Biochemical and Biophysical Research Communications 447 (2014) 413–418

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Reversible inactivation of CO dehydrogenase with thiol compounds

Oliver Kreß [a], Manuel Gnida [b], Astrid M. Pelzmann [a], Christian Marx [c], Wolfram Meyer-Klaucke [b], Ortwin Meyer [a,*]

[a] Department of Microbiology, University of Bayreuth, 95440 Bayreuth, Germany
[b] Department of Chemistry, University of Paderborn, 33098 Paderborn, Germany
[c] Institute of Biochemistry and Biophysics, Friedrich-Schiller-University of Jena, 07745 Jena, Germany

ARTICLE INFO

Article history:
Received 20 March 2014
Available online 6 April 2014

Keywords:
Carbon monoxide dehydrogenase regulation
Oligotropha carboxidovorans
Thiol
Molybdenum
Copper
X-ray absorption spectroscopy

ABSTRACT

Carbon monoxide dehydrogenase (CO dehydrogenase) from *Oligotropha carboxidovorans* is a structurally characterized member of the molybdenum hydroxylase enzyme family. It catalyzes the oxidation of CO ($CO + H_2O \rightarrow CO_2 + 2e^- + 2H^+$) which proceeds at a unique [CuSMo(=O)OH] metal cluster. Because of changing activities of CO dehydrogenase, particularly in subcellular fractions, we speculated whether the enzyme would be subject to regulation by thiols (RSH). Here we establish inhibition of CO dehydrogenase by thiols and report the corresponding K_i-values (mM): L-cysteine (5.2), D-cysteine (9.7), *N*-acetyl-L-cysteine (8.2), D,L-homocysteine (25.8), L-cysteine–glycine (2.0), dithiothreitol (4.1), coenzyme A (8.3), and 2-mercaptoethanol (9.3). Inhibition of the enzyme was reversed by CO or upon lowering the thiol concentration. Electron paramagnetic resonance spectroscopy (EPR) and X-ray absorption spectroscopy (XAS) of thiol-inhibited CO dehydrogenase revealed a bimetallic site in which the RSH coordinates to the Cu-ion as a third ligand {[Mo^{VI}(=O)$OH_{(2)}SCu^{I}$(SR)S-Cys]} leaving the redox state of the Cu(I) and the Mo(VI) unchanged. Collectively, our findings establish a regulation of CO dehydrogenase activity by thiols *in vitro*. They also corroborate the hypothesis that CO interacts with the Cu-ion first. The result that thiol compounds much larger than CO can freely travel through the substrate channel leading to the bimetallic cluster challenges previous concepts involving chaperone function and is of importance for an understanding how the sulfuration step in the assembly of the bimetallic cluster might proceed.

1. Introduction

Carbon monoxide dehydrogenase (CO dehydrogenase) from the Gram-negative α-proteobacterium *Oligotropha carboxidovorans* OM5 is a molybdenum- and copper-containing iron–sulfur flavoenzyme, which has been crystallized and structurally characterized in various states [1,2]. It enables *O. carboxidovorans* to use CO as a sole source of carbon and energy by catalyzing the oxidation of carbon monoxide according to the following equation: $CO + H_2O \rightarrow CO_2 + 2e^- + 2H^+$ [3]. The subunit structure is a dimer of LMS heterotrimers. The L-subunit is a molybdo-copper-protein and accommodates the [CuSMo(=O)OH] active site which is buried ~17 Å below the solvent accessible surface of CO dehydrogenase [4]. The Mo and the Cu ion are coordinated by the molybdopterin cytosine dinucleotide (MCD) cofactor and a cysteine residue of the active site loop VAYRC^{388}SFR, respectively. The two metals are bridged by a μ-sulfido ligand. Flavin adenine dinucleotide (FAD)- and [2Fe–2S]-cofactors are coordinated in the M- and S-subunit, respectively [2,4,5]. These cofactors establish an intramolecular electron transfer. Electrons generated through the oxidation of CO at the [CuSMo(=O)OH] cluster are delivered to [2Fe–2S] I, [2Fe–2S] II and finally to FAD, from where they are fed into a CO-insensitive respiratory chain to generate a membrane potential. The oxidation of CO ($^-|C{\equiv}O|^+$) is irreversibly inhibited at the [CuSMo(=O)OH]-active site by the isoelectronic n-butylisocyanide ($^-|C{\equiv}N^+{-}R$) [2]. The isocyanide group is covalently bound to the μ-sulfido ligand, the hydroxo ligand and the Cu atom forming a thiocarbamate, the alkyl chain extends into the substrate channel. CO dehydrogenase is inactivated by n-butylisocyanide in the oxidized state, whereas the reduced enzyme is not affected [2]. Xanthine oxidase which is another member of the molybdenum hydroxylase enzyme family, is closely related to CO dehydrogenase and also carries a sulfur substituent at the Mo ion [6]. It exists in various interconvertible forms, an NAD^+-dependent dehydrogenase and a reversible and irreversible oxygen-dependent oxidase [7–9]. A number of these interconversions are mediated by thiol reagents, while the mechanism still remains unclear [7,9]. Enzyme

