Insights into the posttranslational assembly of the Mo-, S- and Cu-containing cluster in the active site of CO dehydrogenase of *Oligotropha carboxidovorans*

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**Abstract** *Oligotropha carboxidovorans* is characterized by the aerobic chemolithoautotrophic utilization of CO. CO oxidation by CO dehydrogenase proceeds at a unique bimetallic [CuSMoO₂] cluster which matures posttranslationally while integrated into the completely folded apoenzyme. Kanamycin insertional mutants in *coxE*, *coxF* and *coxG* were characterized with respect to growth, expression of CO dehydrogenase, and the type of metal center present. These data along with sequence information were taken to delineate a model of metal cluster assembly. Biosynthesis starts with the MgATP-dependent, reductive sulfuration of [MoVIO₃] to [MoVO₂SH] which entails the AAA-ATPase chaperone CoxD. Then Mo V is reoxidized and Cu İ ion is integrated. Copper is supplied by the soluble CoxF protein which forms a complex with the membrane-bound von Willebrand protein CoxE through RGD–integrin interactions and enables the reduction of CoxF-bound Cu²⁺, employing electrons from respiration. Copper appears as Cu²⁺-phytate, is mobilized through the phytase activity of CoxF and then transferred to the CoxF putative copper-binding site. The *coxG* gene does not participate in the maturation of the bimetallic cluster. Mutants in *coxG* retained the ability to utilize CO, although at a lower growth rate. They contained a regular CO dehydrogenase with a functional catalytic site. The presence of a pleckstrin homology (PH) domain on CoxG and the observed growth rates suggest a role of the PH domain in recruiting CO dehydrogenase to the cytoplasmic membrane enabling electron transfer from the enzyme to the respiratory chain. CoxD, CoxE and CoxF combine motifs of a DEAD-box RNA helicase which would explain their mutual translation.

**Keywords** Carbon monoxide dehydrogenase · Molybdoenzyme maturation · Copper · Phytase · DEAD-box protein

**Abbreviations**

AAS Atomic absorption spectroscopy  
EPR Electron paramagnetic resonance  
FAD Flavin adenine dinucleotide  
MCD Molybdopterin cytosine dinucleotide  
MIDAS Metal ion-dependent adhesion site  
OD Optical density  
PAGE Polyacrylamide gel electrophoresis  
PHP Pleckstrin homology  
SDS Sodium dodecyl sulfate  
UV/VIS Ultraviolet/visible  
VWA Von willebrand factor A  
XdhC Xanthine dehydrogenase C

**Introduction**

Carbon monoxide dehydrogenases (CO dehydrogenases) are enzymes which catalyze the oxidation of CO to CO₂ yielding two electrons and two protons (CO + H₂ → CO₂ + 2e⁻ + 2H⁺) or the reverse reaction [1, 2]. They are key to the generation of a proton motive force across the cytoplasmic membrane for ATP synthesis or cooperate with acetyl-CoA synthase in the biosynthesis of acetyl-CoA. Two principal types of the enzyme have been...
identified and structurally characterized. The homodimeric CO dehydrogenase of anaerobic bacteria or archaea (e.g. Carboxydothermus hydrogenoformans, Rhodospirillum rubrum or Moorella thermoacetica) is an iron–sulfur protein with a [Ni-4Fe-4S]-cluster in the active site [3–8]. The heterohexameric CO dehydrogenase of the α-proteobacterium Oligotropha carboxidovorans and other representatives of the aerobic CO-utilizing (carboxidotrophic) bacteria is a molybdo iron–sulfur flavoprotein [9–12]. The enzyme can be grouped into the molybdenum hydroxylase (xanthine oxidase) family of Mo enzymes on the basis of its overall structural properties [9, 13]. In contrast to the mononuclear Mo enzymes and nitrogenase, CO dehydrogenase accommodates in its catalytic site a bimetallic [Mo-O2-S-Cu1, S-Cys] cluster, which is unprecedented in nature [10, 11, 14]. The Mo ion of the cluster is coordinated by the ene-dithiolate of the molybdopterin cytosine dinucleotide cofactor (MCD) [10, 15–18]. The Cu ion is ligated by the γS of the cysteine residue 388 [10] which is part of the unique signature VAYRCSFR [19, 20]. 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cassette isolated from plasmid pUC4KIXX (Amersham Pharmacia Biotech, Freiburg, Germany) from E. coli DH5α [34]. The recombinant plasmid is referred to as pETEkm. The fragment of coxE was isolated from pETEkm and cloned into the suicide plasmid pSUP 201-1 isolated from E. coli S17-1 [35]. The resulting plasmid pSUPEkm2 was transformed into E. coli S17-1. Finally, coxEkm was transferred from E. coli S17-1 to O. carboxidivorans OM5 by conjugation. Successful recombination was checked by Southern blotting employing a KIXX probe, which was directed against the kanamycin resistance cassette and a specific probe for coxE. For the KIXX probe the 1.2 kb KIXX fragment from the pUC4KIXX was isolated and labeled with digoxigenin. For the specific coxE probe, the plasmid pCAC1 [19], which carries an EcoRV fragment of pHCG3 with the coxE fragment, was isolated from E. coli DH5α. The sequence was amplified using PCR, and purity was checked by agarose gel electrophoresis. The PCR product was hydrolyzed with MscI, and the resulting fragments were separated by agarose gel electrophoresis. A 0.75 kb fragment was eluted from the gel and labeled with digoxigenin. The plasmid DNA of the O. carboxidivorans wild type and mutant was isolated and restricted with EcoRV and HindIII. Agarose gel electrophoresis was used to separate the fragments. After transferring the fragments onto a nylon membrane, digoxigenin-labeled probes were added and detection was performed. A 3.2 kb EcoRV fragment of pETEkm was used as a positive control.

