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Characterization of the Region Encoding the CO-Induced Hydrogenase of *Rhodospirillum rubrum*

JEFFREY D. FOX,[1] YIPING HE,[2] DANIEL SHELVER,[2] GARY P. ROBERTS,[2] AND PAUL W. LUDDEN[1]*

Departments of Biochemistry[1] and Bacteriology,[2] University of Wisconsin—Madison, Madison, Wisconsin 53706

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In the photosynthetic bacterium *Rhodospirillum rubrum*, the presence of carbon monoxide (CO) induces expression of several proteins. These include carbon monoxide dehydrogenase (CODH) and a CO-tolerant hydrogenase. Together these enzymes catalyze the following conversion: $CO + H_2O \rightarrow CO_2 + H_2$. This system enables *R. rubrum* to grow in the dark on CO as the sole energy source. Expression of this system has been shown previously to be regulated at the transcriptional level by CO. We have now identified the remainder of the CO-regulated genes encoded in a contiguous region of the *R. rubrum* genome. These genes, *cooMKLXU*, apparently encode proteins related to the function of the CO-induced hydrogenase. As seen before with the gene for the large subunit of the CO-induced hydrogenase (*cooH*), most of the proteins predicted by these additional genes show significant sequence similarity to subunits of *Escherichia coli* hydrogenase 3. In addition, all of the newly identified *coo* gene products show similarity to subunits of NADH-quinone oxidoreductase (energy-conserving NADH dehydrogenase I) from various eukaryotic and prokaryotic organisms. We have found that dicyclohexylcarbodiimide, an inhibitor of mitochondrial NADH dehydrogenase I (also called complex I), inhibits the CO-induced hydrogenase as well. We also show that expression of the *cooMKLXUH* operon is regulated by CO and the transcriptional activator CooA in a manner similar to that of the *cooFSCTJ* operon that encodes the subunits of CODH and related proteins.

In the presence of carbon monoxide (CO), *Rhodospirillum rubrum* induces synthesis of a CO-oxidizing system. This system catalyzes the net reaction $CO + H_2O \rightarrow CO_2 + H_2$. The reaction is carried out by two enzymes: CO dehydrogenase (CODH) and a CO-tolerant hydrogenase. CODH is a well-characterized nickel-iron enzyme (10) that carries out the oxidation of CO to CO_2, producing two reducing equivalents. Hydrogenase then consumes these reducing equivalents by the reduction of two protons to H_2 (9, 17). Intermediate electron carriers (including the ferredoxin-like small subunit of CODH [17]) may also be involved in the reaction.

The hydrogenase is tightly membrane bound and has yet to be purified, although the sequence of its large subunit (CooH) and several of its properties have been described previously (22). A wide variety of hydrogenases from other organisms, however, have been purified and characterized. These hydrogenases have been found to fall into three distinct categories: Fe-only, Ni-Fe, and Ni-Fe-Se enzymes (for reviews, see references 1, 4, and 56). The CO-induced hydrogenase from *R. rubrum* apparently belongs to the Ni-Fe class, on the basis of its protein sequence and the requirement of Ni for activity (22).

There seem to be two CO-regulated transcripts in *R. rubrum*. The first, *cooFSCTJ*, encodes CODH and related proteins (27, 29, 43) and has been shown to be regulated by the product of the *cooA* gene (43). CooA appears to bind to a specific region of DNA upstream of the *cooF* promoter in a CO-dependent manner (25). The coding properties and regulation of the second transcript, which is composed of six hydrogenase-related open reading frames (ORFs), including *cooH*, are described in this paper. In addition, the effects of several inhibitors, including *N*,*N*′-dicyclohexylcarbodiimide (DCCD), on hydrogenase activity are described, and the possibility of hydrogenase conserving energy through proton translocation is discussed.

* Corresponding author. Mailing address: Department of Biochemistry, University of Wisconsin—Madison, 420 Henry Mall, Madison, WI 53706. Phone: (608) 262-6859. Fax: (608) 262-3453. Electronic mail address: ludden@biochem.wisc.edu.

MATERIALS AND METHODS

Culture of bacterial strains and bacteriophage. *Escherichia coli* was routinely grown in LC medium or 2× LC medium (27) or Terrific Broth (41) at 37°C with vigorous shaking. Bacteriophage lambda was propagated and manipulated as described by Silhavy et al. (44). *R. rubrum* was grown and the *coo* genes were induced as previously described (22). Membrane preparations from the *R. rubrum* cells grown in the presence of CO were obtained as previously described (22).

DNA isolation and manipulation. DNA from *E. coli* and bacteriophage lambda was purified and manipulated as described by Shelver et al. (43).

DNA sequencing. The cloned DNA sequenced in this work included the inserts on plasmids pRP14 (37), pCO21 (30), pCO7 (30), and pJDF1 (Fig. 1). To extend the cloned region upstream of pCO7, a lambda EMBL4 library of *R. rubrum* DNA (21) was screened as previously described (43), resulting in the identification of λL7. pJDF1 (Fig. 1) was created by ligating a 7.7-kbp *Pvu*II-*Kpn*I fragment from λL7 into pBSKS$^-$ (Stratagene) cut with *Kpn*I and *Sma*I. This construct was electrotransformed into *E. coli* XL1-Blue (Stratagene) as described previously (43). DNA sequencing was carried out as described previously (27, 43) with nested deletions of each plasmid and custom primers to fill in the remaining gaps. Deletions from the left end of pJDF1 (as drawn in Fig. 1) were begun from the *Bgl*II site. All other deletions were initiated from restriction sites in the multiple-cloning region of the vector (pBSKS$^-$). Sequencing of pCO7 was limited to the small region not covered by pRP14 and pCO21 and utilized custom sequencing primers. Some regions of DNA proved difficult to sequence by the manual chain termination method because of compression and/or pausing artifacts and were resequenced with the ABI PRISM (Perkin-Elmer Corp.) dye terminator cycle automated sequencing system at the University of Wisconsin—Madison Biotechnology Center employing Ampli*Taq* DNA polymerase, FS.