* Corresponding author. Fax: +49 921 55 2727.
E-mail address: Ortwin.Meyer@uni-bayreuth.de (O. Meyer).

http://dx.doi.org/10.1016/j.bbrc.2014.03.147
0006-291X/

modifying and regulating thiols are widespread among pro- and eukaryotes [10,11]. Mostly, modification or regulation takes places as a formation of mixed disulfides or intramolecular disulfides, increasing or decreasing enzymatic activity [11]. Here, we report on the interaction of thiols at the CO dehydrogenase bimetallic center and conditions under which inhibition is reversed.

2. Materials and methods

2.1. Bacterial strain, cultivation and preparation of CO dehydrogenase

The type strain OM5 of *O. carboxidovorans* (DSM 1227, ATCC 49405) used throughout this study [1,3], was cultivated chemolithoautotrophically with CO as an energy source and CO_2 as a carbon source [12]. CO dehydrogenase was purified according to published protocols [5]. Purified enzyme was beaded in liquid nitrogen and kept at −80 °C until use. The quantity of CO dehydrogenase was determined from its absorbance at 450 nm (ε_{450} = 72 mM^{-1} cm^{-1} [13]) and purity was assessed by the ratios of absorbance at 280, 450, and 550 nm as well as by denaturing polyacrylamide gel electrophoresis. We have always used the as isolated enzyme to avoid artifacts that might be introduced through reconstitution measures.

2.2. Treatment of CO dehydrogenase with thiol compounds and assay of CO dehydrogenase activity

CO dehydrogenase (0.2 mg in 0.5 ml of 50 mM potassium phosphate buffer, pH 7.0) contained in serum-stoppered Wheaton vials (5 ml total volume) was made anoxic by repeated evacuation and gassing with N_2. Assays were adjusted to 0.1 to 100 mM thiol compound by injecting (Hamilton syringe) appropriate volumes from anoxic stocks in the same buffer. Assays were kept with magnetic stirring at 100 rpm at 30 °C. Further anoxic additions of compounds in buffer were made by Hamilton syringe where needed. CO, O_2 or acetylene were supplied by flushing vials with the pure gases. Excess thiol compounds were removed from CO dehydrogenase by anoxic gel filtration (Sephadex G-25, PD10, Amersham Pharmacia, Freiburg, Germany). Aliquots were removed by Hamilton syringe and assayed for the oxidation of CO or H_2. CO dehydrogenase activity was determined spectrophotometrically by following the CO- or H_2-dependent reduction of iodonitrotetrazoliumchloride (INT) [14].

2.3. 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 previously described [5]. Spectra were recorded at 120 K for Mo-EPR or at 100 K for Cu-EPR 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. For thiol inactivation, CO dehydrogenase samples (12 mg/ml in 50 mM HEPES–NaOH, pH 7.2) were treated with 15 mM cysteine or 150 mM 2-mercaptoethanol. Samples were incubated for 25 min at room temperature and subsequently frozen in liquid nitrogen. Inactive CO dehydrogenase was reactivated by flushing with CO for 30 min. For reduced spectra, the enzyme was inactivated with 2-mercaptoethanol followed by subsequent treatment with 5 mM sodium dithionite.