For the insertional mutagenesis of coxF the corresponding fragment was cloned into the vector pET11a (Novagen, Heidelberg, Germany) resulting in the recombinant construct pETFG1, which could be isolated from E. coli DH5α. For in vitro mutagenesis of the coxF gene, a kanamycin resistance cassette was isolated from plasmid pUC4KIXX (Amersham Pharmacia Biotech, Freiburg, Germany) of E. coli DH5α [34], inserted into the coxF fragment and transformed into E. coli DH5α. The recombinant plasmid is referred to as pETFG1 km. The coxFkm fragment isolated from pETFG1 km was cloned into the suicide plasmid pSUP 201-1 isolated from E. coli S17-1 [35], resulting in the plasmid pSUPFkm. After the transformation of pSUPFkm into E. coli S17-1, the coxFkm construct was transferred from E. coli S17-1 to O. carboxidivorans OM5 by conjugation. Mutagenesis of the coxF gene could be confirmed by Southern Blotting employing a KIXX probe directed against the inserted kanamycin resistance cassette. This probe was produced by isolating the 1.2 kb KIXX fragment from the plasmid pUC4KIXX followed by labeling with digoxigenin. Insertional mutagenesis of coxF followed the same procedure as described for coxE.

Translational analysis

For translational analyses, cell-free extracts of wild type and the mutants in coxD (D::km), coxE (E::km) and coxF (F::km) were separated by denaturing PAGE [36]. Protein bands were blotted onto polyvinylidene fluoride (Roth, Karlsruhe, Germany) and used for immunodetection with IgG antibodies (rabbit) directed against the polypeptides CoxD, CoxE and CoxF (Eurogentec, Seraing, Belgium).

Enzyme purification and activity assay

Bacteria suspended in 50 mM KH₂PO₄/KOH (pH 7.2) were disintegrated with a French-pressure cell at maximum pressure (American Instruments Company, Silver Spring, Maryland, USA). The resulting extracts were subjected to ultracentrifugation (2.5 h at 100,000 × g, Centrifan T4510, Kontron, Eching, Germany). Purifications of CO dehydrogenase started from soluble supernatants involving anion exchange chromatography, hydrophobic interaction chromatography, gel filtration and chromatography on hydroxylapatite [10, 14, 29]. Purity was checked by native PAGE (7.5 % acrylamide, 50 mM Tris/384 mM glycine, pH 8.5) stained for protein with Coomassie Brilliant Blue 250. The amount of protein in CO dehydrogenase bands appearing on gels was quantitated by video densitometry (ImageJ: http://rsb.info.nih.gov/ij/). Purified preparations of CO dehydrogenase were frozen in liquid nitrogen and stored at −80 °C until use. CO-oxidizing activity was assayed photometrically employing INT [1-phenyl-2-(4-iodo-phenyl)-3-(4-nitrophenyl)-2H-tetrazolium chloride] as electron acceptor [37] or analyzed by activity staining on native PAGE. One unit of CO dehydrogenase activity is defined as 1 μmol of CO oxidized per min at 30 °C. Protein estimation followed published procedures [38, 39]. Amounts of purified CO dehydrogenase were also determined from the visible absorption spectrum at 450 nm employing INT [1 at 450 nm [40] and a molecular mass of 277,074.37 Da. Ultraviolet/visible spectra were recorded on a spectrophotometer (BioMate 6, Thermo-scientific, Madison WI, USA). Functionalities were determined as follows: The absorption differences at 450 nm of CO dehydrogenase in the air-oxidized state minus the dithionite-reduced state or minus the CO-reduced were determined. The percentage of the latter relative to the former was taken as functionality. Functionality describes the portion of catalytically active CO dehydrogenase in a preparation which also contains inactive enzyme species. The method for the determination of functionality is based on the fact that CO oxidation at the catalytic site releases two electrons which travel to the FAD cofactor, via the iron–sulfur centers. Bleaching of the flavin occurs only in
catalytically active enzyme species and not in inactive enzyme species because CO is not oxidized and consequently electrons are not released. Functionality was determined as follows: Enzyme solution contained in a serum-stoppered cuvette was saturated with pure N₂, and 45 min). Then another sample of the same enzyme in a cuvette was sparged with pure CO for 30 min at room temperature. Subsequently visible spectra were recorded with time until the decrease in absorption at 450 nm came to a standstill (this usually took less than 45 min). Then another sample of the same enzyme in a serum-stoppered cuvette was saturated with pure N₂, and sodium dithionite was injected through the septum to achieve 5 mM final concentration. Again, spectra were recorded until the absorption decrease at 450 nm went to completion (this usually took about 5 min). The quantity of the absorption decrease at 450 nm of the dithionite-reduced enzyme reflects the total flavin content and measures the amount of active and inactive enzyme present. It was set 100 %. The quantity of the absorption decrease at 450 nm obtained in the presence of CO indicates the amount of CO dehydrogenase species capable of oxidizing CO and reducing the FAD cofactor contained in the enzyme. The term “functionality” refers to the percentage of the extent of reduction at 450 nm achieved with CO relative to the reduction obtained with dithionite. Other than the specific activity, which describes μmol CO oxidized min⁻¹ mg⁻¹, functionality of CO dehydrogenase preparations indicates the amount of enzyme species capable of oxidizing CO relative to the sum of catalytically active and inactive species.

Determination of sulfane sulfur, FAD and metals

Sulfane sulfur was determined by treating samples of CO dehydrogenase with potassium cyanide followed by colorimetric analysis of the resulting thiocyanate as FeSCN [12]. FAD was determined from its absorption at 450 nm in supernatants of trichloroacetic acid precipitates of CO dehydrogenase, neutralized with 2.4 mM K₂HPO₄ [9]. Copper, molybdenum and zinc in CO dehydrogenase were determined by flame atomic absorption spectroscopy (model 1100 B, Perkin Elmer, Überlingen, Germany).

