Binding of Flavin Adenine Dinucleotide to Molybdenum-containing Carbon Monoxide Dehydrogenase from *Oligotropha carboxidovorans*

STRUCTURAL AND FUNCTIONAL ANALYSIS OF A CARBON MONOXIDE DEHYDROGENASE SPECIES IN WHICH THE NATIVE FLAVOPROTEIN HAS BEEN REPLACED BY ITS RECOMBINANT COUNTERPART PRODUCED IN *ESCHERICHIA COLI*

(Received for publication, August 16, 1999)

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The carbon monoxide (CO) dehydrogenase of *Oligotropha carboxidovorans* is composed of an S-selanylcytisteine-containing 88.7-kDa molybdoprotein (L), a 17.8-kDa iron-sulfur protein (S), and a 30.2-kDa flavoprotein (M) in a (LMS)₃ subunit structure. The flavoprotein could be removed from CO dehydrogenase by dissociation with sodium dodecylsulfate. The resulting M(LS)₂- or LS₂-structured CO dehydrogenase species could be reconstituted with the recombinant apoflavoprotein produced in *Escherichia coli*. The formation of the heterotrimeric complex composed of the apoflavoprotein, the molybdoprotein, and the iron-sulfur protein involves structural changes that translate into the conversion of the apoflavoprotein from non-FAD binding to FAD binding.

Binding of FAD to the reconstituted deflavo (LMS)₂ species occurred with second-order kinetics (Kₚ = 1350 M⁻¹ s⁻¹) and high affinity (Kᵦ = 1.0 × 10⁻⁹ M). The structure of the resulting flavo (LMS)₂ species at a 2.8 Å resolution established the same fold and binding of the flavoprotein as in wild-type CO dehydrogenase, whereas the S-selanylcytisteine 388 in the active-site loop on the molybdoprotein was disordered. In addition, the structural changes related to heterotrimeric complex formation or FAD binding were transmitted to the iron-sulfur protein and could be monitored by EPR. The type Π 2Fe:2S center was identified in the N-terminal domain and the type I center in the C-terminal domain of the iron-sulfur protein.

Carbon monoxide (CO) dehydrogenase (EC 1.2.99.2) from *Oligotropha carboxidovorans* is a selenium-containing molybd-iron-sulfur flavoprotein that catalyzes the oxidation of CO with H₂O yielding CO₂, two electrons, and two H⁺ (1, 2). In its membrane-associated state, CO dehydrogenase is involved in energy transduction. The electrons formed through CO oxidation are channeled via cytochrome b₅₅₃ into a CO-insensitive respiratory chain that generates a proton motive force (1, 3). In its soluble state, CO dehydrogenase transfers the electrons to O₂, thereby producing hydrogen peroxide (H₂O₂) and superoxide (O₂⁻).

CO dehydrogenase is a prototype of the molybdenum hydroxylase sequence family, which includes the aldehyde oxidoreductase (Mop) from *Desulfovibrio gigas* as well as enzymes, oxidizing aromatic N-heterocyclic compounds such as xanthine dehydrogenases/oxidases from different procarccytic and eucaryotic sources, quinoline 2-oxidoreductase from *Pseudomonas putida*, and nicotine dehydrogenase from *Arthrobacter nicotinovorans* (5, 6). The crystal structure of CO dehydrogenase from *O. carboxidovorans* has been solved at a 2.2-Å resolution (7) and shows a dimer of heterotrimers (see Fig. 1). Each heterotrimer is composed of a 88.7-kDa molybdoprotein (L), a 30.2-kDa flavoprotein (M), and a 17.8-kDa iron-sulfur protein (S).

The molybdoprotein of CO dehydrogenase carries the molybdopterin cytosine dinucleotide (MCD)³⁻-type of molybdenum cofactor and the unique active-site loop Gly³⁸³.Val-Ala-Tyr-Arg-Cys-Ser-Phe-Arg³⁰¹, which positions the catalytically essential S-selanylcysteine 388 in a distance of 3.7 Å to the molybdenum ion. The iron-sulfur protein carries the two 2Fe:2S centers of CO dehydrogenase. One center is located in the N-terminal domain and the other is located in the C-terminal domain of the iron-sulfur protein (see Fig. 1). The N-terminal 2Fe:2S center is positioned at the interface between the iron-sulfur protein and the flavoprotein and is exposed to the solvent. It lies 8.7 Å apart from the FAD in the flavoprotein (Fig. 1). The flavoprotein binds the FAD as a prosthetic group in a fold formed by its N-terminal and middle domains. Binding of FAD involves strong interactions of two vanillyl alcohol oxidase-like double glycine motifs on each of the two domains, mainly with the pyrophosphate moiety of the FAD. On the N-terminal domain a loop between the sheet β2 and the helix α₂ of a βαβ unit (P-loop) containing the motif Ala³⁸⁴-Gly³⁸⁵-Gly³⁸⁴-His³⁸⁵-Ser³⁸⁶ is involved, and on the middle domain a small a-helix (α7) containing the major part of the motif Thr¹¹¹,Ile¹¹²,Gly¹¹³-Gly¹¹⁴-Asn¹¹⁵ is involved. The C-terminal domain of the flavoprotein carries a Tyr¹⁰⁶ residue, which shields the central part of the isoalloxazine ring from the solvent (7).