2.4. X-ray absorption spectroscopy (XAS)

For XAS measurements, 24 µl of enzyme solution (11.6 mg/ml in 50 mM potassium phosphate buffer, pH 7.0), treated with 150 mM 2-mercaptoethanol, was filled into sample cells of HESAR glass covered with Kapton windows. The cells were sealed and kept near liquid nitrogen temperatures at all times. XAS data at the Mo- and Cu-*K*-edges were collected at Beamline B18 of the Diamond Light Source (Didcot, Oxfordshire, UK) using a liquid nitrogen cryostat and a 9-element Ge solid state fluorescence detector. XAS spectra were calibrated using the transmission spectra of reference foils and setting their edge positions (first maximum of first derivative) to 8979 eV (Cu) and 20002 eV (Mo). Data processing such as background removal, normalization and extraction of the extended X-ray absorption fine structure (EXAFS) portion of the XAS spectra was achieved using the ATHENA program [15]. EXAFS data were analyzed using the refinement program EXCURVE 9.310 [16].

3. Results

3.1. Inhibitory effect of thiols on the oxidation of CO or H_2 by CO dehydrogenase

CO dehydrogenase incubated with L-cysteine under anoxic conditions and then assayed for CO oxidizing activity was inactive at the beginning (Fig. 1A). In the activity assay a slow time-dependent reactivation of CO dehydrogenase was observed which can be ascribed to dilution and oxidation of the thiol in combination with enzyme reactivation by CO. These subjects will be discussed later in this paper. The oxidation of H_2, which is a side activity of CO dehydrogenase, was also inhibited by L-cysteine (data not shown). Similar to L-cysteine, numerous other thiols were also identified as potent inhibitors of CO- or H_2-oxidation by the enzyme (Fig. 1B). Inhibition depended on the substrate concentration and followed Michaelis–Menten kinetics. Inhibition constants (K_i in mM) were determined from plots of enzyme activity in the presence of different thiol concentrations *versus* time (L-cysteine, 5.2; D-cysteine, 9.7; *N*-acetyl-L-cysteine, 8.2; D,L-homocysteine, 25.8; L-cysteine–glycine, 2.0; dithiothreitol, 4.1; coenzyme A, 8.3; 2-mercaptoethanol, 9.3). The K_i-values do not correlate with physical properties of the thiols such as molecular weight, molecule size or polar surface. The list of inhibitory thiols includes L-cysteine, L-glutathione and coenzyme A which naturally occur in intact cells of *O. carboxidovorans*. In contrast to thiols with a free SH-group, their methylated or acetylated counterparts (S-methyl-L-cysteine, L-methionine, acetyl-coenzyme A) had no effect on CO dehydrogenase activity which identifies the SH-group as the reactive constituent. The concentration of some thiols (e.g. glutathione) could not be increased to the extent of complete inhibition of CO dehydrogenase activity (Fig. 1B).

3.2. Reactivation of thiol-inhibited CO dehydrogenase

Inhibition of CO dehydrogenase by thiols was fully or partially reversed under various conditions (Fig. 1C) including removal of the thiol (e.g. by gel filtration), exposure to CO, alkylation of the thiol SH-group with *N*-ethylmaleimide and oxidation by O_2. The fact that O_2 reactivates inhibited CO dehydrogenase (Fig. 1C) can be ascribed to the oxidation of thiols to the corresponding disulfides and explains why inactivation of the enzyme by thiols proceeds under anoxic conditions. Reactivation of thiol-inactivated CO dehydrogenase by CO can be explained by binding of CO to Cu(I) with the concomitant displacement of the thiol from the metal.