Reconstitution of apo-CO dehydrogenase

CO dehydrogenases in their as isolated state (5 mg ml⁻¹ in 50 mM HEPES, pH 7.2) were treated under anoxic conditions with 150 μM Cu²⁺(thiourea)₃ or with sodium sulfide and sodium dithionite (5 mM each) first, followed by 150 μM Cu¹⁺(thiourea)₃. Assays treated with sodium sulfide and sodium dithionite were incubated in the dark at 37 °C for 10 h. Assays treated with Cu¹⁺(thiourea)₃ were incubated at 37 °C for 2–4 h in the dark. For the removal of Cu and sulfane sulfur, enzyme (~10 mg ml⁻¹) was incubated with 5 mM potassium cyanide for 24 h and then, gel filtered on PD10 ready-to-use columns (Sephadex G25, GE Healthcare, Little Chalfont, UK). Finally, CO dehydrogenase in the excluded volume was treated with sulfide/dithionite and copper as above. The procedures have been adopted from [41].

Electron paramagnetic resonance (EPR) spectroscopy

X-band EPR spectra were recorded on a Bruker EMX spectrometer equipped with an ESR 900 helium cryostat (Oxford Instruments, Oxon, UK) as described [14]. Spectra were recorded at 120 K applying a microwave frequency of 9.47 GHz, 1 mT modulation amplitude and 10 mW microwave power. The magnetic field was calibrated with a diphenylpicrylhydrazin sample. Assays containing CO dehydrogenase (12 mg ml⁻¹ in 50 mM HEPES, pH 7.2) were made anoxic by sparging with N₂ and then reduced with 5 mM sodium dithionite or sparged with pure CO for 30 min and frozen in liquid N₂. Where indicated, assays were amended with 15 mM cysteine, 15 mM Na₂S or 15 mM 2-mercaptoethanol and kept at room temperature for 25 min. Samples were kept in liquid nitrogen until use.

Miscellaneous methods and chemicals

All chemicals employed were of analytical grade and purchased from the usual commercial sources. Bioinformatic tools employed: The online server SABLE (Solvent AccessiBiLiEs of amino acid residues in proteins and improved prediction of secondary structures http://sable.echmc.org/; [42]) for secondary structure predictions; the Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi; [43]) for domain search on Cox proteins; T-Coffee (http://www.tcoffee.org/) for structural sequence alignments of CoxG.

Results

Bioinformatic characterization of the proteins CoxDEFG

The CoxD protein is a novel AAA+ ATPase (Fig. 1) which has the key elements of an AAA+ domain in the same arrangement and same positions as in the BchI component of Mg²⁺-chelatase [29]. CoxD operates in the maturation of the CO dehydrogenase bimetallic cluster, particularly in the sulfuration of the [MoO₄]²⁻site and in ATP-dependent chaperone functions [29, 44]. In addition to the AAA-signatures, the CoxD sequence reveals five homologues of conserved motifs suggestive of the DEAD-box protein family of RNA helicases (Fig. 1). DEAD-box
proteins have been shown to support the maturation of RNA molecules and to be required for translation initiation [45]. CoxE (399 aa; 44,235 Da; seventeen predicted α-helices and six β-sheets) is remarkably high in arginine (12.5 %, [19]) and contains an integrin I domain at its C-terminus (Fig. 1). Von willebrand proteins function in processes such as cell adhesion or multiprotein complex formation [46]. Furthermore, CoxE also carries two DEAD-box protein motifs (Fig. 1). CoxF (280 aa; 29,346 Da; nine α-helices and ten β-sheets) reveals an XdhC protein family motif at its N-terminus in combination with an XdhC Rossman domain near the C-terminus, a putative Cu-binding motif [47], an integrin-binding motif [46], a histidine acid phytase motif [48] at the C-terminus, and two scattered DEAD-box motifs (Fig. 1). Phytases (myo-inositol hexakisphosphate phosphohydrolases) from fungi, bacteria, yeasts or plants catalyze the partial or complete hydrolytic removal of orthophosphates from phytates (myo-inositol hexakisphosphates) [48]. Phytic acid is a potent chelator of divalent cations [48] with the order of affinity Cu$^{2+} >$ Zn$^{2+} >$ Mn$^{2+} >$ Mg$^{2+} >$ Co$^{2+} >$ Ni$^{2+}$ [49]. The release of phosphate groups from phytate by phytase results in the release of metal ions [48]. CoxG (205 aa; 21,559 Da; six predicted α-helices and eight β-sheets) contains a PH domain (pleckstrin homology domain) (Fig. 1). The PH domain encompasses about 100 loosely conserved amino acids and is found in numerous different types of mostly eukaryotic proteins with functions in intracellular signaling, cellular membrane dynamics and the cytoskeleton [50, 51].
PH domains can bind phosphatidylinositol membrane lipids, membrane proteins or both. They determine the membrane localization of the proteins in which they reside, thus presenting them to required cellular compartments or enabling them to interact with other components of the signal transduction pathways.

The functions of the genes coxE and coxF are essential for the utilization of CO by *O. carboxidovorans*, whereas coxG is not required.

In a previous paper, the D::km mutant has been studied [29]. Disruption of coxD led to a phenotype of D::km which was impaired in the utilization of CO, whereas the utilization of H₂ plus CO₂ was not affected. Under appropriate induction conditions, bacteria synthesized a fully assembled apo-CO dehydrogenase, which could not oxidize CO. Apo-CO dehydrogenase contained a [MoO₃] site in place of the [CuSMoO₂] cluster. Employing sodium sulfide first and then the Cu¹⁺-(thiourea)₃ complex, the non-catalytic [MoO₃] site could be reconstituted in vitro to a [CuSMoO₂] cluster capable of oxidizing CO. Sequence information suggested that CoxD is a MoxR-like AAA⁺ ATPase chaperone related to the hexameric, ring-shaped BchI component of Mg²⁺-chelatases. Similar to D::km, the mutants E::km and F::km also had lost the ability to utilize CO as a sole source of carbon and energy under aerobic chemolithoautotrophic conditions (Fig. 2a, b). This shows that the genes coxE and coxF are both obligatory for the utilization of CO as a growth substrate. As will be shown later in this paper the genes are functional in the maturation of CO dehydrogenase which is the enzyme catalyzing the oxidation of CO [1, 31]. On the other hand, the chemolithoautotrophic utilization of H₂ plus CO₂ by both mutant strains was not impaired, neither in the absence nor in the presence of CO (Fig. 2a, b). This is because H₂ is oxidized by a NiFe-hydrogenase [34]. The ability to grow on H₂ plus CO₂ also indicates that ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) and all other enzymes of the Calvin–Benson–Bassham (CBB) cycle employed for autotrophic CO₂ fixation are functional in the mutants. The deletion of coxG led to a phenotype which was still able to utilize CO, although the generation time increased considerably from 21 h (wild type) to 149 h (G::km) (Fig. 2c).

