JOURNAL OF BACTERIOLOGY, Apr. 1995, p. 2197–2203
0021-9193/95/$04.00+0

Vol. 177, No. 8

Molecular Characterization of the Gene Cluster *coxMSL* Encoding the Molybdenum-Containing Carbon Monoxide Dehydrogenase of *Oligotropha carboxidovorans*

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Received 17 October 1994/Accepted 4 February 1995

The CO dehydrogenase structural genes (*cox*) and *orf4* are clustered in the transcriptional order *coxM*→ *coxS*→ *coxL*→ *orf4* on the 128-kb megaplasmid pHCG3 of the carboxidotroph *Oligotropha carboxidovorans* OM5. Sequence analysis suggested association of molybdopterin cytosine dinucleotide and flavin adenine dinucleotide with CoxL and of the [2Fe-2S] clusters with CoxS.

The molybdenum-containing iron-sulfur flavoprotein CODH (26) is the key enzyme in the chemolithoautotrophic utilization of CO by *Oligotropha carboxidovorans* OM5 (27, 29). The large, medium, and small subunits of CODH are encoded by the structural genes *coxL*, *coxM*, and *coxS*, respectively (27). They reside on the 128-kb megaplasmid pHCG3 (20, 21). We report here the structural characteristics of the *cox* genes and the analysis of the deduced amino acid sequences.

Abbreviations. The following terms have been abbreviated in this report (abbreviations are shown in parentheses): carbon monoxide dehydrogenase (CODH), aldehyde oxidoreductase (MOP), xanthine dehydrogenase (XDH), flavin adenine dinucleotide (FAD), molybdopterin cytosine dinucleotide (MCD), and amino acid (aa).

The strains employed were *O. carboxidovorans* OM5 (DSM 1227 [29]), *Escherichia coli* DH5α (15), and *E. coli* K38 (32).

The basic recombinant DNA techniques used followed standard protocols (3, 25). Plasmids were isolated as described previously (4, 21). The *coxS* probe (oligonucleotide S) was the 64-fold degenerate 17-mer ATRTGNGCYTTNGCCAT derived from the N-terminal protein sequence MAKAHI of CoxS (12, 20). The *coxM* probe (oligonucleotide M) was the 128-fold degenerate 17-mer ATNCKRTGRTARTCRAA derived from the protein sequence FDYHRI of CoxM (12, 20). Deoxyoligonucleotides were 3′ end labeled with digoxigenin-11-ddUTP. Restriction fragment gene probes were labeled with digoxigenin-11-dUTP by the use of random primers (7). With the 1.45-kb *Bam*HI-*Eco*RV fragment of pHCG3 (Fig. 1), hybridizations were carried out at 60°C and washes were carried out with 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (containing 0.1% sodium dodecyl sulfate) at 68°C.

The 4.86-kb *Bam*HI-*Hin*dIII fragment of pHCG3 carrying the structural genes *coxMSL* was inserted into the vector phagemid pBluescript I KS+ (Stratagene, Heidelberg, Germany), yielding phagemid pCDH1 (Fig. 1). Sets of nested deletions were introduced bidirectionally into the cloned region by treatment with exonuclease III-S1 nuclease (10) of the Erase-a-Base kit (Promega, Madison, Wis.). Sequencing of both strands of cloned DNA with the primers T3 and T7 (Stratagene, La Jolla, Calif.) was performed with the *Taq* DyeDeoxy Terminator Cycle Sequencing kit in the model 373A DNA sequencing system (Applied Biosystems, Foster City, Calif.). Each nucleotide position was determined with a redundancy of four. The HUSAR program package (Deutsches Krebsforschungszentrum, Heidelberg, Germany) was employed for sequence analysis.

DNA sequence and molecular organization of the *coxMSL* gene cluster. Southern hybridizations with the oligonucleotides M and S identified the 5′ coding regions of *coxM* on a 0.23-kb *Bam*HI-*Eco*RI fragment and of *coxS* on a 0.4-kb *Eco*RI-*Hin*cII fragment derived from plasmid pHCG3 (Fig. 1). The fragments are part of a 4.86-kb *Bam*HI-*Hin*dIII fragment which was cloned as pCDH1 and completely sequenced in both directions (Fig. 1 and 2). *cox*-positive fragments of pHCG3 were cloned into the T7 RNA polymerase-dependent vector pT7-5. Recombinant plasmids were transformed in *E. coli* K38 containing pGP1-2 (34). Labeling with [^{35}S]methionine and Western blots (immunoblots) revealed expression of CoxS and CoxL.