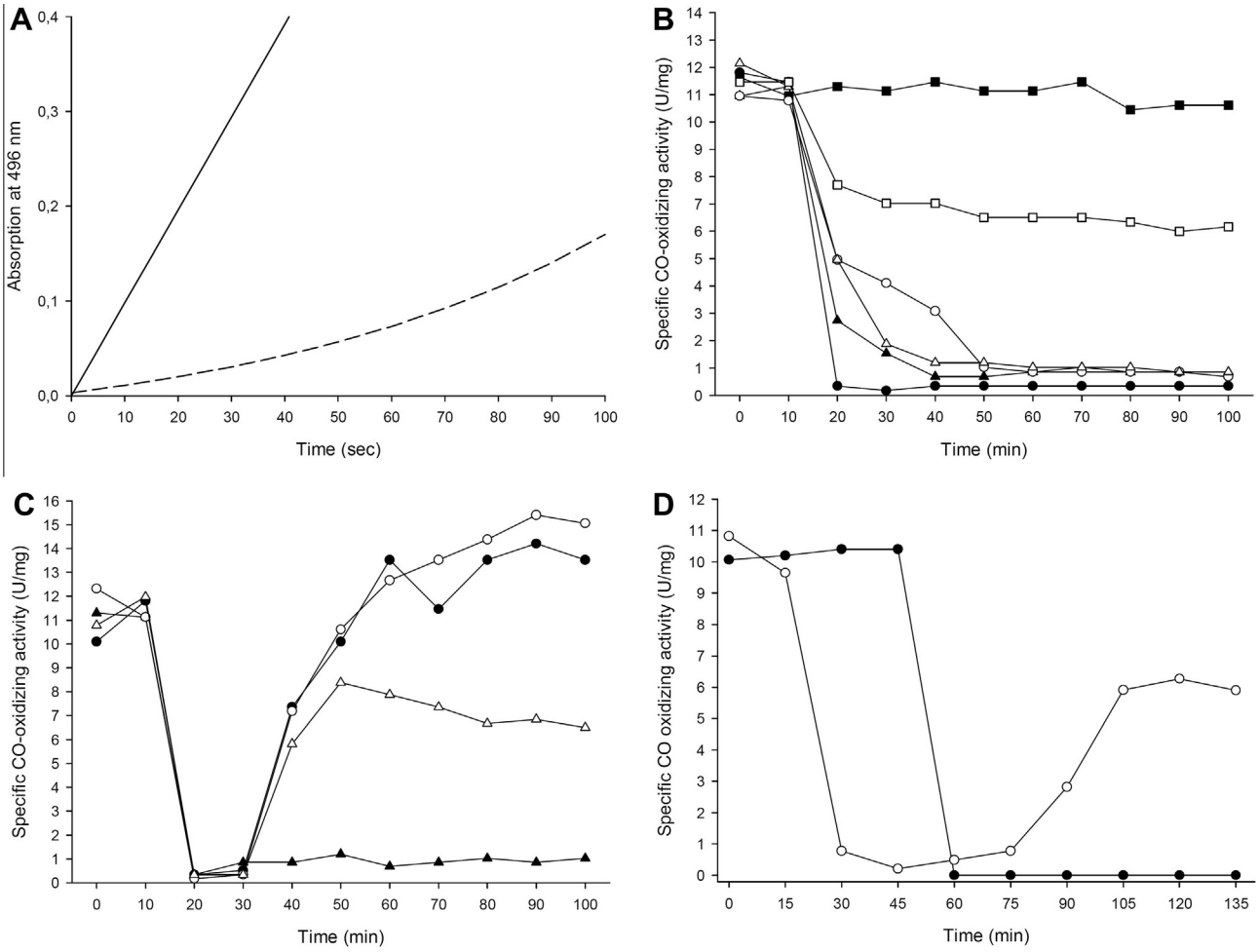

Fig. 1. Inactivation of CO dehydrogenase by thiols. (A) CO dehydrogenase (0.4 mg/ml) in phosphate buffer was inactivated by incubation with 10 mM L-cysteine for 60 min and then analyzed for the reduction of iodonitrotetrazoliumchloride (INT) by CO (dashed line). The untreated enzyme is shown as a control (solid line). (B) Effect of thiol compounds on CO dehydrogenase activity. CO dehydrogenase (0.4 mg/ml) in phosphate buffer was kept for 10 min under N_2 and then supplied with 10 mM of 2-mercaptoethanol (●), L-cysteine (○), L-glutathione (□), coenzyme A (▲), dithiothreitol (△), or S-methyl-L-cysteine (■). CO dehydrogenase activity was assayed as in (A). (C) Reactivation of CO dehydrogenase. The enzyme solution (0.4 mg/ml) kept in N_2-saturated phosphate buffer was supplied with 10 mM 2-mercaptoethanol after 10 min of incubation. At 30 min the N_2 in the gas atmosphere was replaced by CO (△) or O_2 (▲). Alternatively, at 30 min assays were freed from excess mercaptoethanol by gel filtration (○) or alkylation with *N*-ethylmaleimide (●) and incubation under N_2 was continued. (D) Effect of n-butylisocyanide (n-BIC) on the reactivation of thiol-inactivated CO dehydrogenase. At 15 min the enzyme solution (0.4 mg/ml) kept in N_2-saturated phosphate buffer was supplemented with 10 mM 2-mercaptoethanol (○). At 45 min 1 mM n-butylisocyanide was added. In the control experiment the CO dehydrogenase solution under N_2 was supplied with 1 mM n-BIC at 45 min but did not receive 2-mercaptoethanol (●). At 75 min the N_2 was replaced by CO. *Abbreviation used:* U/mg – 1 μmol CO oxidized per min at 30 °C per milligram protein (Specific enzyme activity).