This paper presents a structural and functional analysis of FAD binding in CO dehydrogenase employing a CO dehydro-
genase species in which the native flavoprotein has been replaced by a recombinant one. The FAD binding site on the free flavoprotein is not able to bind FAD. However, in the heterotricmeric complex of the flavoprotein with the iron-sulfur protein, the flavin binding site is capable to integrate FAD.

MATERIALS AND METHODS

Bacterial Strains, Culture Conditions, and Overexpression—*O. carboxydovorans* (DSM 1227) (formerly *Pseudomonas carboxydovorans* OM5 (8)) was mass-cultivated in 50-liter fermentors under chemolithoautotrophic conditions with CO as a sole source of carbon and energy (9). *Escherichia coli* DH5α (10) and *E. coli* BL21(DE3) (11) were grown in LB medium (12) supplemented with ampicillin (200 μg ml⁻¹) and kanamycin (100 μg ml⁻¹). The plasmids pREP4 (Qlegen), pBluescript I KS⁺ (13), and pET11a (Novagen) were used for heterologous expression, and pBluescript I KS⁺ and pET16b (Novagen) were used for sequence analysis.

A 0.95-kilobase BamHI-HincII fragment of pCDH1 containing the CO dehydrogenase structural gene coxM (5) was cloned into the vector pBluescript I KS⁺, yielding the plasmid pSK7. This plasmid was used as a template for polymerase chain reaction to amplify the first 244 nucleotides of coxM, using the following primers. The 54-mer 5’-TGTAGAGGCCTCCATATGATCCTGGTTTACATTGTGAT-3’ was used to generate a NdeI site by replacing the start codon GTG by ATG and to alter the second codon for isoleucin from ATA to ATC, resulting in a suitable codon usage for expression in *E. coli* (14). The 25-mer 5’-ATTATCGCATCCTGAGTGGTCTCATG-3’ contained an XhoI fragment from pSK7, which contained the remaining part of coxM, yielding pETS1. The NdeI-BamHI fragment from pETS1 was cloned into pET11a, yielding pETSK2, which was then transformed into *E. coli* BL21(DE3)/pREP4 (11) yielding *E. coli* BL21(DE3)/pREP4/pETSK2. Overexpression of CoxM was induced with 2 mM isopropyl-β-D-thiogalactopyranoside in LB medium at 20 °C and at an A₆₀₀ of 0.7.

**Assay of CO Dehydrogenase**—The oxidation of CO or H₂ by CO dehydrogenase was coupled to the reduction of 50 μM methylene blue (2), 100 μM 1-phenyl-2-(4-isodophenyl)-3-(4-nitrophenyl)-2H-tetrazolium chloride (INT), 20 μM 1-methoxyphenazine methosulfate (MPMS) (15), or 100 μM 2,6-dichlorophenolindophenol in 50 mM NaH₂PO₄-NaOH (pH 7.2) and followed spectrophotometrically at 30 °C employing ε₆₅₀ of 37.11 mm⁻¹ cm⁻¹ (methylene blue), ε₄₆₀ of 17.98 mm⁻¹ cm⁻¹ (INT/MPMS), and ε₆₃₀ of 16.10 mm⁻¹ cm⁻¹ (2,6-dichlorophenolindophenol). Assays of 1-ml total volume were flushed with pure CO or H₂ in screw-capped cuvettes provided with a rubber septum. Reactions were
initiated by injection of 20 μl of enzyme.