Our hypothesis is that the sevenfold increase in generation time is caused by limited interaction of CO dehydrogenase with the cytoplasmic membrane controlled by diffusion. Compared to exponential wild-type bacteria, which contain about 50 % of the CO dehydrogenase pool at the inner aspect of the cytoplasmic membrane [52],
immunocytochemical localization revealed most of the enzyme in the cytoplasm ([20], unpublished data).

However, contact of CO dehydrogenase with the membrane is required for electron transfer to drive energy transduction by the respiratory chain. This points to a role of CoxG in recruiting CO dehydrogenase to the membrane, which is further corroborated by the PH domain present on CoxG. The utilization of H₂ and CO₂ was not affected in the mutant G::km.

Under inducing growth conditions (i.e. H₂ plus CO₂ in the presence of CO) the bacteria utilize H₂ as an energy source employing hydrogenase and CO₂ as a carbon source employing the CBB cycle [1, 31]. The function of CO is to induce the transcription of the cox gene cluster on the plasmid pHCG3 [20]. Under these conditions, the mutant D::km synthesized an apo-CO dehydrogenase in which the [CuSMoO₂] cluster was replaced by a [MoO₃] site [29]. It was, therefore, tempting to assume that under same conditions the mutants E::km and F::km also would be able to synthesize an inactive apo-CO dehydrogenase, however, with a metal cluster representing a different stage in cluster biosynthesis. The fact that the mutant G::km was able to utilize CO under chemolithoautotrophic conditions is indicative for the presence of a catalytically active and thus fully assembled enzyme.

Overall properties of CO dehydrogenases purified from the mutants E::km, F::km or G::km

O. carboxidovorans and its mutants E::km, F::km and G::km were cultivated under chemolithoautotrophic conditions employing H₂ plus CO₂ as sources of energy and carbon and CO as an inducer of cox gene transcription (Fig. 2). Employing the protocol described in the methods section, the four CO dehydrogenases produced under these conditions were purified about 17-fold with an average yield of about 20 %. CO dehydrogenase amounted to approximately 6 % of the cytoplasmic proteins. The CO dehydrogenases showed the same mobilities on native PAGE, and their purity was apparent from a single protein band (Fig. 4a). Activity staining indicated the absence of CO-oxidizing activity in the CO dehydrogenases from the mutants E::km and F::km (Fig. 4b, lanes 2 and 3), whereas the enzymes from wild-type bacteria and the mutant G::km both displayed significant activities (Fig. 4b lanes 1 and 4). These results are corroborated by specific activities of the purified CO dehydrogenases (μmol CO oxidized mg⁻¹ min⁻¹) from wild-type bacteria and the mutants E::km, F::km and G::km of 8.529, 0.036, 0.136, or 4.360, respectively. The four CO dehydrogenases contained the required stoichiometric amounts of Mo and the FAD cofactor (Table 1).

In addition, the UV/VIS absorption spectra were indistinguishable (Fig. 5) and showed absorption maxima indicative of FAD (~ 450 nm) and FeS (~ 550 nm). The observed absorption ratios of A₂80/A₄₅₀ = 6.1 and A₁₅₀/A₅₅₀ = 3.1 indicate an iron–sulfur to flavin ratio of 4:1 present in wild-type CO dehydrogenase as well as in the enzymes from the mutants. The iron contents of the CO dehydrogenases from wild-type bacteria, E::km or F::km were (mol Fe per mol of enzyme) 7.67 ± 0.5, 7.67 ± 0.5, and 7.57 ± 0.5, which indicates that CoxE and CoxF are not involved in the incorporation of iron–sulfur clusters. The absorption maxima of FAD and the iron–sulfur centers of all four CO dehydrogenases were bleached by sodium dithionite (Fig. 5). Solely the enzymes from E::km and F::km were not bleached by CO (Fig. 5b, c) which
indicates a defect in the catalytic site. The functionalities calculated from the spectra in Fig. 5 of the CO dehydrogenases from wild-type bacteria and the mutants E::km, F::km and G::km were 57, <0.1, <0.1, and 47 %, respectively. Obviously, the preparations of catalytically active CO dehydrogenase contain about 50 % of non-functional forms of the enzyme which has been recognized before [12]. These data show a specific involvement of the genes coxE and coxF in the maturation of a form of CO dehydrogenase which is already folded to the regular 3-D structure and has received molybdenum, the flavin cofactor and the iron–sulfur centers. Therefore, the defect in catalytic activity must be located in the bimetallic cluster.

Constituents and reactivity of the molybdenum site of the CO dehydrogenases from the mutants E::km, F::km and G::km. In agreement with their ability to oxidize CO, the CO dehydrogenases from wild-type O. carboxidovorans cultivated with CO plus CO2 or with H2 plus CO2 in the presence of CO as an inducer of cox gene expression exhibited similar contents of Mo, Cu, and sulfane sulfur which was applied to the enzyme from the G::km mutant as well (Table 1). In contrast, copper was completely absent from the CO dehydrogenases of the mutants E::km and F::km, although they were complete in Mo and revealed stoichiometric amounts of sulfane sulfur (Table 1). Contaminating Zn was present in all preparations (Table 1).