The nucleotide sequence included four open reading frames arranged as a gene cluster in the transcriptional order (5′) *coxM*→ *coxS*→ *coxL*→ *orf4* (3′) (Fig. 1 and 2). *coxM* (867 bp), *coxS* (501 bp), and *coxL* (2,430 bp) encode the CODH sub-

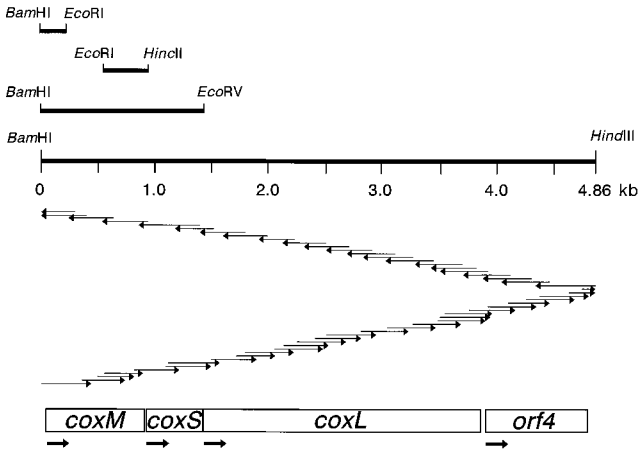

FIG. 1. Physical and genetic map of the *cox* region from pHCG3 of *O. carboxidovorans* OM5. Depicted are fragments detected by or employed for hybridizations, the cloned *Bam*HI-*Hin*dIII fragment of pCDH1, the lengths and orientations of the sequences obtained from the *Bam*HI-*Hin*dIII fragment and its deletion derivatives (thin arrows), and the transcriptional arrangement of *coxMSL* and *orf4* (heavy arrows). coxS and *coxL* overlap by 4 nucleotides (Fig. 2).

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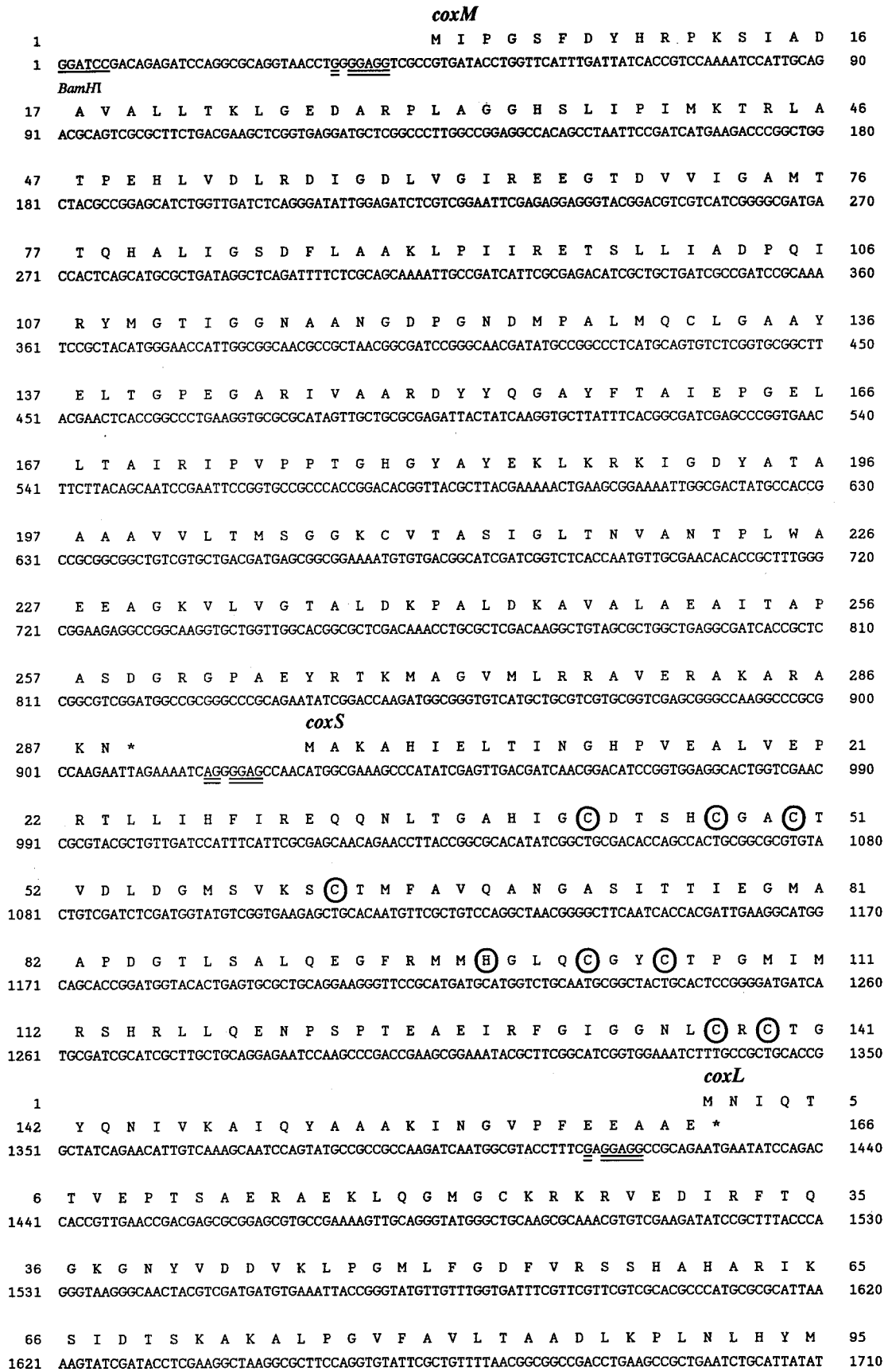
coxM
BamHI
coxS
coxL