**Preparation and Purification of Proteins**—All purification steps were carried out below 4 °C. For purification of CO dehydrogenase cell paste of CO-grown *O. carboxidovorans* (about 100 g wet mass) was suspended in 200 ml of 50 mM Hepes/NaOH (pH 7.5) (buffer A) containing 1 mM Na2EDTA, 0.2 mM phenylmethanesulfonyl fluoride, and 5 mM DTT, disrupted in a high pressure homogenizer (Rannie AS), and subjected to low spin centrifugation, yielding crude extracts. Cytoplasmic fractions were prepared from crude extracts by ultracentrifugation for 2 h at 100,000 × g. Anion exchange chromatography was on Source 30 Q (Amersham Pharmacia Biotech) equilibrated with buffer A. Cytoplasmic fractions were applied to the column (dimensions 15 cm by 5 cm) and eluted with 640 ml of buffer A followed by 1440 ml of a linear gradient of 0 to 1 M NaCl in buffer A. Fractions with CO dehydrogenase activity were pooled, supplemented with 1.3 mM ammonium sulfate and 1 mM Na2EDTA, and gently stirred for 60 min. Precipitated protein was removed by low spin centrifugation, and the supernatant (220 ml) was loaded onto a hydrophobic interaction chromatography column (butyl-Sepharose 4 Fast Flow) (Amersham Pharmacia Biotech) (dimensions 6.5 cm by 5 cm) that has been equilibrated with 0.85 mM ammonium sulfate in buffer A containing 1 mM Na2EDTA. Proteins were desorbed with 360 ml of equilibration buffer followed by 840 ml of a linear gradient combining decreasing ammonium sulfate concentrations (0.85 to 0 mM) with increasing 2-propanol concentrations (0 to 20%, vol/vol) in buffer B containing 1.5 mM diithothreitol. The compartments were pooled, loaded onto Source 30 Q column (dimensions 12.5 cm by 2.6 cm), and eluted with 540 ml of a linear gradient of 0 to 1 M NaCl in buffer A. Purified CO dehydrogenase species with the subunit compositions (LMS)2, M(LS)2, and (LS)5 proteins were pooled separately, concentrated, desalted by gel filtration in buffer A, and concentrated by ultrafiltration. The purified enzyme was frozen in liquid nitrogen and kept at −80 °C until use.

For the preparation of enzyme dissociation of the flavoprotein, typically 200 mg of CO dehydrogenase in 75 ml of buffer A containing 1% (mass/vol) SDS were incubated for 45 min at 30 °C followed by gel filtration on Sephadex G-75 (Amersham Pharmacia Biotech, column dimensions 35 cm by 5 cm). Excluded CO dehydrogenase species were pooled, loaded onto a Source 30 Q column (dimensions 12.5 cm by 2.6 cm), and eluted with 540 ml of a linear gradient of 0 to 1 M NaCl in buffer A. Purified CO dehydrogenase species with the subunit compositions (LMS)2, (M(LS)2), and (LS)5 proteins were pooled separately, concentrated, desalted by gel filtration, and stored as described above.

Recombinant apoflavoprotein was purified from *E. coli* BL21(DE3)/pREP4/pETSK2 as follows. 12 g of cell paste suspended in 40 ml of buffer B (50 mM Tris/HCl, pH 8.7) containing 2 mg of DNase I were ultrasonicated (model Labsonic U, probe tip 40 T, Braun Biotech), centrifuged, desalted, and stored as described above.

CD spectra were recorded on a spectropolarimeter (J-600, Jasco). Contents of secondary structural elements were calculated as described (26).

RESULTS AND DISCUSSION

**The Recombinant M Subunit of CO Dehydrogenase Produced in E. coli Is Deflavo and Does Not Bind FAD**—The medium (M) subunit of CO dehydrogenase was heterologously overexpressed. After induction of *E. coli* BL21(DE3)/pREP4/pETSK2 with isopropyl-β-D-thiogalactopyranoside for at least 6 h, the recombinant protein comprised 30 to 40% of the total cell protein and comigrated with the authentic M subunit (Fig. 2). Induction at 20 °C and a pH of 8.7 of the sonication buffer were essential to obtain maximum yields of the soluble recombinant M subunit (25% soluble and 75% aggregated).

The recombinant M subunit was purified 8-fold by ultracentrifugation, anion exchange chromatography, and gel filtration on a Bio-Gel P-60 column (Bio-Rad) with an electrophoretic purity of 50% and a yield of 25% (Fig. 2B, lane 4). The protein was almost exclusively in the dimeric form and exhibited the flavin and iron, molybdenum, and acid-labile sulfur content characteristic of the wild-type flavoprotein (5). The apparent molecular masses of the recombinant M subunit determined from SDS-
Protein (with the intermediate formation of a partially active 247-kDa two 30-kDa M subunits (Fig. 3, band IV) (mass/vol) SDS and analyzed by nondenaturing PAGE (0.18 nmol of CO dehydrogenase species/lane) (C). Lane 1, native CO dehydrogenase; lane 2, M(LS)₂ species prepared from native CO dehydrogenase; lane 3, (LS)₂ species prepared from native CO dehydrogenase; lane 4, recombinant M subunit from E. coli; lane 5, (LMS)₂, reconstituted from M(LS)₂ and the recombinant M subunit; lane 6, (LMS)₂, reconstituted from (LS)₂ and the recombinant M subunit; lane 7, native CO dehydrogenase. CoxL, CoxM, and CoxS refer to the 89-, 30-, and 18-kDa CO dehydrogenase polypeptides, respectively.

