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Lilith Domnik, Meriem Merrouch, Sebastian Goetzl, Jae-Hun Jeoung, Christophe Léger, et al.. CODH-IV: A High-Efficiency CO-Scavenging CO Dehydrogenase with Resistance to O₂. *Angewandte Chemie International Edition*, 2017, 56 (48), pp.15466-15469. 10.1002/anie.201709261 . hal-01696167

HAL Id: hal-01696167

<https://hal.science/hal-01696167>

Submitted on 3 Feb 2020

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A high efficiency CO-scavenging CO dehydrogenase with increased resistance to O₂

Lilith Domnik^[a], Meriem Merrouch^[b], Sebastian Goetzl^[a], Jae-Hun Jeoung^[a], Christophe Léger^[b], Sébastien Dementin^[b], Vincent Fourmond^{*[b]}, Holger Dobbek^{*[a]}

Abstract: CO dehydrogenases (CODHs) catalyse the reversible conversion between CO and CO₂. Genomic analysis indicated that the metabolic functions of CODHs vary. The genome of *Carboxydothemus hydrogenoformans* encodes five CODHs (CODH-I – V), of which CODH-IV is found in a gene cluster near a peroxide reducing enzyme. Using a combination of solution assays and protein film voltammetry experiments, we show that CODH-IV differs from other CODHs by characteristic properties: it has a very high affinity for CO, oxidizes CO at diffusion-limited rate over a wide range of temperatures, and is more tolerant to oxygen than CODH-II. Thus, our observations support the idea that CODH-IV is a CO scavenger in defence against oxidative stress and highlight that CODHs are more diverse in reactivity than expected. (802/800)

Anaerobic microorganisms, both bacteria and archaea, employ Ni- and Fe-containing carbon monoxide dehydrogenases (CODHs) to reversibly reduce CO₂ to CO.^[1-3] Ni,Fe-CODHs are homodimeric enzymes containing a Ni,Fe,S-cluster, termed cluster C, at their active site. Cluster C is a [NiFe₃S₄OH_x]-cluster, where a Ni(II) ion is integrated into an Fe/S scaffold forming a distorted NiFe₃S₄-heterocubane with an additional Fe(II) ion with water/hydroxide coordination linked in *exo*. Additional [4Fe4S]-clusters, termed clusters B and D form with cluster C a V-shaped chain of Fe/S-clusters. Ni,Fe-CODHs are attractive targets both for employing CO as electron source and for efficient CO₂ reduction with minimal driving force.^[2] However, their inactivation by dioxygen as well as their moderate affinity for CO limit their potential applications in biotechnology.^{[4],[5]} The degree of sensitivity of Ni,Fe-CODHs against O₂ has not been systematically studied and the mechanism of O₂-dependent inactivation of Ni,Fe-CODHs is unknown. A recent comparison of the Ni,Fe-CODHs from the sulfate-reducing, microaerotolerant bacterium *Desulfovibrio vulgaris* (Dv-CODH) and *Carboxydothemus hydrogenoformans* (CODH-II) revealed that both react with O₂ to form an inactive state, but Dv-CODH reacts more slowly with O₂ than CODH-II and, unlike CODH-II, it can be fully reactivated by reduction.^[6] This unexpected observation prompted us to investigate and characterize the kinetic properties as well as the O₂-sensitivity of other Ni,Fe-CODHs.

C. hydrogenoformans is a hydrogenogenic bacterium found in a hot spring of Kunashir Island.^[7] Its genome revealed five Ni,Fe-CODH genes in different genomic contexts, of which only three (CODH-I, CODH-II and CODH-III) have been biochemically characterized.^[8-10] The gene encoding CODH-IV is part of an operon encoding enzymes typically found in aerobic stress response; it was therefore suggested that electrons derived from CO-oxidation by CODH-IV may be used to reduce reactive oxygen species (ROS) by ROS-detoxifying enzymes.^[8] However, CODH-IV had never been isolated from *C. hydrogenoformans* and its properties were unknown.