Sequence assembly and analysis were performed with the Wisconsin package Genetics Computer Group software. The identities of start codons for each ORF were determined by analysis of the output of the CODONPREFERENCE program and by the presence of likely ribosome binding sites for *R. rubrum* (7). Sequence comparison and alignments were performed with the GAP and PILEUP utilities. Database searches were performed with the BLAST electronic mail service of the National Center for Biotechnology Information (National Institutes of Health). Protein secondary structure predictions were carried out through the Protein Structure Analysis World Wide Web server (http:\\bmerc-

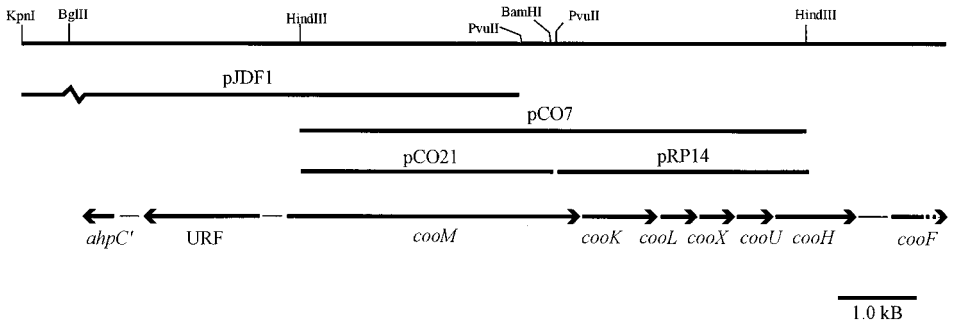

FIG. 1. Organization of genes in the hydrogenase portion of the *R. rubrum coo* operon. Arrows indicate the direction of transcription for each gene.

www.bu.edu\psa\welcome.html) of the BioMolecular Engineering Research Center (Boston University) by type 2 (minimal assumptions) analysis (48, 55).

Primer extension and DNase I protection. CO induction of *R. rubrum*, RNA isolation, and primer extension of mRNA were performed as described by He et al. (25), except that a synthetic oligonucleotide (primer 1, 5′-CTCGCCGATAA GCATGAT-3′), which is complementary to the coding strand in region +40 to +57 relative to the translational start codon of *cooM*, was used in the primer extension assay.

In DNase I footprinting and gel retardation experiments (25), a PCR-amplified DNA fragment containing the promoter sequence of *cooM* was used. The PCR amplification was performed with ^{32}P-labelled primers 1 and 2 (5′-CGCA ATGTCCACAATATGG-3′). Primer 2 is approximately 250 nucleotides upstream from primer 1. The sequencing marker for footprinting analysis was obtained with ^{32}P-primer 1.

Inhibitor studies. Studies with various inhibitors were carried out by adding inhibitor directly to a reduced methyl viologen (MV_{red})-dependent or CO-dependent H_2 evolution assay (17), where 20 μl of a membrane suspension from CO-induced cells (31 mg of protein ml^{-1}) was the enzyme source. Injection of only the inhibitor solvent into the assay was used as the negative control. The following solvents were used: ethanol (for DCCD), 20% chloroform–80% ethanol (for rotenone), and acetone (for oligomycin and antimycin A). Several levels of each inhibitor were tried, ranging from 0 to 25 μg per assay. The inhibition by DCCD was further characterized in a time course experiment as described in the legend to Fig. 7.

Northern (RNA) blot analysis. The Northern blot analysis was performed with mRNA purified from CO-induced and uninduced *R. rubrum* (25). Electrophoretic separation, blotting, probe labeling, hybridization, and washes were performed as described previously (24). The probes were pRP14 (Fig. 1) and a deletion clone containing ~600 bp of URF and extending to the left end of pJDF1, each cut with *Pvu*II.

Nucleotide sequence accession number. The nucleotide sequence data reported in this article will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession no. U65510.

RESULTS AND DISCUSSION

The large subunit of the CO-induced hydrogenase of *R. rubrum* is encoded by the *cooH* gene (22, 27). An additional ~8.4 kb of DNA adjacent to *cooH* has now been sequenced (Fig. 1), and five more genes that encode hydrogenase-related proteins have been identified. Also, two other ORFs that seem to be unrelated to the CO-regulated genes have been found at the 5′ end of the region and are transcribed in the opposite direction (Fig. 1).

CooH. Previously, CooH was shown to have good sequence similarity with the large subunit of hydrogenase 3 (formate hydrogenlyase [FHL]) of *E. coli* and somewhat less similarity to other Ni-Fe hydrogenases such as the periplasmic hydrogenase from *Desulfovibrio gigas* (22, 27). It was also shown to be somewhat similar to a subunit of the energy-conserving NADH-quinone oxidoreductase complex (NADH dehydrogenase I [NDH-I]) of various organisms (e.g., subunit Nqo4 of *Paracoccus denitrificans*) (22, 27). The similarity of Ni-Fe hydrogenase large and small subunits to NDH-I subunits has been noted elsewhere (3).

All of the *coo* gene products reported in this paper exhibit some sequence similarity to subunits of NDH-I (Table 1). In addition, all but one (CooU) show similarity to gene products from the *E. coli* hydrogenase 3 operon. In the results given below, the sequence database searches and comparisons are presented, highlighting the similarity of each gene to hydrogenase 3 genes, to other Ni-Fe hydrogenase genes, and to NDH-I genes.