N-butylisocyanide is inhibitory to CO dehydrogenase because it forms a stable thiocarbamate at the active site of the enzyme. Inhibition requires Mo in the +VI oxidation state whereas the Mo(IV)-enzyme is not affected. Treatment of CO dehydrogenase with 2-mercaptoethanol produced an inactive enzyme which was reactivated by CO although *N*-butylisocyanide was present (Fig. 1D). On the other hand, CO dehydrogenase was readily inhibited by *N*-butylisocyanide when 2-mercaptoethanol was omitted under otherwise same conditions (Fig. 1D). This data indicates that the thiol outcompetes the isocyanide-group and interacts with the enzyme's [$CuSMoO_2$] cluster. The various conditions suitable to reactivate CO dehydrogenase show that the activity of the thiol-inactivated enzyme can be fully restored provided that the thiol has been removed or its SH-group has been rendered ineffectual.

3.3. Interaction of thiols at the CO dehydrogenase catalytic site

The biochemical data point towards an interaction of thiols with the [$CuSMoO_2$] cluster (Section 3.2). To obtain structural information on this potential interaction, we performed XAS on thiol-inactivated CO dehydrogenase at the Mo- and Cu-*K*-edges. Although we were able to co-crystallize CO dehydrogenase with 2-mercaptoethanol (100 mM), X-ray diffraction data were limited to resolutions exceeding 3.5 Å and therefore did not provide the anticipated structural insights.

3.3.1. Absorption edges and redox states of metals and cofactors

Significant differences of the Cu-*K*-edges of CO dehydrogenase in the presence or absence of 2-mercaptoethanol indicate changes of the Cu environment (Fig. 2A). In the absence of thiol, the appearance of a pronounced $1s \rightarrow 4p$ peak (intensity 0.8) in the rising edge of the spectrum, along with the absence of a $1s \rightarrow 3d$ pre-edge peak characteristic of Cu(II), is in agreement with the previously established non-linear 2-coordinate Cu(I) in oxidized CO dehydrogenase [17]. Thiol-inactivated CO dehydrogenase does not have a pre-edge peak either and its edge position corresponds to the one in the absence of thiols. Accordingly, thiol-inactivation leaves the Cu(I) oxidation state unchanged. However, the absence of a pronounced $1s \rightarrow 4p$ peak after addition of thiol, and the drop in

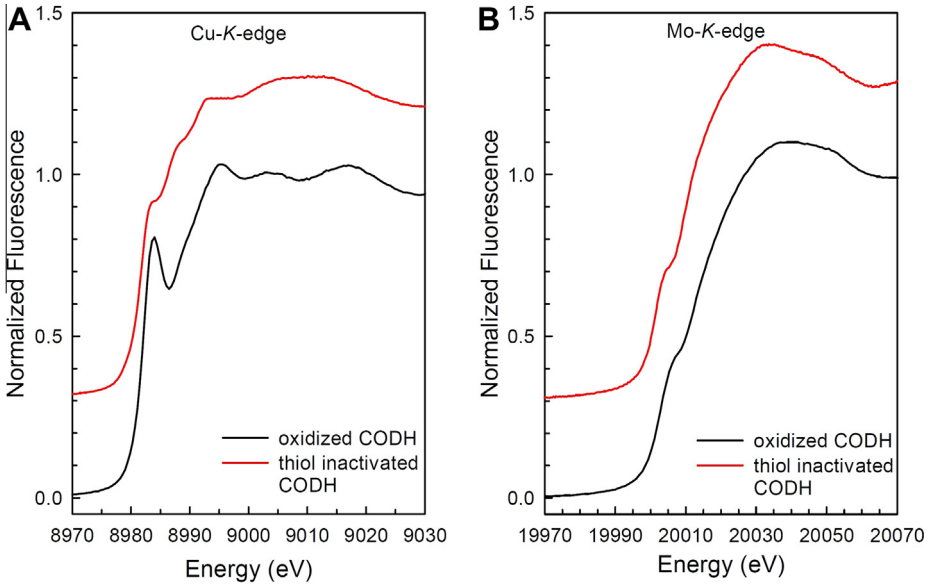

Fig. 2. Normalized Cu- and Mo-*K*-edges of CODH before (black curve) and after (red) inactivation with thiol. Data for oxidized CODH were previously reported [17]. An offset of 0.3 fluorescence units has been introduced into the red curves for better clarity.

intensity in this energy range (0.6) indicates a raise in coordination number (3- or 4-coordinate) due to the interaction with thiol [18].