PAGE (30 kDa, Fig. 2B, lane 4) or by gel filtration (41 kDa) indicate a monomeric subunit structure. The recombinant M subunit revealed a far-UV CD spectrum with a maximum at 190 nm and a trough extending from 210 to 225 nm, which is typical of α-helix/β-sheet proteins and indicates that it was folded. The recombinant M subunit revealed the following percentages of secondary structural elements: α-helices (24 ± 1), antiparallel β-sheets (20 ± 1), parallel β-sheets (4 ± 1), β-turns (22 ± 1), and other elements (30 ± 1). Its isoelectric point was 7.4. The recombinant M subunit was deflavo on the basis of its UV-visible absorption spectrum (protein concentration of 0.8 mg/ml, ε₂₇₇ = 19.6 mM⁻¹ cm⁻¹) or the spectrum of trichloroacetic acid supernatants. When recombinant M protein was incubated for 18 h at 4 °C with a 50-fold molar excess of FAD or FMN and subjected to gel filtration, and the protein fraction was analyzed spectrophotometrically, no flavins were identified. These data indicate that the recombinant M subunit is devoid of flavins and has no affinity for FAD or FMN.

Removal of the M Subunit from Native CO Dehydrogenase and Characterization of the Resulting Protein Species—The anionic detergent SDS was found to remove specifically one or both M subunits from CO dehydrogenase, resulting in the formation of proteins of different mobility, designated band I to IV (Fig. 3A). The formation of the band I to IV proteins was paralleled by a time-dependent, gradual loss of CO dehydrogenase activity (Fig. 3B). The 277-kDa band (Fig. 3, band I) represented catalytically competent CO dehydrogenase. It was converted into an inactive 217-kDa protein (Fig. 3, band III) with the intermediate formation of a partially active 247-kDa protein (band II). The data indicate a sequential release of the two 30-kDa M subunits (Fig. 3, band IV) from native (LMS)₂-structured CO dehydrogenase, resulting in the formation of an intermediate M(LS)₂ species (band II) and a final (LS)₂ species (band III). Band IV (Fig. 3) was identified as the M subunit on the basis of its correct N terminus of MIPGSFDY (5) and migration as a 30-kDa polypeptide upon SDS-PAGE (data not shown). The low mobility of the M subunit (Fig. 3, band IV) indicates that it became partially or fully unfolded under the experimental conditions applied.

The purified band I protein (Fig. 2C, lane 1) had a (LMS)₂ subunit structure on the basis of 89-, 30-, and 18-kDa polypeptides appearing at a 1:0.8:0.8 molar ratio upon SDS-PAGE (Fig. 2B, lane 1) and represents native CO dehydrogenase. The purified band II protein (Fig. 2C, lane 2) had an M(LS)₂ subunit structure on the basis of 89-, 30-, and 18-kDa polypeptides appearing at a 1:0.5:0.9 molar ratio (Fig. 2B, lane 2). The purified band III protein (Fig. 2C, lane 3) had a (LS)₂ subunit structure on the basis of 89- and 18-kDa polypeptides appear-
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Specific activities are given in milliunits mg⁻¹, corresponding to 1 nmol of CO or H₂ reduced min⁻¹ mg⁻¹ of protein. The species M(LS)₂ and (LS)₂ were produced by controlled dissociation of native CO dehydrogenase with SDS; the flavo (LMS)₂ species was obtained by reconstitution of (LS)₂ with the recombinant M subunit and FAD. The contents of form A are given relative to native CO dehydrogenase, set to 100; all other contents are in nmol/ml of protein; values with S.D. are the mean from three independent determinations. DCPIP, 2,6-dichlorophenolindophenol.

| Table I | Activities and cofactor contents of different CO dehydrogenase species |
|---------|---------------------------------------------------------------------|
| Species of CO dehydrogenase | Native | M(LS)₂ | (LS)₂ | Flavo (LMS)₂ |
| Specific activities | | | | |
| CO → INT/MPMS | 4026 ± 115 | 1464 ± 73 | <1 | <1 |
| CO → DCPIP | 528 ± 18 | 204 ± 11 | <1 | <1 |
| H₂ → methylene blue | 197 ± 12 | 77 ± 8 | <1 | <1 |
| Contents | | | | |
| FAD | 1.97 ± 0.09 | 0.84 ± 0.07 | <0.02 | 2.10 ± 0.06 |
| Iron | 7.71 ± 0.37 | 7.70 ± 0.27 | 8.34 ± 0.51 | 7.91 ± 0.34 |
| Acid-labile sulfur | 7.79 ± 0.27 | 8.10 ± 0.14 | 8.97 ± 0.47 | 7.96 ± 0.22 |
| Molybdenum | 1.74 ± 0.11 | 1.85 ± 0.09 | 1.23 ± 0.05 | 1.21 ± 0.08 |
| Selenium | 0.43 ± 0.01 | 0.29 | 0.27 | 0.26 |
| 5'-CMP | 1.65 ± 0.06 | 1.46 ± 0.04 | 1.22 ± 0.10 | 1.24 ± 0.12 |
| Form A | 100 ± 18 | 92 ± 11 | 79 ± 9 | 74 ± 7 |