CooU. The *cooU* gene is immediately 5′ of *cooH*, and its sequence predicts a protein with a length of 172 amino acids (18.7 kDa) and with a pI of 5.13. A search of the protein sequence databases yielded no similarity to *hyc* (hydrogenase 3) gene products. However, the protein did show similarity to Nqo5 (*P. denitrificans*) (57), bovine mitochondrial complex I 30-kDa protein (38), and NuoC (*E. coli*) (53), which are all NDH-I subunits. These three genes all correspond to a family of 30-kDa NDH-I subunits that are believed to be located in the soluble (peripheral to the membrane) portion of the com-

TABLE 1. Summary of the hydrogenase-related *coo* gene products and their homologs

Gene product	Homolog of:		Proposed function[c]		
	Hyc[a]	NDH-I[b]	Coo	Hyc	NDH-I
CooH	HycE	Nqo4, NuoD, 49-kDa subunit	Hydrogenase large subunit	Hydrogenase large subunit	?
CooU		Nqo5, NuoC, 30-kDa subunit	?		?
CooX	HycF	Nqo9, NuoI, 23-kDa subunit	Fe/S	Fe/S	Fe/S
CooL	HycG	Nqo6, NuoB, 20-kDa subunit	Hydrogenase small subunit	Hydrogenase small subunit	Fe/S?
CooK	HycD	Nqo8, NuoH, ND1	H^+ translocation	Membrane spanning?	H^+ translocation
CooM	HycC	Nqo12, Nqo13, Nqo14 NuoL, NuoM, NuoN ND2, ND4, ND5	Membrane spanning	Membrane spanning	Membrane spanning

[a] Hyc (hydrogenase 3) gene products originate from *E. coli.*
[b] NDH-I (NADH-ubiquinone oxidoreductase) gene products originate from *P. denitrificans* (Nqo), *E. coli* (Nuo), or bovine complex I (the remainder).
[c] Proposed functions of hydrogenase 3 (Hyc) subunits are taken from reference 42, and proposed functions of NDH-I subunits are taken from references 53 and 60.

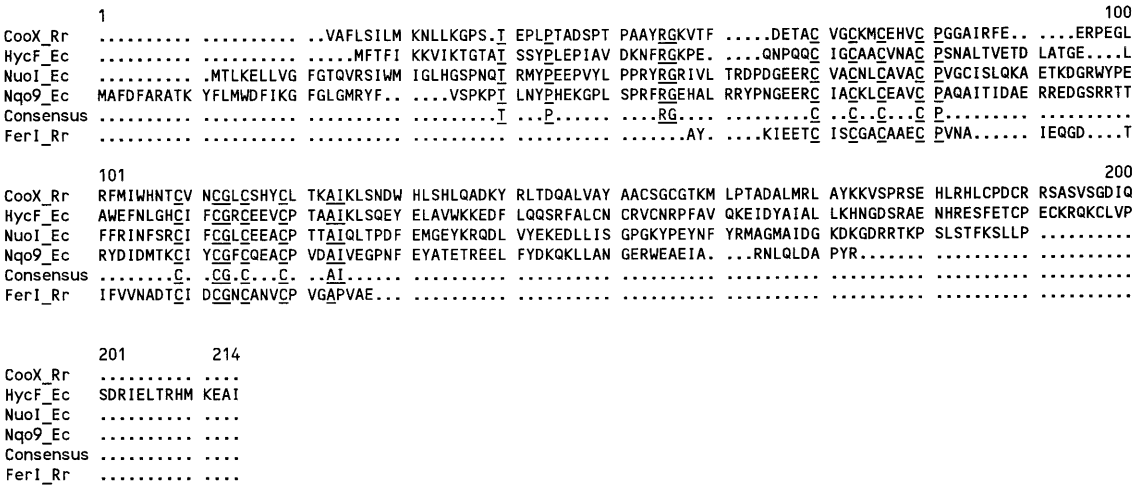

FIG. 2. Multiple sequence alignment of CooX and its related proteins. The consensus line indicates residues conserved in the first four sequences. Residues matching the consensus are underlined in each sequence. The following sequences are included: CooX, *R. rubrum* (Rr); HycF, *E. coli* (Ec) (8, 42); NuoI, *E. coli* (53); and FerI, ferredoxin I of *R. rubrum* (33).

plex (20). However, secondary structure predictions with the CooU sequence indicate the possibility of a membrane-spanning domain (data not shown). The function of the 30-kDa subunit family is unknown in NDH-I (38, 53), and neither the 30-kDa family nor CooU contains a conserved cysteine motif which might indicate metal cluster binding (data not shown).

CooX. *cooX* is predicted to encode a protein with a length of 166 amino acids (18.3 kDa) and with a pI of 8.13. Translation of this gene appears to begin at a GTG start codon, whereas all other *coo* genes utilize ATG (data not shown). Secondary structure analysis predicts that CooX does not contain any membrane-spanning segments (data not shown). This protein resembles a number of Fe_4S_4 cluster-containing proteins, including ferredoxins. It may be functionally analogous to HycF of *E. coli* hydrogenase 3 (8, 42), showing 49% similarity and 28% identity. HycF has also been postulated to be an Fe_4S_4 cluster-containing ferredoxin-like protein for hydrogenase 3 (8).

Interestingly, a database search with CooX also yielded moderate similarity to the HycB (8, 42) protein of hydrogenase 3 (43% similarity, 23% identity). However, alignment analysis shows HycB to be more similar to CooF (the ferredoxin-like small subunit of CODH) (27) in its amino acid sequence and cysteine arrangement than to CooX. The significance of this is unknown, although Sauter et al. (42) propose a model in which HycB is the immediate electron acceptor for formate dehydrogenase in the FHL complex. This is similar to CODH, in which CooF acts as the immediate electron acceptor (17).

CooX shows similarity to the NDH-I 23-kDa (TYKY) subunit family. It aligns well with Nqo9 (58), NuoI (53), and the bovine complex I 23-kDa subunit (16), which have been postulated to be Fe_4S_4-containing proteins in NDH-I. A multiple sequence alignment (Fig. 2) shows how these proteins conserve two cysteine-containing Fe_4S_4 cluster binding motifs [CxxCxx CxxxC(P)] and compares these motifs with the two Fe_4S_4 motifs of ferredoxin I from *R. rubrum* (33). Since they resemble ferredoxins, these proteins are most likely all involved in electron transfer in the hydrogenase or NDH-I systems.

CooL. *cooL* is predicted to encode a protein with a length of 142 amino acids (15.5 kDa) and with a pI of 8.11. Secondary structure predictions indicate that this protein most likely does not contain transmembrane domains (data not shown). CooL shows low similarity to Ni-Fe hydrogenase small subunits such as HyaA of *D. gigas* (32, 39, 52) (55% similarity, 21% identity). CooL has better similarity (68% similarity, 40% identity) to HycG (8, 42), which was postulated to be the small subunit of *E. coli* hydrogenase 3 on the basis of its moderate similarity with other Ni-Fe hydrogenase small subunits (42).