We have not been able to generate a paramagnetic EPR-signal at 100 K from CO dehydrogenase inactivated with 150 mM 2-mercaptoethanol (Supplementary Fig. 1) which further adds to a Cu ion in the +I oxidation state. As for Mo, the EPR spectra of CO dehydrogenase at 120 K in the presence of the above thiols did not reveal a paramagnetic signal either which can be taken as evidence for the absence of a paramagnetic Mo(V) species (Supplementary Fig. 1). The UV–vis absorption spectra of CO dehydrogenase in the presence or absence of 2-mercaptoethanol (10 mM) were indistinguishable (data not shown) which again indicates that under these conditions thiols are not oxidized and do not serve as electron donors to CO dehydrogenase. Obviously, the interaction of CO dehydrogenase with thiols does not involve any redox chemistry.

The overall shape of the normalized Mo-*K*-edges of CO dehydrogenase in the presence or absence of 2-mercaptoethanol is strikingly similar (Fig. 2B) and, hence, similar Mo coordination environments can be expected. However, a difference is observed for the intensity of the shoulder in the rising edge (so-called oxo-edge feature), indicating a lower oxo content (Mo=O) in the thiol-inactivated species [19].

3.3.2. Cu EXAFS

The Fourier transforms of the Cu EXAFS of CO dehydrogenase treated with 2-mercaptoethanol reveal a larger sulfur content and an increase in the average Cu–S distance from 2.176 Å in oxidized CODH [17] to 2.222 Å in thiol-incubated CODH (Fig. 3A and B). An elongation of the average Cu–S distance is expected for an increase in coordination number [20].

3.3.3. Mo EXAFS

The k^3-weighted Mo EXAFS of thiol-treated CO dehydrogenase in the presence or absence of 2-mercaptoethanol are different (Fig. 3C and D). Although the Cu content of the enzyme remains unchanged upon thiol treatment, the Mo environment is very similar to the one reported previously for the Cu-deficient species [17]. The direct Mo environments in oxidized and Cu-deficient CO dehydrogenase mostly differ in the oxo content [17], and the present results indicate that the oxo content in thiol-treated CO dehydrogenase is similar to the one in the Cu-deficient enzyme. Consequently, the Mo environment in thiol-inactivated CODH can be fitted with a model that is very close to the Mo environment in Cu-deficient CODH (Fig. 3D). Also note that the partial replacement of one of the two oxo groups in oxidized CODH corresponds well to the less pronounced oxo-edge feature discussed above (Fig. 2).

4. Discussion

Our data can be summarized in the model shown in Fig. 4. Thiol compounds (RSH) can bind to the bimetallic cluster of CO dehydrogenase resulting in a 3-coordinate Cu ion. The remainder of the structure mostly corresponds to the $[Mo^{VI}S(=O)OH_{(2)}]$ cluster present in Cu-deficient CO dehydrogenase [2,17]. As is apparent from EPR (Supplementary Fig. 1) the thiol has no effect on the oxidation state of Mo and Cu and leaves them at +VI or +I, respectively. It has been proposed that CO interacts with the bimetallic cluster through the formation of a thiocarbonate intermediate bridging Mo and Cu [2]. Although this view has been challenged by quantum chemical modeling of CO oxidation by the active site of CO dehydrogenase, it is generally accepted that a natural position to place CO is on Cu, which turned out to be the energetically best position [21,22]. It seems that thiol compounds and CO compete for the same position at Cu(I) which explains the reactivation of thiol-inhibited enzyme by CO.

Bacteria can contain free thiols (e.g. L-cysteine or coenzyme A) at concentrations ranging approximately from 20 to 200 μM [23,24]. Coenzyme A is an essential cofactor in the biosynthetic metabolism of *O. carboxidovorans*. The K_i of CO dehydrogenase for coenzyme A of 8.3 mM is much higher which explains why the compound does not permanently inhibit CO oxidation in intact cells of *O. carboxidovorans*. On the other hand, the intracellular concentration of the thiol pool in *O. carboxidovorans* might well approach a level not far from the K_i-values displayed by CO dehydrogenase. Regulation of CO dehydrogenase activity by the intracellular pool of free coenzyme A (CoASH), L-cysteine and/or other thiols would establish a mechanism to downregulate ATP synthesis under conditions where the bacteria must reduce the production of amino acid precursors in the citric acid cycle.