![Fig. 4](image-url)

**Fig. 4.** UV-visible absorption spectra of protein species obtained by controlled dissociation of CO dehydrogenase (A) or reconstitution with recombinant M and FAD (B). A, spectra of purified CO dehydrogenase species. a, native CO dehydrogenase; b, M(LS)₂; c, (LS)₂; d, difference of spectrum a minus spectrum c; e, difference of spectrum a minus spectrum b. B, spectra of reconstituted CO dehydrogenase species. e, (LS)₂; f, deflavo (LMS)₂ reconstituted from recombinant M subunit and (LS)₂; g, flavo (LMS)₂ reconstituted from deflavo (LMS)₂ and FAD; h, difference of spectrum g minus spectrum f. Spectra were recorded on solutions of 2.6 μM CO dehydrogenase species (panel A) or 1.69 μM CO dehydrogenase species (panel B) in 50 mM Hepes/NaOH (pH 7.2), respectively.

We have shown that controlled dissociation of CO dehydrogenase with SDS produces a rather rigid deflavo (LS)₂ substructure from which both M subunits have been removed (Figs. 2 and 4). The noncovalent interactions of the individual L and S subunits and of two LS monomers are apparently rather tight and are contrasted by only weak binding of the M subunits to (LS)₂. The existence of a discrete M(LS)₂ species containing FAD at a 1:1 molar ratio (Figs. 2 and 4) and exhibiting about 50% full enzymic activity (Table I) indicates that each LMS heterotrimer in CO dehydrogenase represents an independent catalytic unit as concluded before from a molybdenum to molybdenum distance of 53 Å (7). The hierarchy of subunit interactions resolved here for CO dehydrogenase is also apparent with Mop from *D. gigas*. Mop is composed of two covalently linked protein domains that are equivalent to the L and the S subunits of CO dehydrogenase. The flavodoxin, employed by Mop as electron acceptor, is not a permanent constituent of the enzyme (33). (LS)₂ derived from CO dehydrogenase and Mop show high sequence similarities in their small subunit or domain (70%, S of CO dehydrogenase and amino acids 1 to 166 of Mop) as well as their large subunit or domain (58%, L of CO dehydrogenase and amino acids 167 to 809 of Mop). Circular dichroism measurements on the (LS)₂ species of CO dehydrogenase revealed a content of 30% α-helices and 25% β-sheets, which compares to the content of secondary structural elements of Mop (28% α-helices, 21% β-sheets) and agrees with a very similar fold of Mop and LS in (LMS)₂-structured CO dehydrogenase (7).

Reconstitution of the (LS)₂ Species with the Recombinant M Subunit Leads to a deflavo (LMS)₂ Species That Binds FAD—The 217-kDa (LS)₂ species (Fig. 2C, lane 3) and the recombinant 30-kDa M subunit (Fig. 2C, lane 4) spontaneously reconstituted to a 277-kDa protein (Fig. 2C, lane 6) that contained M in a (LMS)₂ subunit composition (Fig. 2B, lane 6) and was devoid of FAD on the basis of its UV-visible absorption spectrum typical of deflavo CO dehydrogenase (Fig. 4B, spectrum f). Therefore, the reconstituted protein is termed deflavo (LMS)₂. Reconstitutions of M(LS)₂ (Fig. 2C, lane 2) with the recombi-
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Fig. 5. Fluorometric analysis of FAD binding to the reconstituted deflavo (LMS)$_2$ species of CO dehydrogenase. Interactions of FAD with the indicated CO dehydrogenase species were examined at 525-nm fluorescence emission (excitation at 450 nm, temperature 8 °C). Panel A: a, 6.47 μM FAD; b, 6.47 μM FAD plus 3.24 μM deflavo (LMS)$_2$ added at 240 s; c, 3.24 μM CO dehydrogenase. Inset of panel A, determination of the second-order rate constant of FAD binding to reconstituted deflavo (LMS)$_2$. Linearization analysis was applied to the fluorescence kinetics of curve b. [H] concentration of the reconstituted LMS monomer (holoenzyme); $c_0$, initial concentration of FAD. For details, see “Materials and Methods.” Panel B: a, 6.47 μM FAD; b, 6.47 μM FAD plus 6.47 μM recombinant M subunit added at 240 s; c, 6.47 μM FAD plus 6.47 μM recombinant M added at 240 s plus 3.24 μM (LMS)$_2$ added at 480 s.