When the CO dehydrogenases from the mutants E::km or F::km were treated with potassium cyanide to establish a [MoO3]-ion and then sulfurated followed by the introduction of Cu1+, significant CO dehydrogenase activities were obtained (Fig. 6; Table 2). Treatment of the two as isolated enzymes with Cu1+-thiourea reconstituted the CO-oxidizing activity to only 14 % (F::km) or 35 % (E::km) which was much lower than achieved by reconstitution of the [MoO3]-enzyme (set 100 %) or expected from the sulfane sulfur contents (Fig. 6; Table 2). The observation that obviously only a small fraction of the sulfane sulfur could bind Cu1+ identifies a separate fraction of sulfur which is reactive with potassium cyanide but cannot integrate Cu1+ into the active site. Although the two enzymes contained stoichiometric amounts (E::km) or nearly stoichiometric amounts (F::km) of cyanolyzable sulfur (Table 2), treatment with sodium sulfide plus sodium dithionite was necessary to achieve the reconstitution of substantial enzyme activity through the addition of copper (Fig. 6; Table 2). The treatment with sodium sulfide plus sodium dithionite did not increase the total cyanolyzable sulfur contents of the enzymes before the addition of Cu1+ which points to reactions at the Mo

| Table 1 | Analysis of CO dehydrogenases for FAD, metals and sulfane sulfur |
|---------|---------------------------------------------------------------|
| Source of CO dehydrogenase | Wild type (CO) | Wild type (H2) | E::km (H2) | F::km (H2) | G::km (H2) |
| FAD    | 1.93 ± 0.08 | 1.98 ± 0.03 | 2.15 ± 0.06 | 1.92 ± 0.06 | 1.79 ± 0.04 |
| Mo     | 1.85 ± 0.07 | 1.73 ± 0.10 | 1.95 ± 0.05 | 1.85 ± 0.03 | 1.91 ± 0.08 |
| Cu     | 1.62 ± 0.05 | 1.47 ± 0.03 | 0.00 ± 0.00 | 0.043 ± 0.00 | 1.48 ± 0.08 |
| ‘S’    | 1.93 ± 0.05 | 1.53 ± 0.01 | 2.0 ± 0.020 | 1.40 ± 0.13 | 2.17 ± 0.08 |
| Zn     | 0.17 ± 0.01 | 0.22 ± 0.00 | 0.22 ± 0.00 | 0.076 ± 0.00 | 0.31 ± 0.01 |

Purified enzymes were obtained from O. carboxidovorans OMS5 and its mutants E::km, F::km and G::km. Bacteria were cultivated under chemolithoautotrophic conditions employing a gas atmosphere of (v/v) 45 % CO, 5 % CO2, and 50 % air (referred to as “CO”) or 30 % H2, 5 % CO2, 30 % CO, and 35 % air (referred to as “H2”). All figures are in mol per mol of CO dehydrogenase determined from the absorption at 450 nm. FAD was estimated spectrophotometrically, metals by atomic absorption spectroscopy, and cyanolyzable sulfur (‘S’, sulfane sulfur) through cyanalysis. For details see “Materials and methods”. Standard deviations are based on at least three independent determinations.
ion that are capable of improving the competence for Cu binding and activation (Table 2). Nevertheless, such treatment did not activate CO dehydrogenase to the same level as that obtained after reconstitution of the \([\text{MoO}_3\text{-}]\) enzyme with sulfide/dithionite/copper (Fig. 6; Table 2). It was also peculiar that the contents of cyanolyzable sulfur in the CO dehydrogenases from E::km and F::km dropped by up to 45 % upon exposure to Cu, whereas cyanide-treated and resulfurated enzymes kept their full complement of S\(^{-}\) after the addition of Cu (Table 2). This might be taken as an indication of different forms of cyanolyzable sulfur, Mo-SH which allows restoration of activity in the CO dehydrogenases from E::km and F::km.

Fig. 5 UV/VIS absorption spectra of CO dehydrogenases purified from wild type (a) or from the mutants in E::km (b), F::km (c) and G::km (d). Traces: a, air-oxidized; b, sparged with pure CO for 30 min; c, reduced with 650 \(\mu\)M dithionite under N\(_2\) for 4 min. The insets show the visible part of the spectra at greater detail.

Fig. 6 CO dehydrogenases from the mutants in coxE (a) or coxF (b) were treated with Cu\(^{1+}\)-(thiourea)\(_3\) (filled circle) or with sodium sulfide and sodium dithionite first followed by Cu\(^{1+}\)-(thiourea)\(_3\) (open circle). For the removal of Cu and cyanolyzable sulfur, CO dehydrogenases were incubated with potassium cyanide and then treated with sulfide/dithionite and copper as above (filled triangle). For details see the “Methods” section.
through the addition of Cu\textsuperscript{1+} and another sulfur (presumably Mo-S-SH) which is of the wrong kind in this respect. The increased Cu contents of CO dehydrogenases treated with that metal of more than 2 Cu per mol of enzyme indicate the presence of so far unidentified Cu-binding sites in addition to the active site (Table 2). The additional Cu-binding sites might not be true sites but artifactual sites that have no physiological importance. Cu treatment is known to irreversibly damage the activity of other enzymes [53].

**Table 2** Sulfane sulfur and copper in purified CO dehydrogenases which had received different treatments. E::km and F::km are the *O. carboxidovorans* mutants in coxE or coxF, respectively

| Source of CODH | Treatment | S before (left) and after (right) the addition of Cu | Cu | Activity (\(\mu\text{mol CO min}^{-1}\text{ mg}^{-1}\)) |
|----------------|-----------|-----------------------------------------------------|----|---------------------------------------------|
| Wild type      | A         | 1.95 ± 0.09                                         |    | 8.07 ± 0.34 8.350                           |
| E::km          | A         | 1.72 ± 0.16                                         | 1.98 ± 0.15 7.74 ± 0.22 8.617                |
|                | B         | 1.93 ± 0.13                                         | 0.87 ± 0.09 7.53 ± 0.63 5.113                |
|                | C         | 2.00 ± 0.02                                         | 1.12 ± 0.04 13.90 ± 0.38 3.016                |
| F::km          | A         | 1.47 ± 0.11                                         | 1.46 ± 0.26 8.85 ± 0.10 7.890                |
|                | B         | 1.58 ± 0.08                                         | 1.05 ± 0.11 8.40 ± 0.05 6.750                |
|                | C         | 1.40 ± 0.13                                         | 1.02 ± 0.35 11.60 ± 0.08 1.116                |