The crystal structure of the periplasmic Ni-Fe hydrogenase from *D. gigas* has been determined (51). That work shows how three Fe-S clusters in the small subunit form an apparent electron pathway from the Ni-containing active site in the large subunit to the exterior surface of the protein dimer. The sequence alignment of CooL (and HycG) with the *D. gigas* small subunit HyaA (Fig. 3) indicates that CooL and HycG conserve only the first of three Fe-S cluster binding motifs (cysteines labeled 1 in Fig. 3) in HyaA.

Volbeda et al. (51) showed that cluster 1 in the *D. gigas* small subunit is very near the Ni site of the large subunit and thus termed it the "proximal" Fe_4S_4 cluster. Cluster 3 (Fe_3S_4) and cluster 2 (Fe_4S_4) form a pathway to the protein surface where cluster 2 (the "distal" cluster) might interact with external electron carriers (51). Since cluster 1 is apparently the only cluster present in CooL and HycG, these proteins must be able to carry out their function as hydrogenase small subunits with only a single iron-sulfur cluster. A few other Ni-Fe hydrogenases also fail to conserve clusters 2 and 3 in the small subunit. These include the NAD^+-reducing hydrogenase from *Alcaligenes eutrophus*, whose small subunit is too short to contain these clusters, and several F_{420}-reducing hydrogenases from methanogens, which contain two classical ferredoxin-type Fe_4S_4 cluster binding motifs in place of clusters 2 and 3 (3). Cluster 1, however, is conserved in nearly all Ni-Fe hydrogenase small subunits (51).

Most membrane-bound or periplasmic hydrogenase small subunits contain an N-terminal leader sequence thought to be involved in protein targeting (for review, see reference 56). However, the short distance from the first conserved cysteine in CooL to the N terminus (Fig. 3) would suggest that no leader sequence is present. The CO-induced hydrogenase activity is tightly bound to the membrane after cell breakage (22). This lack of a leader sequence in CooL might be taken as evidence for the CO-induced hydrogenase being associated with the cytoplasmic side of the membrane. This is consistent

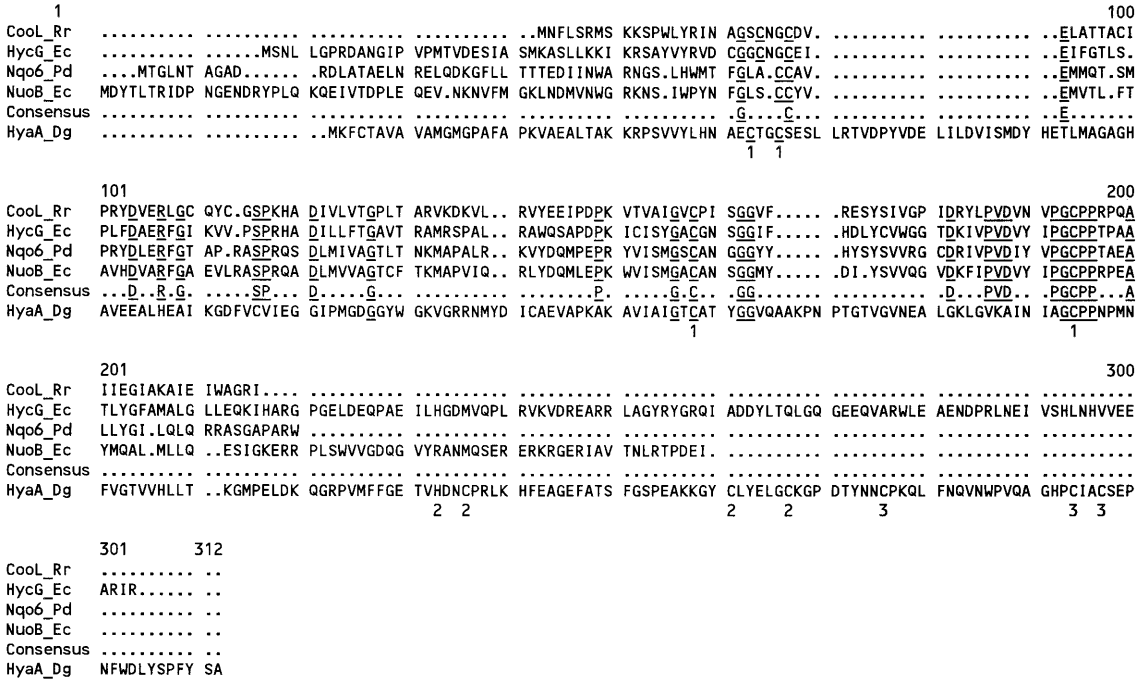

FIG. 3. Multiple sequence alignment of CooL and its related proteins. The consensus line indicates residues conserved in the first four sequences. Residues matching the consensus and cysteines suspected to be involved in metal center binding are underlined in each sequence. The numbers (1, 2, and 3) indicate amino acid ligands to the three iron sulfur clusters of the *D. gigas* protein, as identified in the X-ray crystal structure (51). The following sequences are included: CooL, *R. rubrum* (Rr); HycG, *E. coli* (Ec) (8, 42); Nqo6, *P. denitrificans* (Pd) (57); NuoB, *E. coli* (53); and HyaA, *D. gigas* (Dg) (32, 39, 52).

with the observation in *Rhodocyclus gelatinosus*, a close relative of *R. rubrum*, that the CO-induced hydrogenase (and CODH) activity is localized on the cytoplasmic side of the plasma membrane (13).

CooL exhibits comparable similarity to HycG (39% identity) and to the NDH-I 20-kDa (PSST) subunit family (e.g., Nqo6 [57] and NuoB [53]; ~35% identity), but somewhat less similarity to the *D. gigas* Ni-Fe hydrogenase small subunit, HyaA (21% identity). The NDH-I 20-kDa subunit family has been postulated to contain an Fe_4S_4 cluster (6). Here, too, only cluster 1 seems to be conserved, but with a slightly altered spacing of the cysteines (Fig. 3). It is unknown whether this arrangement of cysteines allows for formation of an intact Fe_4S_4 cluster in these NDH-I proteins. If in fact the 20-kDa subunit family contains an iron-sulfur cluster, then it is likely that these subunits, in both NDH-I and hydrogenase, are all involved in electron flow through the enzymes.