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 96  P T L A G D V Q A V L A D E K V L F Q N Q E V A F V V A K D  125
1711 GCCGACGCTGGCTGGCGATGTGCAGGCAGTGCTTGCAGACGAGAAGGTTCTTTTCCAGAATCAGGAGGTTGCCTTTGTAGTGGCGAAAGA 1800

126  R Y V A A D A I E L V E V D Y E P L P V L V D P F K A M E P  155
1801 TCGTTACGTTGCGGCGGACGCGATCGAATTGGTCGAAGTCGATTATGAGCCGCTGCCGGTTCTAGTCGACCCATTCAAGGCAATGGAACC 1890

156  D A P L L R E D I K D K M T G A H G A R K H H N H I F R W E  185
1891 AGATGCACCTCTGCTACGTGAAGATATCAAAGACAAAATGACCGGTGCGCACGGTGCGCGCAAACATCACAACCATATCTTCCGTTGGGA 1980

186  I G D K E G T D A T F A K A E V V S K D M F T Y H R V H P S  215
1981 AATAGGCGATAAGGAAGGCACCGATGCGACCTTCGCCAAAGCCGAAGTCGTGTCAAAAGATATGTTTACCTATCATCGGGTGCATCCGTC 2070

216  P L E T C Q C V A S M D K I K G E L T L W G T F Q A P H V I  245
2071 GCCGCTGGAAACGTGTCAGTGCGTTGCGTCGATGGACAAGATCAAGGGTGAACTGACGTTGTGGGGCACATTCCAGGCGCCGCATGTCAT 2160
                                                    I
246  R T V V S L I S G L P E H K I H V I A P D I G G G F G N K V  275
2161 CCGTACCGTGGTGTCGCTGATCTCGGGTTTGCCGGAGCATAAAATCCACGTCATTGCACCGGACATCGGGGGCGGCTTTGGCAACAAGGT 2250

276  G A Y S G Y V C A V V A S I V L G V P V K W V E D R M E N L  305
2251 GGGCGCTTATTCCGGCTACGTCTGCGCGGTGGTTGCCTCCATCGTGCTGGGCGTGCCCGTGAAGTGGGTCGAAGACCGAATGGAGAACCT 2340

306  S T T S F A R D Y H M T T E L A A T K D G K I L A M R C H V  335
2341 CTCCACGACATCATTTGCGCGCGACTATCATATGACGACAGAACTCGCAGCCACCAAGGACGGCAAGATTCTTGCGATGCGCTGTCACGT 2430

336  L A D H G A F D A C A D P S K W P A G F M N I C T G S Y D M  365
2431 CCTGGCTGATCACGGAGCGTTCGACGCCTGTGCCGATCCATCGAAATGGCCGGCGGGCTTCATGAACATCTGTACCGGCTCCTATGACAT 2520