The association constant of FAD with deflavo (LMS)$_2$ of $1.0 \times 10^9$ M$^{-1}$ ($K_d = 1.0 \times 10^{-9} M$), determined from the quenching of flavin fluorescence in equilibrium binding experiments, groups CO dehydrogenase among the most efficient noncovalently flavin-binding proteins (36).

Binding of Compounds other than FAD to the Deflavo (LMS)$_2$ Species—To check the ability of the deflavo (LMS)$_2$ species to integrate compounds representing structural elements of FAD, the protein was incubated for 300 min with a 50-fold molar excess of these compounds and analyzed for bound compounds. Under these conditions the following relative ranking of binding was observed (mol of compound/mol of (LMS)$_2$): FAD (2.10 ± 0.06) > ADP (1.94 ± 0.10) > FMN (1.52 ± 0.02) > ATP (1.26 ± 0.05). Riboflavin, AMP, CDP, and GDP did not bind, since less than 0.05 mol of these compounds were resolved/mol of (LMS)$_2$. That the strongest interactions of FAD with the protein involve the pyrophosphate linkage is indicated by the ability of deflavo (LMS)$_2$ to bind stoichiometric amounts of ADP and substoichiometric amounts of ATP and FMN and the inability to bind riboflavin and AMP. The isoalloxazine ring, the ribitol, and the mononucleotide provide only weak or no interactions at all. The adenine moiety apparently determines the specificity for FAD since deflavo (LMS)$_2$ has no affinity for CDP or GDP. The binding of 0.99 ± 0.08 mol of 8-azido-ATP/mol of (LMS)$_2$ offers the possibility to establish photochemical cross-links. Native CO dehydrogenase exhibited no binding affinity for ADP or ATP, and both compounds were not hydrolyzed by native CO dehydrogenase or the deflavo (LMS)$_2$ species.

EPR of the Type II Iron Sulfur Center Reports the Interaction of the Subunits M and S in CO Dehydrogenase—The 2Fe:2S center in the N-terminal domain of the S subunit is located at the interface that interacts with the M subunit and is exposed to the solvent (Fig. 1A). The C-terminal 2Fe:2S center is located at the interface of the subunits L and S and is buried in the protein structure 11 Å below the surface of the protein (Fig. 1A). It is, therefore, immediately obvious that the environment of the N-terminal iron-sulfur center is likely to be altered by the presence or absence of the M subunit, whereas the C-terminal center is unlikely to be affected. EPR has been proven a most suitable technique for the analysis of the two iron-sulfur centers in CO dehydrogenase (27–29). Fig. 6A shows the EPR-spectra of native CO dehydrogenase. At 49 K almost only the signals originating from the type I 2Fe:2S center were detected ($g_1 = 2.024, g_2 = 1.947, g_3 = 1.901, g_4 = 1.957$), whereas at 16 K, a combined EPR spectrum originating from the type I ($g_1 = 2.025, g_2 = 1.947, g_3 = 1.900, g_4 = 1.957$) and type II ($g_1 = 2.159$) 2Fe:2S centers was observed. Fig. 6B shows the iron-sulfur EPR spectra of the (LS)$_2$ species, which differ from those of native CO dehydrogenase depicted in Fig. 6A. Fig. 6B shows at 49 K a spectrum that originates from the type I and type II 2Fe:2S centers. The signals of the (LS)$_2$ protein at $g_1 = 2.024, g_2 = 1.948, g_3 = 1.903, g_4 = 1.958$ are the same as in native CO dehydrogenase (Fig. 6A, 49 K) and must, therefore, be ascribed to the type I 2Fe:2S center, whereas the signal at $g = 2.048$ is...
not visible in native CO dehydrogenase (Fig. 6A, 49 K). It is apparent that the removal of the M subunits has no effect on the EPR properties of the type I center in (LS)_2. Fig. 6C shows the EPR difference spectrum obtained by subtracting various amounts of the pure type I spectrum of native CO dehydrogenase at 49 K (Fig. 6A) from the combined EPR spectrum of the (LS)_2 species at 49 K (Fig. 6B). The resulting EPR difference spectrum is rhombic and originates from the type II 2Fe:2S center of (LS)_2. The g values (g_1 = 2.048, g_2 = 1.949, g_3 = 1.915, g_{av} = 1.971) of the difference spectrum are similar to those reported for the type II 2Fe:2S center in Mop (g_1 = 2.057, g_2 = 1.970, g_3 = 1.900, g_{av} = 1.976 (30, 38)), and the temperature dependence of the difference spectrum of the (LS)_2 species (Fig. 6C) and Mop is the same. The type II signal of (LS)_2 was fully sharpened at 43 K and scarcely detectable at 60 K. In Mop, the type II signal was fully sharpened at 45 K and scarcely detectable at 70 K (30, 38). In contrast, the type II signal of native CO dehydrogenase was fully sharpened at 16 K and scarcely detectable at 26 K. The data can be explained by assuming that the removal of the M subunits from native CO dehydrogenase leads to a Mop-like environment of the type II center in (LS)_2. Fig. 6E shows the EPR spectra of the reconstituted flavo (LMS)_2 species at 49 K (g_1 = 2.024, g_2 = 1.951, g_3 = 1.900, g_{av} = 1.958) and 16 K (type I: g_1 = 2.024, g_2 = 1.950, g_3 = 1.900, g_{av} = 1.958, type II: g_1 = 2.165). The spectra are almost indistinguishable from those of native CO dehydrogenase under the same conditions (Fig. 6A), indicating that the Mop-like type II 2Fe:2S center in (LS)_2 has been rearranged to that of native CO dehydrogenase.