Treatments of CO dehydrogenases (5 mg ml\(^{-1}\)): A, for the removal of Cu and sulfane sulfur, anoxic enzymes were incubated with 5 mM KCN for 24 h and then treated with 5 mM sodium sulfide plus 5 mM sodium dithionite first followed by 150 \(\mu\text{M Cu}^{2+}\) (thiourea); B, as isolated enzyme was treated with sodium sulfide and sodium dithionite and then with \(\text{Cu}^{2+}\) (thiourea); C, as isolated enzyme was treated with \(\text{Cu}^{2+}\) (thiourea). The sulfide/dithionite assays were incubated in the dark at 37 °C for 10 h, and the \(\text{Cu}^{2+}\) (thiourea)\(_2\) assays at 37 °C for 2–4 h in the dark. After each treatment the enzyme solution was gel filtered on PD10 ready-to-use columns (Sephadex G25, GE Healthcare, Little Chalfont, UK). All figures are in mol per mol of CO dehydrogenase. Refer to Table 1, Fig. 6 and the “Methods” section for further details.

**Fig. 7** Mo-EPR of CO dehydrogenases (12 mg ml\(^{-1}\); 50 mM HEPES, pH 7.2) from *O. carboxidovorans* wild type and the mutants E::km, F::km, or G::km (from top to bottom). The enzymes were exposed to CO (a) or treated with 5 mM sodium dithionite (b). Spectra were recorded at 120 K at a microwave frequency, modulation amplitude, and microwave power of 9.47 GHz, 1 mT and 10 mW, respectively.
Sulfur compounds as reporters of incomplete forms of the [CuSMoO2] cluster

1-cysteine, 2-mercaptoethanol and sodium sulfide were examined with respect to their ability to generate and/or alter paramagnetic EPR signals from species of CO dehydrogenase modified in the [CuSMoO2] cluster (Fig. 8). Treatment of CO dehydrogenase with potassium cyanide is known to convert the [CuSMoO2] cluster into a [MoO3]-center, a reaction which abolishes the ability of the enzyme to oxidize CO [11, 41]. Without added sulfur compounds, the [MoO3]-CO dehydrogenase prepared by cyanolysis of the wild-type enzyme afforded an EPR spectrum devoid of signals (Fig. 8a, below 3% paramagnetic Mo by spin integration) referring to a mostly diamagnetic Mo(VI) ion. In the presence of cysteine, sodium sulfide or 2-mercaptoethanol the spectrum remained silent (Fig. 8a) which indicates the absence of one-electron redox interactions of the sulfur compounds with the [MoVIIO2]-site. [MoO2S]-CO dehydrogenase was produced by treatment of the [MoO3]-enzyme with sodium sulfide plus sodium dithionite (Fig. 8b). Its weak paramagnetic signal centered at g = 1.975 (about 7% of the total Mo) significantly increased in the presence of sulfur compounds, particularly cysteine (32% of the total Mo) or 2-mercaptoethanol (26% of the total Mo) (Fig. 8b). These data indicate that the SH group of the studied sulfur compounds can bind to the equatorial sulfur of [MoO2S] under formation of a [MoO2S–S–R] mixed disulfide which is reported by EPR. The CO dehydrogenases obtained from E::km (Fig. 8c) or F::km (Fig. 8d) showed paramagnetic Mo(V) EPR signals similar to that of the [MoO2S]-enzyme in the presence of Na2S (Fig. 8b). Furthermore, the signals significantly increased upon the addition of cysteine or 2-mercaptoethanol (Fig. 8c, d). These data exclude a Cys-388 persulfide in the CO dehydrogenases from the two mutants and rather refers to the coexistence of two different types of sulfurated Mo, i.e. [MoO2S] and [MoO2S–S–H]. The proportions of Mo species in the CO dehydrogenases from E::km (first mention) or F::km (second mention) deduced from spin integration were 2 to 21% or 3 to 6% of [MoO2S], 23 or 18% of [MoO2S–S–H] and 56 to 75% or 76 to 79% of [MoO3]. The coexistence of these Mo species explains the different levels of activation obtained upon reconstitution (Fig. 6).

A pleckstrin homology (PH) domain is predicted on CoxG

Conserved domain searches revealed on CoxG a hydrophobic ligand site of the SRPBCC (START/RHO_alpha_C/PITP/Bet_v1/CoxG/CalC) super family. For structural sequence alignments of CoxG the bioinformatics program T-Coffee (http://www.tcoffee.org/) was used. The T-coffee search exhibited 62% overall similarity of CoxG to the human pleckstrin 2 domain with especially good scores in the following patches: M1 to M3, R13 to I36, V48 to P54, F59 to L65, I104 to K110, T128 to K139, E155 to A168, and V205 (Fig. 1, green boxes). Disruption of the coxG gene did not impair the ability of O. carboxidovorans to utilize CO as a substrate for chemolithoautotrophic growth, except that the generation time was markedly increased (Fig. 2c). In addition, the CO dehydrogenase from the G::km mutant was complete in cofactor composition (Table 1). Although CO dehydrogenase from G::km was catalytically active, its specific CO-oxidizing activity was only 51% that of the wild-type enzyme. It has been established that CO dehydrogenase is synthesized in wild-type bacteria as a mix of fully active and inactive species [12]. The inactive ones are complete in Mo and FAD but deficient in cyanozylatable sulfur and/or copper to a different extent. Apparently, this also applies to the CO dehydrogenase expressed in the absence of CoxG as it was complete in cyanozylatable sulfur but deficient in copper and fairly high in Zn which is suspected to occupy the Cu-binding site. Whether CO dehydrogenase expressed in the absence of CoxG might be trapped in a partially active precursor state of the active site requires further research. Evidently, the CoxG protein is not involved in the assembly of the bimetallic cluster. Instead, it is reasonable to assume that the PH domain on CoxG plays a role in recruiting soluble CO dehydrogenase to the inner aspect of the cytoplasmic membrane thus enabling electron transfer from the CO dehydrogenase FAD cofactor to the quinone pool [54] of the respiratory chain [55]. This interpretation is corroborated by the absence of particular CO dehydrogenase in extracts of the G::km mutant [20].