366  P V A H L A V D G V Y T N K A S G G V A Y R C S F R V T E A  395
2521 GCCGGTGGCACATCTGGCCGTGGATGGTGTCTATACCAACAAAGCGTCCGGCGGCGTAGCCTATCGTTGCTCGTTCCGAGTGACGGAAGC 2610

396  V Y A I E R A I E T L A Q R L E M D S A D L R I K N F I Q P  425
2611 GGTTTATGCCATTGAGCGCGCGATCGAGACGCTGGCGCAGCGGCTCGAGATGGACTCAGCCGATCTACGCATCAAGAACTTTATCCAGCC 2700

426  E Q F P Y M A P L G W E Y D S G N Y P L A M K K A M D T V G  455
2701 GGAGCAGTTCCCTTATATGGCGCCGCTGGGCTGGGAGTACGACAGCGGAAATTATCCACTCGCGATGAAGAAAGCGATGGATACGGTCGG 2790

456  Y H Q L R A E Q K A K Q E A F K R G E T R E I M G I G I S F  485
2791 TTATCATCAGCTTCGTGCTGAACAGAAAGCCAAACAGGAAGCCTTCAAGCGCGGCGAGACACGCGAGATTATGGGCATCGGTATCTCGTT 2880

486  F T E I V G A G P S K N C D I L G V S M F D S A E I R I H P  515
2881 TTTCACCGAGATTGTCGGCGCCGGGCCGTCGAAGAATTGCGATATTCTCGGCGTGTCGATGTTTGACTCGGCGGAAATCCGTATCCATCC 2970

516  T G S V I A R M G T K S Q G Q G H E T T Y A Q I I A T E L G  545
2971 AACCGGTTCAGTGATTGCCCGCATGGGCACCAAGAGCCAGGGCCAGGGGCACGAGACGACCTACGCTCAGATCATCGCCACCGAACTCGG 3060
                                                    II
546  I P A D D I M I E E G N T D T A P Y G L G T Y G S R S T P T  575
3061 TATTCCCGCTGACGACATCATGATCGAAGAAGGCAATACCGACACTGCCCCTTATGGCCTTGGCACTTACGGCTCGCGCTCGACGCCGAC 3150

576  A G A A T A V A A R K I K A K A Q M I A A H M L E V H E G D  605
3151 GGCTGGTGCGGCAACCGCTGTGGCCGCGCGCAAAATCAAAGCCAAGGCGCAGATGATTGCGGCGCACATGCTCGAAGTGCATGAGGGCGA 3240

606  L E W D V D R F R V K G L P E K F K T M K E L A W A S Y N S  635
3241 TTTGGAATGGGACGTGGACCGCTTCCGGGTGAAAGGCCTTCCGGAAAAATTCAAGACCATGAAGGAACTCGCCTGGGCGTCCTACAATAG 3330

636  P P P N L E P G L E A V N Y Y D P P N M T Y P F G A Y F C I  665
3331 TCCGCCGCCCAATCTCGAGCCTGGGCTCGAGGCTGTGAACTATTACGACCCTCCGAATATGACTTATCCGTTCGGTGCCTATTTCTGCAT 3420