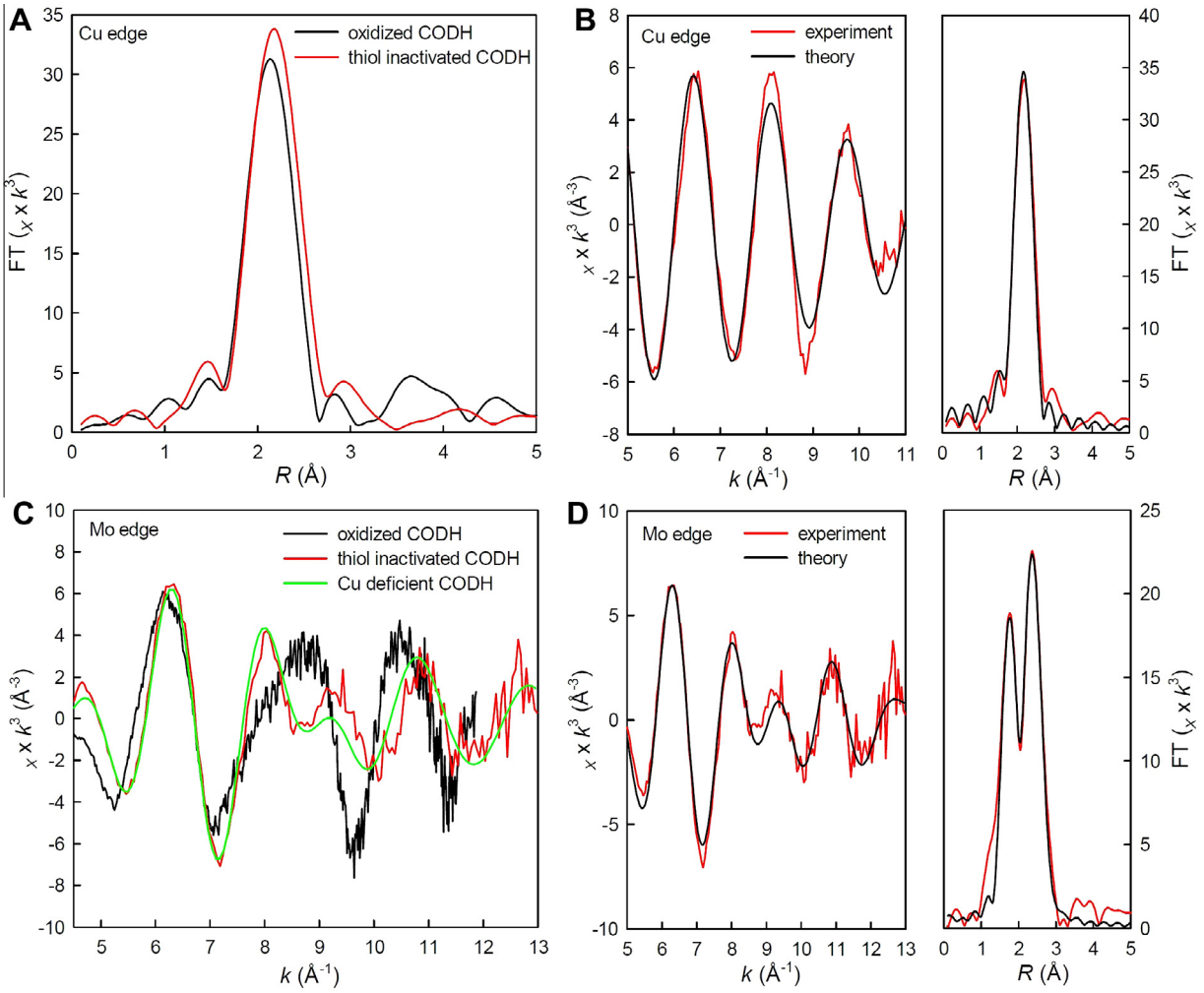

Fig. 3. (A) Comparison of the Fourier transforms of the Cu EXAFS of thiol-inactivated and oxidized CODH. It appears that the addition of thiol leads to an increase in sulfur content as well as an increase in the average Cu–S distance. (B) Cu EXAFS fit for thiol-inactivated CODH: 3 Cu–S with $R = 2.222(5)$ Å, $2\sigma^2 = 0.012(1)$ Å^2, $E_F = -2.4(8)$ eV, *R*-factor 20.7%. Fits with either two or four sulfur atoms are worse (*R*-factors 22.2% and 29.5%, respectively). (C) Demonstration that the k^3-weighted Mo EXAFS of thiol-incubated CODH (red) and oxidized CODH (black) are different. The Mo environment in thiol-inactivated CODH is very similar to the one reported previously for the Cu-deficient species (green). (D) Mo EXAFS fit for thiol-inactivated CODH: 1.5(3) Mo=O at 1.731(11) Å, 0.5 Mo—$OH_{(2)}$ at 2.216(33) Å, 1 Mo—S at 2.354(54) Å, 2 Mo—S at 2.461(15) Å, $2\sigma^2$ (O) = 0.006(3) Å^2, $2\sigma^2$ (S) = 0.009(6) Å^2, $E_F = -17.4(30)$ eV, *R*-factor 29.0%. XAS data for oxidized and Cu-deficient CODH were previously published [17]. *Abbreviations used:* χ – EXAFS amplitude, *k* – wave number; *R* – distance, FT – Fourier transform amplitude, $2\sigma^2$ – Debye–Waller parameter, $E_F - E_0$ correction, *R*-factor – goodness of fit.

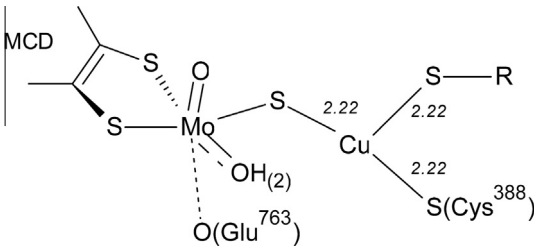

Fig. 4. Plausible structural model for the inactivation of CO dehydrogenase by thiols. EXAFS indicates that thiol compounds (RSH) bind to the bimetallic cluster of CO dehydrogenase resulting in a 3-coordinate Cu ion with an average Cu–S distance of 2.22 Å (Fig. 3). The thiol (R-SH) coordinates via its SH-group to the Cu(I)-ion of the [CuSMo(=O)OH] cluster. Concomitantly, binding of the thiol decreases the oxo content (Mo=O) of the [CuSMo(=O)OH] cluster (Fig. 3). MCD stands for molybdopterin cytosine dinucleotide.