CooK. *cooK* predicts a protein of 323 amino acids (34.4 kDa) with a pI of 7.11. It is predicted to be a transmembrane protein and potentially contains six or seven membrane-spanning helices (data not shown). CooK is 62% similar and 31% identical to HycD of hydrogenase 3 (8, 42) (Fig. 4). HycD was postulated to be a transmembrane subunit of the hydrogenase 3 complex (42), but its function in that complex is unknown. CooK is also ~27% identical and 55 to 60% similar to members of the subunit 1 family of NDH-I. These include Nqo8 (58), NuoH (53), and bovine complex I subunit ND1 (5, 15) (Fig. 4). Subunit 1 has been postulated to be a transmembrane protein and a site of proton translocation and energy coupling (59, 60). In addition, subunit 1 has been proposed as the site for quinone binding (23), although an isolated subcomplex of bovine complex I in which ND-1 was absent was able to couple NADH oxidation to quinone reduction (20). Subunit 1 has been shown to be reactive to DCCD (60), a covalent carboxyl-modifying reagent that attacks specific acidic amino acids in hydrophobic domains (46). Interestingly, DCCD also inhibits CO-induced hydrogenase activity, making CooK a potentially important subunit for energy coupling by the CO-induced hydrogenase complex (see below).

CooM. CooM encodes a very large, hydrophobic protein with a length of 1,264 amino acids, molecular mass of 133.0 kDa, and pI of 9.46. At least 33 membrane-spanning helices are predicted in the structure (data not shown). This gene has similarity to HycC (8, 42) through most of its length, even though HycC is only 608 amino acids in length. A direct alignment does not show the similarity well, because much of the similarity is due to elements of HycC repeated at various positions in CooM. HycC is thought to be a membrane-spanning subunit of hydrogenase 3, but its function is otherwise unknown (42).

The large size of CooM presents some difficulties in its analysis. It shows significant similarity to subunits 2, 4, and 5 (ND2, ND4, and ND5) of bovine complex I (5, 15), *E. coli* (53), and *P. denitrificans* (58) NDH-I, which are also believed to be membrane-spanning proteins. These three subunits have been shown to be similar to one another (31, 34), and this may be a partial explanation of the similarity of CooM to all three. However, a more detailed analysis shows that the relationship to these subunits is complex. CooM is 1,264 amino acids in length, whereas subunits 2, 4, and 5 range in length from about 500 to 700 amino acids. The best similarity between CooM and all three of these subunits occurs in the central portion of CooM. However, on further analysis, various portions of these NDH-I subunits appear to be repeated in the N-terminal and C-terminal regions of the CooM sequence (data not shown).

The function of CooM in the CO-induced hydrogenase reaction is unknown. Because of its large size and hydrophobic nature, it is likely a major factor in anchoring the hydrogenase

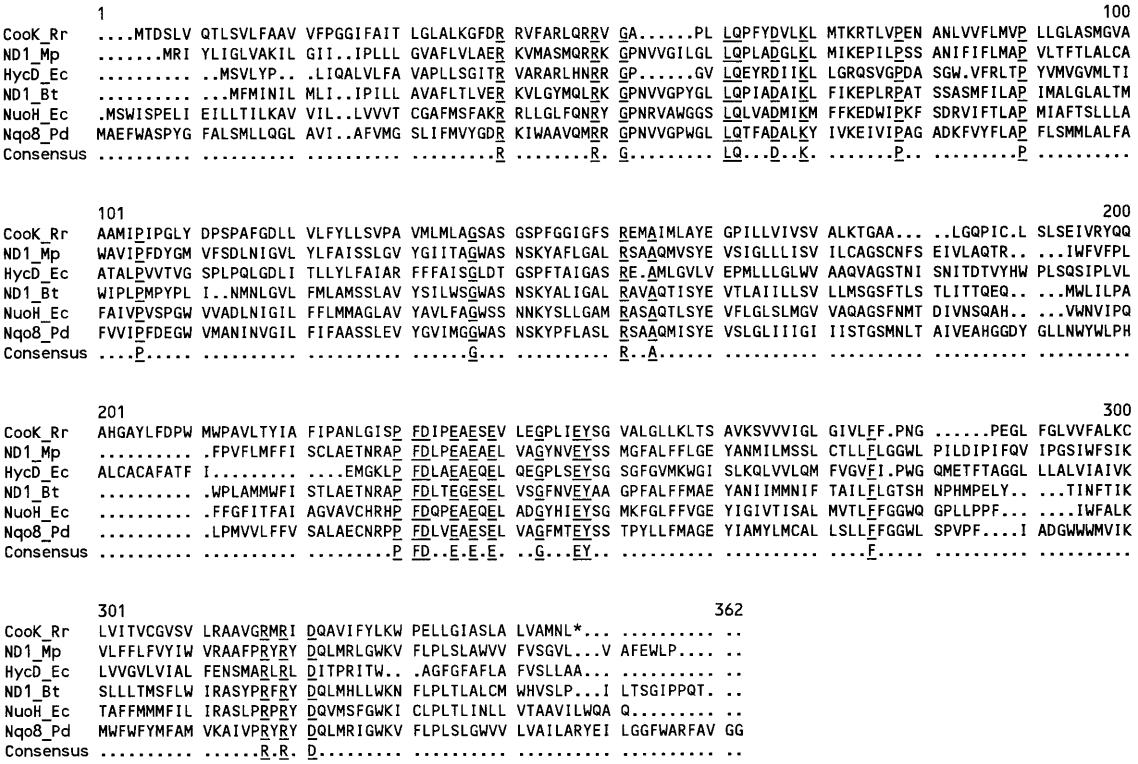

FIG. 4. Multiple sequence alignment of CooK and its related proteins. The consensus line indicates residues conserved in all six sequences. Residues matching the consensus are underlined in each sequence. The following sequences are included: CooK, *R. rubrum* (Rr); ND1, *Marchantia polymorpha* (Mp) (36); HycD, *E. coli* (Ec) (8, 42); ND1, *Bos taurus* (Bt [bovine]) (5, 15); NuoH, *E. coli* (53); and Nqo8, *P. denitrificans* (Pd) (58).

complex to the membrane. Since the functions of subunits 2, 4, and 5 of NDH-I are unknown, it is difficult to speculate on additional roles for CooM.