Discussion

Types of Mo sites appearing in the assembly of the CO dehydrogenase bimetallic site

It is now clear that the genes coxD, coxE and coxF accomplish specific functions in the posttranslational
sulfuration of a [MoO₃]-site and the subsequent introduction of copper yielding a functional [CuSMoO₂] cluster in CO dehydrogenase. The process proceeds at the molybdenum cofactor (Moco), composed of trioxo-Mo(VI) coordinated by the ene-dithiolate of molybdopterin-cytochrome-dinucleotide (MCD), which is buried about 17 Å below the solvent accessible surface of apo-CO dehydrogenase [10]. The latter is properly folded and complete in cofactor composition, as it contains the iron–sulfur centers and the flavin-adenine-dinucleotide (FAD) cofactor. This cofactor composition, as it contains the iron–sulfur centers E::km and F::km (Fig. 3). As a consequence, the mutant E::km and F::km indicates that the corresponding genes operate in different extents and slightly differ in the in vitro activation (Fig. 6). The presence of [Mo(VI)(=O)OH(2)SSH] and [Mo(V)(=O)(OH(2)SSH)] in the CO dehydrogenases from E::km and F::km indicates that the corresponding genes operate in the introduction of copper into a sulfurated molybdenum site.

Prediction of CoxD, CoxE and CoxF as DEAD-box proteins and anticipated functions

Members of the DEAD-box family of RNA helicases use ATP to rearrange RNA and RNA–protein structures [45, 57, 58]. They consist of a helicase core containing 12 conserved motifs involved in ATP binding, RNA binding and ATP hydrolysis [59]. Five homologues appear on CoxD (Ia, I, IV, II, V), two on CoxE (Ib, III) and two on CoxF (III, IV) (Fig. 1) with the corresponding sequences: Ia, P²¹DRDLA²⁶; I, G⁴³EAGVGKT⁵⁰; IV, L⁶⁴IR⁶⁶; II, D¹³⁴EVD¹³⁷; V, A²⁰⁹RII⁵⁷; III, S⁹¹AT⁹³ (CoxE), S¹²⁰L¹²³ and S¹⁷⁸GT¹⁸⁰ (CoxF); IV, L²²⁴TV²²⁶ (CoxF). The motif III is duplicated on CoxF (Fig. 1). Because of the presence of these DEAD-box motifs, it can be envisioned that CoxD can accomplish functions of a RNA-helicase engaged in translation, such as eIF4A from Saccharomyces cerevisiae [60]. CoxE and CoxF carry only a small number of DEAD-box motifs which is in contrast to CoxD (Fig. 1). On the other hand, the motifs Ib and III on CoxE and the motif III on CoxF are special because they are not present on CoxD. This might
point to some sort of cooperation in translation between CoxD, CoxE and CoxF. Indeed, CoxD can hydrolyse MgATP [44], and RNA-helicase functions of the three Cox proteins would explain their mutual requirement for translation (Fig. 3). Furthermore, the insertion of a kanamycin resistance cassette might exert an effect on the stability of the mRNA in the mutants. The mutation in coxF could influence mRNA folding, particularly increase the stability of the coxD message in F::km and/or might enhance the accessibility of the ribosome-binding site. As a result, higher expression levels of CoxD can be achieved (Fig. 3a).

Metal cluster maturation

Solely the mutant G::km synthesized a fully active enzyme completely in sulfur and copper (Table 1) suggesting a [Mo(=O)OH–S–Cu–S-Cys] cluster which indicates that CO dehydrogenase biosynthesis is complete prior to the action of coxG. The post translational assembly of the [Mo(=O)OH–S–Cu–S-Cys] cluster in the active site of CO dehydrogenase is a complex and highly ordered process which involves the introduction of sulfur and copper into a [Mo^{V=O}(OH)_2] site (Fig. 9). It represents the final step of cluster maturation, resulting in a catalytically active enzyme. We have shown herein that the biosynthesis of the Mo/Cu cluster involves the functions of the genes coxE and coxF, in addition to coxD [29, 44]. Cluster biosynthesis starts with the one-electron reduction of [Mo VI(=O)2OH(2)] to [Mo V(=O)2OH(2)] by an unknown mechanism (Fig. 9). However, since CoxD is a membrane protein [44], the shuttle between Mo VI and Mo V can be imagined to involve the electron transport system (ETS). The next step is the MgATP-dependent sulfuration of [Mo V(=O)2OH(2)] to [Mo V(=O)OH(2)SH] which involves the AAA-ATPase chaperone CoxD [29, 44]. It is not known whether CoxD itself acts as a sulfurtransferase and what the actual sulfur source is. However, cysteine or thiosulfate is sulfur donor which must be considered [25, 56, 61]. Our data along with a previous report [62] show that thiol compounds much larger than CO (e.g. l-cysteine or 2-mercaptoethanol) can freely travel through the substrate channel leading to the CO dehydrogenase active site. This challenges previous concepts involving chaperone function of CoxD on apo-CO dehydrogenase [29].
The in vivo oxidation state of Mo in [Mo(=O)OH$_2$SH] must be +VI to enable a transfer of Cu$^{1+}$. This is suggested by the conditions of chemical reconstitution and EPR described previously [41] as well as from the experiments shown in Figs. 6 and 8. The incorporation of Cu$^{1+}$ involves CoxE and CoxF which is indicated by the absence of Cu in the CO dehydrogenases from E::km and F::km (Table 1). Although the exact functions of CoxE and CoxF must await studies at the protein level, sequence information suggests the Cu acquisition shunt depicted in Fig. 9. CoxF reveals signatures of a potential histidine acid phytase (HAPhy, R$^{266}$HGRQRQS, RHGXRXP) and a suspected Cu-binding site (M$^{107}$CPShGTM; [47] (Fig. 1). These could enable CoxF to release phosphate-bound Cu through the hydrolysis of phosphate monoesters with the subsequent transfer to the Cu-binding site. Complex formation of soluble CoxF(Cu) and membrane-bound CoxE through their RGD motif and the VWA domain establishes access to the electron transport system (ETS) for the reduction of Cu$^{2+}$ to Cu$^{1+}$ (Fig. 9). Finally, Cu$^{1+}$ transfer from its escorting protein CoxF to [Mo$^{VI}$(=O)OH$_2$SH] in apo-CO dehydrogenase results in the formation of a complete and functional bimetallic cluster (Fig. 9). CoxE is an integrin with a single metal ion-dependent adhesion site (MIDAS) located in its ligand-binding site.

