		L	Consensus	V ATGC A		Y	K		
<i>Rr</i> CooS	RRVKRLGDLG	LLPHNIDASY	AQMSRTTIVG	CDADPTNLIL	GGLRVAMADL	250	<i>Rr</i> CooS	VMHMGSCVDN	SR....AVAL	ATALANK...	LGVD	LSDLPLVASA
<i>Ct</i> CmbB	GRKEKFRTHN	VVPFGIHASI	SELVNAHMG	MONDPVNLVF	SAIRVALADY	<i>Ct</i> CmbB	VFMHMGSCVDN	SR....AVDL	LMAMAND...	LGVD	TPKVPFVASA	
<i>Ms</i> CdhA	KKPKSLKDFG	EALEYCEEEI	VQLLAACHTG	QEGHYMDFES	KSLHSGMIDS	<i>Ms</i> CdhA	ICNIGSCVAN	AHIGAAIKV	ATIFAHNER	ANYDDIADYI	MSKVACGVA	
Consensus			H G		D	Consensus	GSCV N	A	A		A	
<i>Rr</i> CooS	DGSMILATELS	DALF....GT	PQPVSAAHL	GVMKRGAVNI	AVNGH...PM	300	<i>Rr</i> CooS	PECMSEKALA	IGSMVATTIGL	PTHVGSVPPV	IGSQIVTKL.	
<i>Ct</i> CmbB	TGEHATDIFS	DILF....GT	PQPVSSEAHM	GVLDPPQVNF	VLHGH...PL	<i>Ct</i> CmbB	PEAMSGKAAA	IGTMMVSLGV	PTHVGTMPVP	EGSDLIYSI.		
<i>Ms</i> CdhA	LKKEICDMLQ	TVAYDMPRGA	ADAPLVEIGH	GTLDQMKGV	IAYGHMLAAG	<i>Ms</i> CdhA	WGAYSQKAAS	IATGVNRIGI	PVVQTPSVI	YRRTFMGRTD	KPEDMMVIDA	
Consensus	G		G	G	GHM	Consensus	S KA I	G P V				
<i>Rr</i> CooS	LSDIICDVAA	DLRDE.AIAA	GAAEGINIIG	ICCTGHEVMM	RHGV...L	350	<i>Rr</i> CooS	VTETAK	DLVGGYFIVD	T...DPKSAGD	KLY....AAI	QERRAGLGL*
<i>Ct</i> CmbB	LSEIIVQAAR	EMEGE.AKAA	G.AKGINLV	ICCTGNEVLM	RQGI...L	<i>Ct</i> CmbB	LTQIAS	DVYGGYFIFE	M...DPQVAAR	KIL....DAL	EYRTMKLGHV	
<i>Ms</i> CdhA	AEAMITTEEH	NLWDKVDIGG	VCCTAIDLTR	ITETGRESKI	PANLGPKAKV	<i>Ms</i> CdhA	KNGHMQUIEP	APEAMLYIAE	TKEAMLEMA	KLCFRPSDNT	QGRGKLTHTY	
Consensus	I		I	I TG E		Consensus		I	K		R L	
<i>Rr</i> CooS	ATNYLSQELP	ILTGALEAMV	VDVQCIIMP	SLPRIAECHFTQ	IITDKHMKI	400	<i>Rr</i> CooS					
<i>Ct</i> CmbB	VTSFASQELA	ICTGAIDAMC	VDVQCIIMP	SLSAVAECYHTR	IITTAONAKI	<i>Ct</i> CmbB	KEVAERYETK	LCQGY*				
<i>Ms</i> CdhA	AGAMGWKRM	VRAGIMDTVI	VDQCVFCDV	..LEDCCQRH	IPVIASNDKI	<i>Ms</i> CdhA	CDISMKYFGK	LPDDMHFLVR	DVKDLPLNYQ	TQMKKELEEK	HGMKIDMKAK	
Consensus		G	VD QC	C I	KI	Consensus		Y K L				
<i>Rr</i> CooS		SGATHVPPFD	EHK.....	AVETAKTII	RMAIAAF.GR	450	<i>Rr</i> CooS				878	
<i>Ct</i> CmbB		PGAYHIDYQ	TAT.....	AIESAKTAI	RMAIEAFKER	<i>Ct</i> CmbB						
<i>Ms</i> CdhA	MLGLPORTND	SADAIVEDLV	SFKMPGVAIL	DPVKAGEVAI	RTAVAVKPKR	<i>Ms</i> CdhA	KFISGLRPA	DVSFDPTNIP	RKIRAKK*			
Consensus		A		I R A	R							