URF and AhpC. Two ORFs were found 5′ of *cooM* and appear to be oriented in the opposite direction (Fig. 1). URF predicts a protein of 49.9 kDa with a pI of 6.25, but a database search resulted in no significant similarity with any well-characterized gene products. At the *coo*-distal end of the sequenced region, a partial ORF seems to encode a protein similar to AhpC from both *E. coli* (19) and *Salmonella typhimurium* (49). AhpC is the small subunit of alkyl hydroperoxide reductase, which is induced in response to oxygen stress (along with catalase, superoxide dismutase, and other enzymes) in these organisms (47). Consistent with a similar role in *R. rubrum*, a strain with a large deletion of *ahpC* and part of the *coo* region displayed noticeably poorer growth under aerobic conditions (data not shown). Anaerobic growth, however, was unaffected.

The function of AhpC is most likely unrelated to CO metabolism, but we wished to determine if *ahpC* or URF displayed the same CO-regulated transcription as do the known *coo* genes. Northern blot analysis clearly showed a CO-induced message accumulation with pRP14 (diagrammed in Fig. 1) as a probe (data not shown). However, the results with a probe carrying the region from about 600 bp into URF to the left end of pJDF1 indicated no detectable message accumulation from that region in either the presence or the absence of CO. This is consistent with the hypothesis that neither URF nor AhpC plays a role in CO metabolism. At the 3′ end of the *coo* region, *cooA* is adjacent to *nadB*, an NAD biosynthesis gene (43). Thus, the *coo* genes identified here (*cooMKLXU*), as well as *cooH* (22, 27), *cooFSCTJ* (27, 29), and *cooA* (43), seem to constitute the entire contiguous *coo* coding region.

Primer extension and DNase I protection. Since Northern blot analysis suggested that transcription of the region covered by pRP14 is CO regulated, it was of interest to determine the nature of the promoter and the site of action of the apparent CO-responsive regulator, CooA (25, 43). As an initial step in this characterization, primer extension was performed with a primer that hybridizes to a region within the 5′ end of *cooM*. Total cellular RNA from the CO-induced or uninduced wild type and CO-induced strain UR407, a mutant strain containing a mutation of the regulator gene *cooA* (43), was used for this study.

The results show that the 5′ end of *cooM* mRNA is located 53 nucleotides upstream of the translational start codon (Fig. 5). This mRNA is detected only in the CO-induced wild type and not in the uninduced wild type or CO-induced strain UR407 (*cooA* mutant) (data not shown). This is similar to the results with the *cooFSCTJ* transcript (25) and suggests that CO and CooA are both required for transcription.

We believe that *cooMKLXUH* genes are expressed in a single transcript for the following reasons: (i) Northern analysis with pRP14 as a probe suggests a transcript larger than 5 kbp (data not shown), which would be sufficient to contain most of this region; (ii) there are no significant gaps between the predicted genes in the region; and (iii) the only good candidates for additional CooA binding sites, which fall in *cooX*, were shown by primer extension to have no transcriptional start sites associated with them (data not shown).

To identify the region actually recognized by CooA, a DNase I footprinting experiment was performed with the

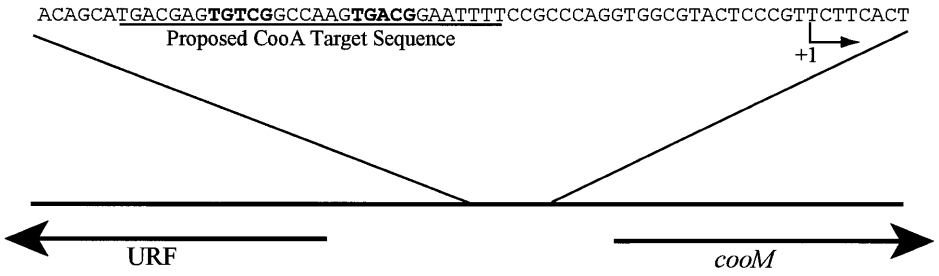

FIG. 5. *cooM* promoter region. The space between the *cooM* and URF genes is 236 bp. The transcriptional start site detected by primer extension assay is indicated as +1. The likely region of CooA binding is underlined. Boldface letters in the CooA target represent a twofold symmetric sequence that is similar to the sequence of the CooA binding site (TGTCA-----CGACA) found in the promoter of *cooF* (25).

crude extracts of UR459 (CooA-overproducing strain [25]) and UR407 (CooA mutant [43]). Figure 6 shows that specific DNase I protection was observed only in the presence of both CO and CooA. The protected region spanned at least 18 bp from −36 to −53 and included a region with a sequence similar to that of the CooA-binding region upstream of *cooF* (Fig. 5) (25). A lack of DNase I sensitivity in part of this region (−36 to −24) under all conditions (Fig. 6) made it difficult to define the extent of the interaction, but it is likely that CooA binds in a uniform manner to the twofold symmetrical sequence (Fig. 5). A gel retardation assay also showed a CooA- and CO-dependent response (data not shown). Interestingly, the pro-