Upregulation of CO dehydrogenase activity would proceed under conditions when the intracellular level of acetyl-CoA and concomitantly the ATP demand for the biosynthesis of amino acids increase.

It has been concluded from the C β of the surrounding residues in the 3D-structure of CO dehydrogenase that the active site is accessible from the outside through a narrow channel that is 17 Å deep with an average diameter of 7 Å [4]. In addition, a restriction of the channel has been assumed to exclude substrates larger than CO. In this paper we have established that rather voluminous molecules, such as L-cysteine, coenzyme A or glutathione, are able to pass through the substrate channel and to enter the bimetallic cluster. This opens the possibility that thiol compounds can act as potential sulfur donors in the sulfuration of [MoO_3] to [MoO_2S], which is the first step in the posttranslational maturation of the bimetallic cluster in apo-CO dehydrogenase [12,25]. In this case, the function of a chaperone, such as the AAA+ ATPase CoxD, which has been envisaged previously to partially unfold CO dehydrogenase for the integration of sulfur into the bimetallic site, would not be required [12,25].

Acknowledgments

This work was financially supported by the University of Bayreuth and by Grants ME 732/11-1 (O.M.) and FOR 1405 (M.G.,

W.M.-K.) from the Deutsche Forschungsgemeinschaft (DFG). We thank Katharina Vogel for preliminary experiments, Ulrike Brandauer for the production of bacterial cell mass, and Brigitte Melzer for the purification of CO dehydrogenase. All these individuals are with the University of Bayreuth, Germany. The authors are grateful to Alina Wetzel and Benjamin Lebsanft (University of Hamburg, Germany) as well as the beamline staff at the Diamond Light Source (UK) for assistance during XAS data collection.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.147.

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