The type II 2Fe:2S centers are also weak reporters of FAD binding to deflavo (LMS)_2. This can be seen by comparing the EPR spectra shown in Fig. 6, D and E, which show at 16 K a shift of the type II signal at g_1 = 2.190 in deflavo (LMS)_2 (Fig. 6D) to g_1 = 2.165 in flavo (LMS)_2 (Fig. 6E). The shift can be explained by an induced fit-like mechanism of flavin binding to the M subunit, where cleft closure effects conformational changes in the environment of the type II center. The FAD binding cleft is formed by the N-terminal and the middle domain of the M subunit (Fig. 1A), where the strongest interactions originate from the FAD pyrophosphate with the double-glycine motifs Ala^32-Gly-Gly-His-Ser^36 in the N-terminal and Thr^111-Ile-Gly-Gly-Asn^115 in the middle domain (7). Since both domains also contribute to the interface interacting with the S subunit, it is likely that they report cleft closure to the N-terminal 2Fe:2S center.

The data discussed so far show that interactions of the proteins (LS)_2 and M are reported only by the type II 2Fe:2S center and have no effect on the type I center. It is apparent from the three-dimensional structure of CO dehydrogenase that the M subunit binds to the N-terminal domain of the S subunit (Fig.
1A). This identifies the type II 2Fe:2S center in the N-terminal domain and the type I center in the C-terminal domain of the S subunit. The intramolecular electron transport pathway from CO to FAD can, therefore, be described as shown in Fig. 1D. This assignment is in accordance with magnetic interaction observed for the molybdenum center and the type I center of xanthine oxidase (39, 40) and the absence of magnetic interaction between the molybdenum center and the type II center or between the molybdenum center and FAD (41).

The N-terminal domain of the S subunit in CO dehydrogenase contains a polypeptide fold that is also found in 2Fe:2S ferredoxins (7). Our data show that the unusual features of the type II center in native CO dehydrogenase (gav of 2.023; detectable at temperatures below 26 K) in comparison to 2Fe:2S ferredoxins (gav around 1.97; detectable at temperatures above 26 K) are due to the interactions of the M subunit with the N-terminal domain of the S subunit.

**Functional and Structural Characterization of the Reconstituted CO Dehydrogenase Species**—The removal of the flavoprotein from native CO dehydrogenase was paralleled by a gradual loss of the catalytic activities of the enzyme (Fig. 2, Table I). Reconstitution of (LS)2 with the recombinant apoflavoprotein and FAD yielded the flavo (LMS)2 species but did not restore amounts (Table I). In the reconstituted flavo (LMS)2 species the catalytic activities, although the cofactors and metals of (LMS)2 species. In addition to the disordered active-site loop, 120 K). Fig. 1, F–H.

Another important function of heterotrimeric complex formation is the structural stabilization of the fragile apoflavoprotein. It is apparent that the formation of the heterotrimeric complex is essential for the proper biological functioning of CO dehydrogenase, perhaps also by modulating the redox potentials of the FAD and the type II iron-sulfur center.

**Acknowledgments**—We thank Dieter Gawlik (Hahn-Meitner-Institut, Berlin) for neutron activation analysis and Franz Xavier Schmid, Christian Scholz, and Volker Sieber (Universität Bayreuth) for help with fluorescence and CD spectroscopy.

**REFERENCES**

1. Meyer, O., Franzeke, K., and Morsdorf, G. (1993) in Microbial Growth on C Compounds (Murrell, J. C., and Kelly D. P., eds) pp. 433–459, Intercept Scientific Publication, Andover, United Kingdom.