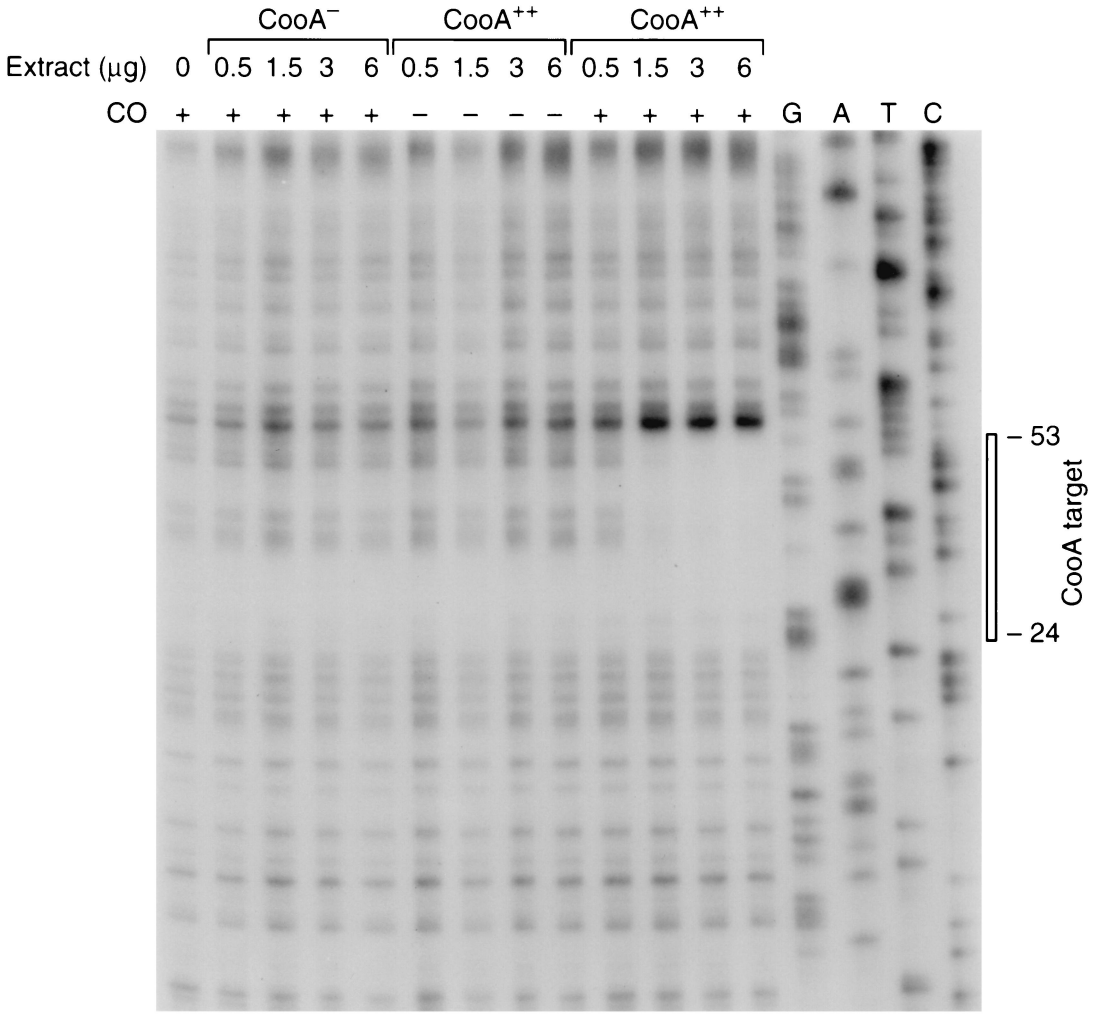

FIG. 6. DNase I footprinting of the binding of CooA to P_{cooM}. The ^{32}P-end-labeled 250-bp fragment used as a probe was obtained by PCR amplification containing P_{cooM} sequences. CooA$^-$ refers to extracts of UR407 (*cooA*::*aacC1*), and CooA^{++} refers to extracts of UR459 (the CooA overproducer with *cooA* under P_{nifH} control). The numbers at the top reflect micrograms of protein in each assay, and the + and − reflect the presence or absence of CO in the binding reactions. The lanes marked G, A, T, and C represent the sequencing marker. The box on the right side indicates the likely region of CooA-DNA interaction.

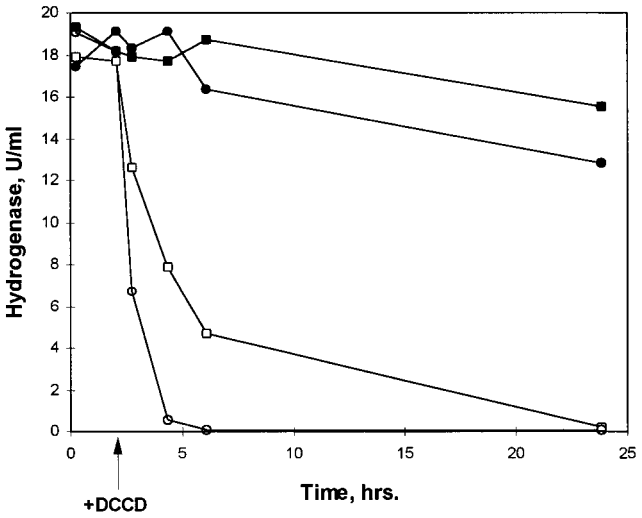

FIG. 7. Effect of DCCD on membrane-bound, CO-induced hydrogenase. Membranes from CO-induced cells (1 ml), suspended in 10 mM triethanolamine (pH 7.8) at a concentration of 6.1 mg of protein ml^{-1}, were mixed with 5 μl of 50 mg of DCCD ml^{-1} (□ and ○) in ethanol (net, 200 nmol of DCCD mg of $protein^{-1}$). Negative controls (■ and ●) were mixed with 5 μl of ethanol. Samples were incubated under a N_2 atmosphere in stoppered vials either on ice (■ and □) or at room temperature (25°C [● and ○]). At the indicated time points, samples from each vial were removed and assayed for MV_{red}-dependent H_2 evolution activity.

tein-DNA complex with the *cooM* promoter also showed the anomalously slow electrophoretic migration property seen with the *cooF* promoter (25), suggesting that activation of these two promoters might involve a similar mode of protein-DNA interaction.