666  M D I D V D T G V A K T R R F Y A L D D C G T R I N P M I I  695
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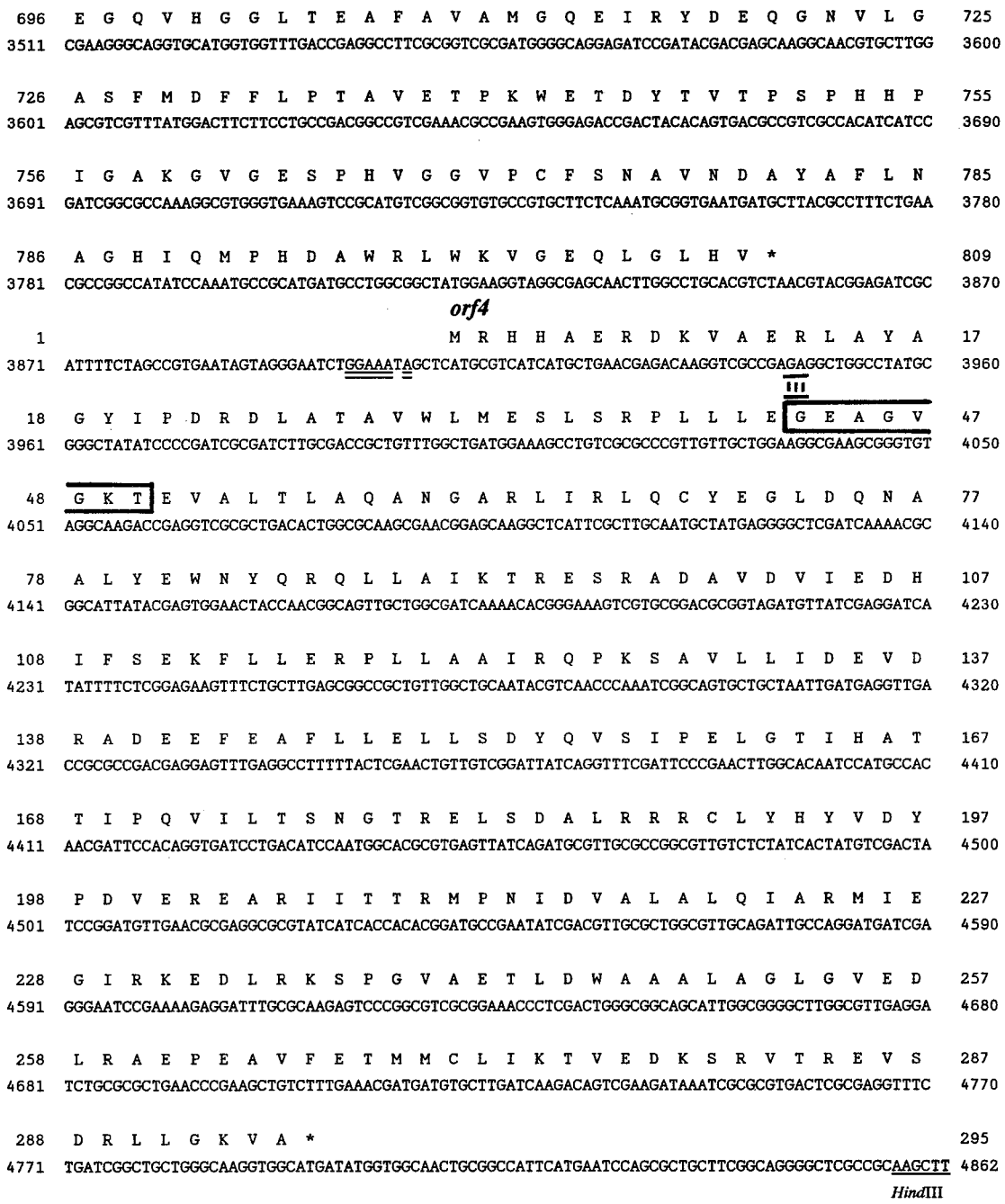

FIG. 2. Nucleotide sequence of the *Bam*HI-*Hin*dIII fragment derived from pHCG3 of *O. carboxidovorans* OM5. The DNA sequence is continuously numbered from the *Bam*HI restriction site to the *Hin*dIII site (both underlined). Deduced amino acid sequences are shown above the corresponding DNA sequences and are separately numbered. Stop codons are indicated by asterisks. The putative ribosomal binding sites of the *cox* genes and *orf4* complementary to the 3′ terminus of the 16S rRNA of *O. carboxidovorans* are double underlined. Presumed ligands of [2Fe-2S] centers are circled. Boxes I to III refer to the MCD-, FAD-, and mononucleotide-binding motifs discussed in the text.

units. The ribosomal binding sites of *coxM*, *coxS*, *coxL*, and *orf4* (888 bp) could be precisely identified on the nucleotide sequence (Fig. 2), since they are complementary to the 3′ terminus of the 16S rRNA of *O. carboxidovorans* (2).

coxM begins with the alternative translational start codon GTG (Fig. 2). The corresponding protein is composed of 288 aa (Fig. 2) and has a molecular weight of 30,239, which agrees with the mean molecular weight of CoxM determined by denaturing polyacrylamide gel electrophoresis of independent preparations of CODHs from *O. carboxidovorans* (molecular weight, 29,700 [27]) or other CO-oxidizing bacteria (molecular weight, 30,200 [27]). The N terminus of the deduced CoxM protein (Fig. 2) matches that of the medium CODH subunit (20), except for the methionine at position 2.