2. Meyer, O. (1982) J. Biol. Chem. 257, 1333–1341

3. Cypionka, H., and Meyer, O. (1983) J. Bacteriol. 156, 1178–1187

4. Ribe, M. Gauffi, D., and Meyer, O. (1997) J. Biol. Chem. 272, 26627–26633

5. Schubel, U., Kraut, M., Morsdorf, G., and Meyer, O. (1995) J. Bacteriol. 177, 2197–2203

6. Hille, R. (1996) Chem. Rev. 96, 2757–2816

7. Denke, H., Gremer, L., Meyer, O., and Huber, R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 8884–8889

8. Meyer, O., Stackebrandt, E., and Auling, G. (1993) Syst. Appl. Microbiol. 16, 390–395

9. Kisselting, M., and Meyer, O. (1982) FEBS Microbiol. Lett. 13, 333–338

10. Jessee J. (1986) in Focus 8/4 pp. 9–10, Life Technologies, Inc., Gaithersburg, MD

11. Studier, F. W., and Moffatt, B. A. (1986) J. Mol. Biol. 189, 113–130

12. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

13. Short, J. M., Fernandez, J. M., Sorge, J. A., and Huse, W. D. (1988) Nucleic Acids Res. 16, 7653–7660

14. Grosjean, H., and Fiers, W. (1982) Gene 18, 199–209

15. Kraut, M., Hugendieck, I., Herwig, S., and Meyer, O. (1989) Arch. Microbiol. 152, 335–341

16. Nishikimi, M., and Kyogoku, Y. (1973) J. Biochem. (Tokyo) 73, 1233–1242

17. Zanetti, G., Cidaria, D., and Curti, B. (1982) Eur. J. Biochem. 126, 453–458

18. Johnson, J. L., and Rajagopalan, K. V. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 6583–6586

19. Gremer, L., and Meyer, O. (1996) Eur. J. Biochem. 238, 862–866

20. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254

21. Buescher, G., Bolze, H. G., Bacher, C., Czok, R., Garbade, K. H., Meyer-Arendt, E., and Pfleiderer, G. (1953) Z. Naturforsch. 8B, 555–557

22. Kaelin, U. K. (1979) Nature 227, 680–683

23. Fish, W. W. (1988) Methods Enzymol. 158, 357–364

24. Cardenas, J., and Mortenson, L. E. (1974) Anal. Biochem. 60, 372–381

25. Fogo, J. K., and Pogowsky, M. (1949) Anal. Chem. 21, 732–735

26. Manavalan, P., and Johnson, W. C., Jr. (1987) Anal. Biochem. 167, 76–85

27. Bray, R. C., George, G. N., Lange, R., and Meyer, O. (1983) Biochem. J. 211, 687–694

28. Hanzebmohl, P., and Meyer, O. (1986) Eur. J. Biochem. 255, 755–765
Flavin Binding to Carbon Monoxide Dehydrogenase

29. Hanzelmann, P. Hofmann, B., Meisen, S., and Meyer, O. (1999) *FEMS Microbiol. Lett.* **176**, 139–145
30. Moura, J. J. G., and Barata, B. A. S (1993) *Methods Enzymol.* **243**, 24–42
31. Romão, M. J., Archer, M., Moura, I., Moura, J. J. G., LeGall, J., Engh, R., Schneider, M., Hof, P., and Huber, R. (1995) *Science* **270**, 1170–1176
32. Huber, R., Hof, P., Duarte, R. O., Moura, J. J. G., Moura, I., Liu, M.-Y., LeGall, J., Hille, R., Archer, M., and Romão M. J. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 8846–8851
33. Barata, B. A. S., LeGall, J., and Moura, J. J. G. (1993) *Biochemistry* **32**, 11559–11568
34. Komai, H., Massey, V., and Palmer, G. (1969) *J. Biol. Chem.* **244**, 1692–1700
35. Kanda, M., Brady, F. O., Rajagopalan, K. V., and Handler, P. (1972) *J. Biol. Chem.* **247**, 765–770
36. Muller, F., and van Berkel, W. J. H. (1990) in *Chemistry and Biochemistry of Flavoenzymes* (Muller, F., ed) , Vol. 1, pp. 261–274, CRC Press, Boca Raton, Inc., FL
37. Muller, F. (1990) in *Chemistry and Biochemistry of Flavoenzymes* (Muller, F., ed) , Vol. 1, pp. 1–71, CRC Press, Boca Raton, Inc., FL
38. Bray, R. C., Turner, N. A., LeGall, J., Barata, B. A. S., and Moura, J. J. G. (1991) *Biochem. J.* **280**, 817–820
39. Lowe, D. J., Lynden-Bell, R. M., and Bray, R. C. (1972) *Biochem. J.* **130**, 239–249
40. Lowe, D. J., and Bray, R. C. (1978) *Biochem. J.* **169**, 471–479
41. Barber, M. J., Salerno, J. C., and Siegel, L. M. (1982) *Biochemistry* **21**, 1648–1656