FIG. 7. Alignments of CODH subunits. Sequence designations refer to *R. rubrum* CooS (*Rr* CooS; this article), *C. thermoacetica* β subunit (*Ct* CmbB [40]), and *M. soehngenii* α subunit (*Ms* CdhA [18]). The consensus line indicates conserved residues. Conserved histidines and cysteines are overlined. Two cysteine clusters present only in the *M. soehngenii* sequence are marked with carets. Asterisks indicate stop codons.

in size to CooF, but the former peptide is devoid of cysteine residues (compared with 18 in the 190-amino-acid CooF) and shows only 13% identity to it. Similarly, CooF is only 17% identical to the *C. thermoacetica* α subunit (729 residues, 10 cysteines) in an alignment that introduces 10 gaps and conserves none of the cysteine positions (data not shown). Finally, in agreement with the absence of interaction of the *R. rubrum* CODH with acetyl-CoA, the putative CoA-binding site in the *C. thermoacetica* α subunit (40) is not evident in either CooF or CooS.

Taken as a whole, the *R. rubrum* *cooHFS* region seems to encode functions analogous to those of the formate hydrogenlyase pathway of *E. coli* (8, 35, 52). (*R. rubrum* also couples formate oxidation with H_2 synthesis [25]). In *E. coli*, the oxidation of formate by the selenomolybdoenzyme formate dehydrogenase (*fdhF* gene product) is presumably coupled via an Fe/S protein (HycB) to the reduction of H^+ catalyzed by the Ni-containing hydrogenase (HycE); in *R. rubrum*, the oxidation of CO by the Ni-CODH (CooS) is coupled via an Fe/S protein (CooF, similar to HycB, Fig. 5) to H^+ reduction by a hydrogenase (tentatively CooH, similar to HycE). Presumably there is additional complexity to the *coo* system, as, unlike the formate hydrogenlyase pathway, CO oxidation sustains growth of phototrophs, including *R. rubrum*, in the absence of an additional energy source (56, 57; data not shown). In this vein, the *coo* system may be analogous to the process that catalyzes CO oxidation and H^+ reduction in extracts of methanogenic bacteria (53).

Given the ubiquitous production of CO by biological and

abiological processes (reviewed in references 15 and 61), it is not surprising that numerous bacterial species are able to oxidize this compound. Phototrophs like *R. rubrum* exist in environments where CO may be produced metabolically and by photooxidative processes. Presumably these bacteria acquired CODH and associated functions to provide an additional source of reductant, energy, and carbon and to serve as a protective mechanism. While the *coo* mutants described in this article displayed very modest growth alterations in the presence of CO, it is reasonable to expect more dramatic effects on the phenotype under conditions in which CO-inhibited metabolic processes (e.g., H_2 metabolism and N_2 fixation) are essential. The normal growth of all strains under aerobic and photoheterotrophic growth conditions is consistent with the inducible nature of the *R. rubrum* *coo* system and is in contrast to the expected deleterious effect of similar mutations in the constitutively expressed Ni-CODHs of other anaerobic bacteria. In addition, the inducible nature of the system implies the presence of a CO-dependent regulatory process.

That CooS should be similar to the catalytically more elaborate *C. thermoacetica* acetyl-CoA synthase/CODH (and to a lesser extent the *M. soehngenii* enzyme) is intriguing. It will be interesting to learn which functions of these enzymes are catalyzed by which subunit (or only by their interaction) and whether the *R. rubrum* enzyme might be more metabolically versatile under certain conditions. Finally, the relative ease with which *R. rubrum* DNA may be mutated in vitro and replaced as a single copy into the *R.*

rubrum chromosome offers opportunities for instructive manipulation of the *coo* gene products in a nonheterologous background.

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