coxS starts 18 nucleotides transcriptionally downstream from the 3′ end of *coxM* (Fig. 2). The peptide inferred from *coxS* consists of 166 aa (Fig. 2). Its molecular weight of 17,792 is comparable to that of 16,000 or 16,800 obtained by denaturing

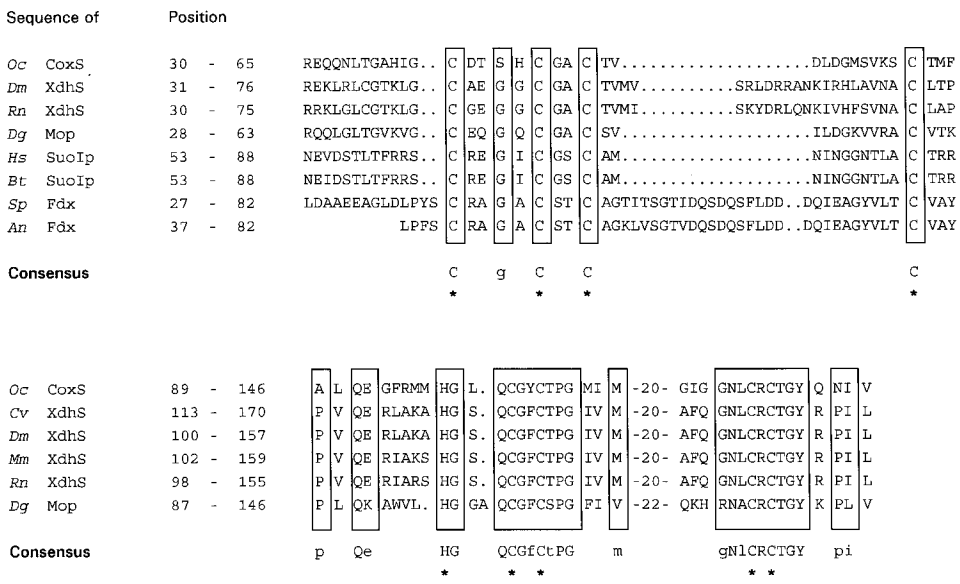

FIG. 3. Comparison of cysteine clusters of CoxS with those of other [2Fe-2S] proteins. The sequences of the CODH small subunit from *O. carboxidovorans* (*Oc* CoxS [Fig. 2]), the XDH 20-kDa fragment of *D. melanogaster* (*Dm* XdhS [24]) or rat liver (*Rn* XdhS [1]), the domain for MOP FeS centers I and II of *D. gigas* (*Dg* Mop [36]), the succinate-ubiquinone oxidoreductase iron-sulfur (Ip) subunit of human liver mitochondria (*Hs* SuoIp [19]) or bovine heart (*Bt* SuoIp [19]), and the [2Fe-2S] ferredoxin of *Spirulina platensis* (*Sp* Fdx [37]) or *Anabaena* strain 7120 (*An* Fdx [33]), as well as the 20-kDa XDH fragment of *C. vicina* (*Cv* XdhS [11]) or mouse liver (*Mm* XdhS [35]), are shown. The positions of the sequences in the individual proteins are indicated. Conserved amino acids are boxed. The letters representing consensus amino acids are capitalized if the amino acids are present in all sequences or in lowercase if the amino acids are present in all except one sequence. Conserved ligands of [2Fe-2S] centers are indicated by asterisks.

polyacrylamide gel electrophoresis of CODH from *O. carboxidovorans* or other CO-oxidizing bacteria, respectively (27). The N termini of CoxS (Fig. 2) and the small CODH subunit (20) are identical.

The first two nucleotides of the translational stop codon TGA of *coxS* are part of the start codon ATG of *coxL*, indicating an overlap of 4 nucleotides between these genes (Fig. 2). The corresponding CoxL protein contains 809 aa (Fig. 2). Its molecular weight of 88,735 matches that of the large CODH subunit from *O. carboxidovorans* (molecular weight, 86,000 [27]) or other carboxidotrophic bacteria (molecular weight, 84,400 [27]). The N terminus of the peptide inferred by *coxL* (Fig. 2) matches that of $CoxL_1$ in most positions (20).

orf4 starts 54 nucleotides transcriptionally downstream from the 3′ end of *coxL* (Fig. 2). The codon usage of *orf4* and that of the *cox* genes are similar. The Orf4 polypeptide is composed of 295 aa (Fig. 2) and has a molecular weight of 33,267. The GGAAATA sequence is considered the ribosomal binding site since it is complementary to the 3′ terminus of the 16S rRNA of *O. carboxidovorans* (2) and is arranged 4 nucleotides upstream from the initiation codon ATG (Fig. 2). The GEAG VGKT motif of Orf4 (Fig. 2, box III, residues 43 to 50) corresponds to the consensus sequence, GXXXXGKT (or S or G) (30), referring to a putative mononucleotide binding site (see Fig. 4).