Inhibitor studies. The resemblance of hydrogenase proteins to NDH-I subunits implies some shared structural or functional properties. Several inhibitors of mitochondrial electron-transport complexes have been characterized (45). Of these inhibitors, initial experiments indicated that rotenone (an electron transfer inhibitor), oligomycin (an inhibitor of proton translocation by ATPase), and antimycin A (a quinone oxidation inhibitor) had little if any effect on CO-dependent H_2 evolution activity in CO-induced membranes (data not shown). In contrast, a ubiquinone-reducing H_2-uptake hydrogenase from *Bradyrhizobium japonicum* has been shown to be inhibited by antimycin A (18). However, addition of DCCD (an inhibitor that covalently modifies carboxyl groups and that has been shown to modify a number of proteins involved in proton translocation [46]) resulted in significant inhibition of both MV_{red}-dependent (Fig. 7) and CO-dependent (data not shown) H_2 evolution by the *R. rubrum* CO-induced enzyme. CODH activity in these membranes was unaffected (data not shown). Data in Fig. 7 show that MV_{red}-dependent H_2 evolution is completely inactivated within approximately 1 h after DCCD addition at room temperature. The inactivation seen with this level of DCCD (200 nmol mg of $protein^{-1}$) is similar to the results of Yagi (59), in which NDH-I from bovine heart submitochondrial particles was inactivated nearly 100% after a 4-h treatment with about 250 nmol of DCCD mg of $protein^{-1}$.

The likely target for modification by DCCD is CooK, which is similar to the subunit 1 family of NDH-I. Bovine subunit 1 has been shown to react specifically with DCCD (60). Inactivation of this bovine subunit by DCCD is correlated with loss of proton translocation by bovine complex I (59, 60), and thus inhibition of CO-induced hydrogenase by DCCD may indicate the presence of a proton-translocating site in the enzyme.

Energy conservation by the CO system. The CO-induced, hydrogenase-related proteins encoded by *cooMKLXUH* genes in *R. rubrum* seem to be most similar to proteins involved in the FHL (hydrogenase 3) pathway of *E. coli*. At first glance, these two systems seem to carry out similar metabolic processes (27). The *E. coli* FHL pathway oxidizes formate to CO_2 with two reducing equivalents being passed to the reduction of two protons to H_2. The *R. rubrum* CODH/hydrogenase system oxidizes CO to CO_2 and also directs two reducing equivalents to the reduction of protons to H_2. However, while the FHL reaction is an isopotential, non-energy-conserving pathway (2) and presumably lacks any proton pumping ability, the CODH/hydrogenase system has been shown to allow *R. rubrum* to grow on CO as the sole energy source (28). It might be expected, then, that the CO-induced hydrogenase complex would possess the ability to conserve energy, perhaps through a proton translocation mechanism. In fact, Champine and Uffen (13) have proposed that the CO oxidation system in *R. gelatinosus* translocates protons and have shown low, but measurable levels of in vivo CO-dependent ATP synthesis in that organism. The proteins and genes related to this system in *R. gelatinosus* have not been further characterized, however. Also, in some methanogenic bacteria, CO-dependent H_2 production (50) supports growth (35) and has been implicated in the formation of a proton gradient and in driving ATP synthesis (11, 12). CODH in these organisms, however, is constitutively expressed and is generally involved in acetate metabolism. CODH in *R. rubrum* and *R. gelatinosus* is specifically CO induced (9, 14) and appears to have no function in acetate metabolism (10).

NADH-quinone oxidoreductase (NDH-I) carries out an energy-conserving reaction in various organisms as four protons are translocated across the membrane while quinone is reduced at the expense of NADH (54). The gene products encoded by the *coo* operon lack any similarity to NADH-binding proteins (such as the 51-kDa mitochondrial complex I subunit [54]), but the similarity between several *coo*-encoded proteins and other NDH-I proteins could indicate some shared mechanism of proton translocation and energy conservation. However, many of the subunits of the non-energy-conserving *E. coli* hydrogenase 3 also share similarity to NDH-I proteins (8). Therefore, it is useful to analyze the similarity of the CO-induced hydrogenase, hydrogenase 3, and NDH-I protein sequences in an attempt to understand what fundamental difference allows the CO-induced hydrogenase complex, but not hydrogenase 3, to conserve energy.

CooU, CooK, and CooL are the most likely candidates for critical functions in energy conservation. CooU does not have an analog in the hydrogenase 3 operon but is similar to the 30-kDa NDH-I subunit. Although the function of the 30-kDa subunit is unknown, it may be involved in proton translocation and could allow *R. rubrum* to conserve energy during CO metabolism. CooK is similar to the membrane-spanning NDH-I subunit 1, which contains the postulated proton translocation site, but the presence of a similar protein (HycD) in *E. coli* complicates the issue. CooK may possess multiple functions in the complex, such as acting as a membrane anchor or being involved in electron transfer in the membrane. The other membrane-spanning subunit, CooM, shows some similarity to HycC, but it is significantly larger than that protein and thus may possess functionality not found in HycC which allows energy conservation. In summary, subunits CooU, CooM, and, especially, CooK are the likely candidates to possess the key properties which allow the energy conservation not found in the hydrogenase 3 system. Future mutagenesis of these subunits will directly address this issue.

Other gene products required for hydrogenase activity. The genes *cooCTJ* have been implicated in the processing of CODH to its mature, Ni-containing form (28), but they have been shown not to be required for the formation of active CO-induced hydrogenase (22). It is interesting, then, that no genes for hydrogenase-processing have been found in the *coo* operon.

In the *E. coli hyc* operon, the *hycH* and *hycI* genes have been shown to be required for cleavage of a C-terminal peptide during hydrogenase 3 maturation (40, 42). However, CooH does not contain the C-terminal peptide found in other Ni-Fe hydrogenases (22). Thus, the absence of *hycH* and *hycI* homologs in the *coo* operon is consistent with the lack of C-terminal processing of CooH.

In *E. coli*, the *hyp* operon, found adjacent to the *hyc* operon, was shown to be required for the activities of all three Ni-Fe hydrogenases (hydrogenases 1, 2, and 3), and mutations in *hyp* genes prevented further processing and peptide cleavage from the hydrogenase 3 large subunit (HycE) (26). Thus, it is possible that *hyp*-like genes are found at some distant location in the *R. rubrum* chromosome and are required for formation of active Ni-Fe hydrogenases (including the CO-induced enzyme and others) in this organism.

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