CoxE is likely to form an intermediate ternary complex with a cation and CoxF to regulate ligand binding (Fig. 1). The types of cations involved in this process, and particularly any role of Cu, are currently not known. Characterizing CoxD, CoxE and CoxF at the protein level and identifying the missing factors in the maturation of the bimetallic site of CO dehydrogenase are important challenges for the future.

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References

1. Moersdorf G, Frunzke K, Gadkari D, Meyer O (1992) Biodegradation 3:61–82.
2. Ragsdale SW, Kumar M (1996) Chem Rev 96:2515–2539.
3. Svetlitchnyi VA, Sokolova TG, Gerhardt M, Ringpfeil M, Kostrikina NA, Zavarzin GA (1991) Syst Appl Microbiol 14:254–260.
4. Svetlitchnyi V, Peschel C, Acker G, Meyer O (2001) J Bacteriol 183:5134–5144.
5. Dobbek H, Svetlitchnyi V, Gremer L, Huber R, Meyer O (2001) Science 293:1281–1285.
6. Drennan CL, Heo J, Sintchak MD, Schreiter E, Ludden PW (2001) Proc Natl Acad Sci 98:11973–11978.
7. Doukov TI, Iverson TM, Seravalli J, Ragsdale SW, Drennan CL (2002) Science 298:567–572.
8. Darnault C, Volbeda A, Kim EJ, Legrand P, Vernède X, Lindahl PA, Fontecilla-Camps JC (2003) Nat Struct Biol 10:271–279.
9. Meyer O (1982) J Biol Chem 257:1333–1341.
10. Dobbek H, Gremer L, Meyer O, Huber R (1999) Proc Natl Acad Sci 96:8884–8889.
11. Dobbek H, Gremer L, Kiefersauer R, Huber R, Meyer O (2002) Proc Natl Acad Sci 99:15971–15976.
12. Guida M, Ferner R, Gremer L, Meyer O, Meyer-Klaucke W (2003) Biochemistry 42:222–230.
13. Hille R, Hall J, Basu P (2014) Chem Rev. doi:10.1021/cr400443z.
14. Gremer L, Kellner S, Dobbek H, Huber R, Meyer O (1990) J Biol Chem 275:1864–1872.
15. Krüger B, Meyer O (1987) Biochim Biophys Acta 912:357–364.
16. Johnson JL, Rajagopalan KV, Meyer O (1990) Arch Biochem Biophys 283:542–545.
17. Meyer O, Stackebrandt E, Auling C, Meyer O (1999) Gene 236:115–124.
18. Fuhrmann S, Ferner M, Jelfke T, Henne A, Gottschalk G, Meyer O (2003) Gene 322:67–75.
19. Wilcoxen J, Snider S, Hille R (2011) J Am Chem Soc 133:12934–12936.
20. Siegbahn PE, Shestakov AF (2005) J Comput Chem 26:888–898.
21. Hofmann M, Kassube JK, Graf T (2005) J Biol Inorg Chem 10:490–495.
22. Dos Santos PC, Smith AD, Frazzon J, Cash VL, Johnson MK, Dean DR (2004) J Biol Chem 279:19705–19711.
23. Neumann M, Leimühler S (2011) Biochem Res Int. doi:10.1155/2011/850924.
24. Mendel RR (2013) J Biol Chem 288:13165–13172.
25. Hu Y, Ribe MW (2013) J Biol Chem 288:13173–13177.
26. Darnault C, Volbeda A, Kim EJ, Legrand P, Vernède X, Lindahl PA, Fontecilla-Camps JC (2003) Nat Struct Biol 10:490–495.
27. Laemmli UK (1970) Nature 227:680–685.
28. Simon R, Priefer U, Pühler A (1983) Biotechnology 1:7784–7790.
29. Pelzmann A, Ferner M, Gnida M, Meyer-Klaucke W, Maisel T, Kiefersauer R, Gremer L, Meyer O (2002) Proc Natl Acad Sci 99:15971–15976.
30. Meyer OO (2005) In: Garrity GM (ed) Genus VII. Oligotropha. Bergey’s Manual of Systematic Bacteriology, vol 2. Springer, New York, pp 468–471.
31. Meyer O, Schlegel HG (1983) Annu Rev Microbiol 37:277–310.
32. Volland S, Rachinger M, Strittmatter A, Daniel R, Gottschalk G, Meyer O (2011) J Bacteriol 193:5043.
33. Paul D, Bridges SM, Burgess SC, Dandass YS, Lawrence ML (2010) BMC Genom. doi:10.1186/1471-2164-11-511.
34. Santiago B, Meyer O (1997) J Bacteriol 179:6053–6060.
35. Simon R, Prierer U, Pühler A (1983) Biotechnology 1:7784–7790.
36. Laemmli UK (1970) Nature 227:680–685.
37. Kraut M, Hugendieck I, Herwig S, Meyer O (1983) Biotechnology 1:7784–7790.
38. Bradford MM (1976) Anal Biochem 72:248–254.
39. Beisenherz G, Boltze HJ, Büchler TR, Czok K, Garbade H (1953) Z Naturforsch 8:555–577.
40. Meyer O, Rajagopalan KV (1984) J Biol Chem 259:5612–5617.
41. Darnault C, Volbeda A, Kim EJ, Legrand P, Vernède X, Lindahl PA, Fontecilla-Camps JC (2003) Nat Struct Biol 10:271–279.