Refined molecular weight of CODH. The molecular weights of the deduced Cox proteins add up to 273,532, assuming an $(LMS)_2$ subunit structure of CODH. CODH contains FAD, MCD, molybdate (two molecules each), and four [2Fe-2S] centers. The molecular weight of 277,436 of catalytically competent CODH is in agreement with a mean molecular weight of 266,800 suggested by biochemical methods (27).

CoxM, -S, and -L and Orf4 are considered hydrophilic proteins on the basis of hydropathy indexes between 0.04 and −0.15 (moving segment of 7 aa [23]).

CoxS contains motifs indicative of type I and II [2Fe-2S] clusters. Chemical analysis (26) and electron paramagnetic resonance (5) of CODH revealed two distinct [2Fe-2S] clusters. Four of the eight cysteines of CoxS (residues 42, 47, 50, and 62) are arranged in the motif $C\text{-}X_4\text{-}C\text{-}X_2\text{-}C\text{-}X_{11}\text{-}C$ (Fig. 2), which is equivalent to $C\text{-}X_4\text{-}C\text{-}X_2\text{-}C\text{-}X_n\text{-}C$ in structurally characterized ferredoxins (9) and other [2Fe-2S] proteins (Fig. 3). The $C\text{-}X_4\text{-}C\text{-}X_2\text{-}C\text{-}X_{11}\text{-}C$ motif of CoxS (Fig. 3) is homologous to a sequence in *Drosophila melanogaster* XDH which binds the type I [2Fe-2S] center (13, 14).

Putative ligands of the type II [2Fe-2S] center in CoxS are the cysteines 102, 105, 137, and 139 as well as histidine 98, since they are clustered as motifs $H\text{-}X_3\text{-}C\text{-}X_2\text{-}C$ and C-X-C (Fig. 2 and 3). The motifs, their amino acid environment, and their position in the protein chain are highly conserved in CoxS, XDHs, and MOP (Fig. 3). Both iron-sulfur motifs were absent from CoxM and CoxL. The above considerations point to CoxS being the iron-sulfur protein of CODH.

CoxL contains dinucleotide-binding motifs which can be assigned to the MCD-type molybdenum cofactor and FAD. CODH contains the dinucleotide cofactors MCD (16, 28) and FAD (26). The binding sites of dinucleotide cofactors exhibit a βαβ fold of two parallel β strands with glycine residues conserved as GXGXXG/A (30, 40).

The GGGFGN motif of CoxL (Fig. 2, box I, residues 268 to 273) is similar to the dinucleotide-binding consensus sequence described above and to GGGFGG conserved in the 85-kDa domain of XDHs from rats and *Drosophila* species (Fig. 4). We consider GGGFGN the binding site of MCD in CoxL, since replacement of the first glycine of GGGFGG by glutamate in XDH of the *D. melanogaster* mutant G-800 → E resulted in a molybdenum deficiency of the mutant enzyme (14).

The GLGTYG sequence (Fig. 2, box II, residues 564 to 569) in CoxL is identical to dinucleotide-binding motif GXGX XG/A (Fig. 4). It is, by exclusion, the putative FAD binding

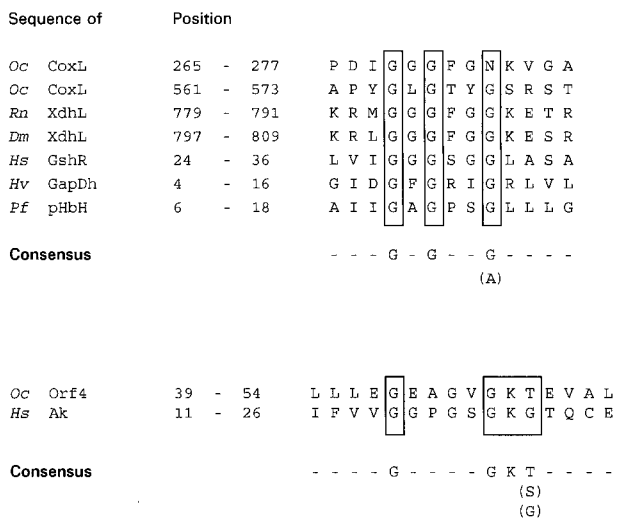

FIG. 4. Comparison of the predicted phosphate-binding sequences in CoxL and Orf4 with those in other nucleotide-binding proteins. The sequence designations refer to the large CODH subunit from *O. carboxidovorans* (*Oc* CoxL [Fig. 2]), the 85-kDa XDH fragment of rat liver (*Rn* XdhL [1]) or *D. melanogaster* (*Dm* XdhL [18]), the glutathione reductase FAD-binding domain of human erythrocytes (*Hs* GshR [22]), the glyceraldehyde 3-phosphate dehydrogenase NAD-binding domain of lobster muscle (*Hv* GapDh [6]), the *p*-hydroxybenzoate hydroxylase FAD-binding domain of *Pseudomonas fluorescens* (*Pf* pHbH [39]), Orf4 from *O. carboxidovorans* (*Oc* Orf4 [Fig. 2]), and the adenylate kinase ATP-binding domain of human skeletal muscle (*Hs* Ak [38]). The positions of the sequences in the individual proteins are indicated. Especially conserved amino acids (30) are boxed.

site. The FAD-binding domain of the ferredoxin-$NADP^+$ reductase type (17) is absent.

CODH is a prototype for the new sequence family of molybdenum hydroxylases. CODH revealed significant sequence similarities to the three domains of XDHs from *D. melanogaster* (aa 1 to 159, 227 to 531, and 1003 to 1277 [18, 24]), *Drosophila pseudoobscura* (aa 8 to 163, 235 to 539, and 1034 to 1284 [31]), and *Calliphora vicina* (aa 29 to 172, 245 to 549, and 1021 to 1295 [11]). Similarities were 76% for CoxS (35% identity) and 68% for CoxM (21% identity). The sequence of CoxL extending from aa 488 to 773 revealed significant similarity (61%) to and identity (26%) with the matching sequences in XDHs. CoxM, CoxS, and CoxL (Fig. 2) and their equivalents in nicotine dehydrogenase of *Arthrobacter nicotinovorans* (8) exhibited similarities of 74% (32% identity), 83% (42% identity), and 71% (33% identity), respectively. CoxS is 74% similar (41% identical) to aa 1 to 166 of MOP from *Desulfovibrio gigas* (36). The homology of CoxL to MOP is 64% (28% identity) for aa 22 to 427 and 72% (27% identity) for aa 645 to 780. CoxM and MOP are not homologous.

The primary structures discussed define a family of closely related prokaryotic and eukaryotic molybdenum hydroxylases. Therefore, the arrangement of molybdenum enzymes into prokaryotic and eukaryotic sequence families (41) seems to be obsolete.

Conserved protein building blocks constitute CODH and the other molybdenum hydroxylases. The subunits of CODH represent conserved building blocks, referred to as M, S, and L. They reappear as subunits in nicotine dehydrogenase or as domains in XDH and MOP; a combined subunit domain structure in a molybdenum hydroxylase has not yet been described. Building blocks S and L are consistently present in the sequenced molybdenum hydroxylases. Building block M is optional, since it is absent from MOP. The M-encoding DNA is inserted between the sequences for building blocks S and L of XDH, whereas it precedes the S- and L-encoding genes of CODH and nicotine dehydrogenase. Accordingly, the genes or nucleotide sequences of molybdenum hydroxylases are arranged in the transcriptional order (5′) (*M*)→*S*→(*M*)→*L* (3′).

Nucleotide sequence accession number. The *cox* and *orf4* sequences are available from the EMBL nucleotide sequence database under the accession number X82447.

We thank Kenneth N. Timmis and Edward Moore for their hospitality during DNA sequence analysis at the Gesellschaft für Biotechnologische Forschung, Braunschweig-Stöckheim, Germany, as well as Annette Krüger and Angelika Arnscheidt for expert technical assistance.

U.S. is a recipient of a travel grant from the Deutsche Forschungsgemeinschaft graduate program III 0 GK-Sp243/3-4. This study was supported by Deutsche Forschungsgemeinschaft grant Me 732/5-2 and the Fonds der Chemischen Industrie (Frankfurt am Main, Germany).

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