Therefore, we produced and investigated the reactivity and O₂-sensitivity of CODH-IV and compared its properties and structure to those of other Ni,Fe-CODHs, particularly CODH-II. Using protein film voltammetry, we showed that CODH-IV combines a high affinity for CO with a low sensitivity for O₂, while the crystal structure of CODH-IV reveals small, but remarkable differences from the more O₂-sensitive CODH-II. Most surprisingly, in contrast to other CODHs, CO oxidation by CODH-IV is diffusion-limited over a wide range of temperature, making it one of the few “perfect” catalysts.^[11]

We could achieve stable heterologous expression of highly active CODH-IV in *E. coli*. We determined the Michaelis constant (K_m) for CO using electrochemistry, by monitoring the CO oxidation current following injections of aliquots of CO-saturated buffer. This method takes advantage of the exponential decrease in the concentration of CO after the injection to determine the catalytic response over a large range of concentrations in a single experiment.^[12] Figure 1 shows typical signals obtained with CODH-II and -IV. The current instantly increases after the injection (at $t = 0$), it remains constant while the enzyme is still saturated with CO, and, eventually, decreases exponentially when the concentration of CO drops below the value of K_m . This final decrease in current occurs much later for CODH-IV than for CODH-II, which shows that the K_m value for CODH-IV is smaller than that of CODH-II. Fitting the model in ref^[13] to the data (dotted lines in figure 1) yielded K_m values of $8 \pm 1 \mu\text{M}$ (CODH-II) and $47 \pm 3 \text{ nM}$ (CODH-IV) (at 25°C).

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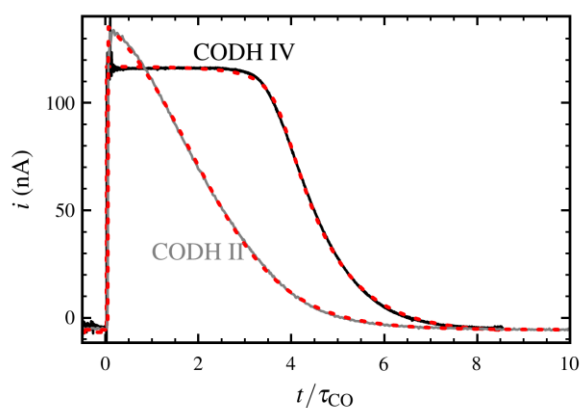
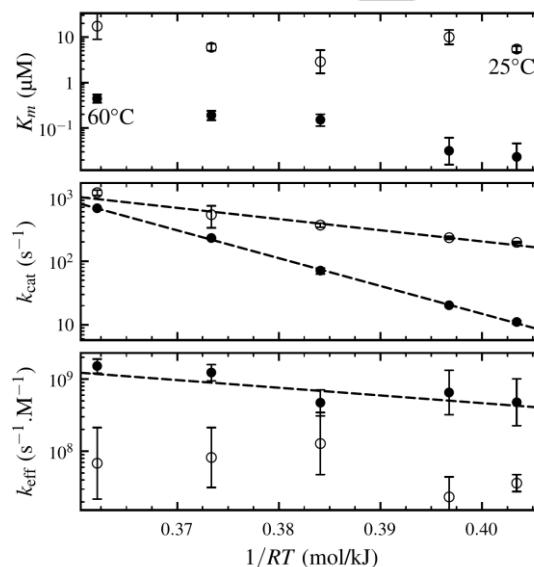


Figure 1: Electrochemical determination of the Michaelis constants of CODH-II and -IV. The figure shows the response in current of films of CODH-II and -IV after injecting 50 μM CO in the electrochemical cell, together with the fit of equation 9 in ref [13] (dashed lines). To ease the comparisons, the time has been normalized by the time constant τ_{CO} of the exponential decrease in the concentration of CO. Conditions: pH 7, $T = 25^\circ\text{C}$. Fit parameters: CODH-IV: $K_m = 47 \pm 3 \text{ nM}$, $nFAM = 118 \pm 4 \text{ nA}$, $nFAG k_{\text{eff}} > 2 \mu\text{A}$, $t_{\text{CO}} = 36.9 \pm 0.3 \text{ s}$; CODH-II: $K_m = 8 \pm 1 \mu\text{M}$, $nFAM > 10 \mu\text{A}$, $nFAG k_{\text{eff}} = 20 \pm 2 \text{ nA}$, $t_{\text{CO}} = 6.27 \pm 0.05 \text{ s}$.

Figure 2 shows the values of K_m (determined using electrochemical experiments), k_{cat} (determined using solution assays) and $k_{\text{eff}} = k_{\text{cat}}/K_m$ for temperatures ranging from 25 $^\circ\text{C}$ to 60 $^\circ\text{C}$. The dependence of k_{cat} on temperature follows Arrhenius law (dashed lines in figure 2), with an activation energy of 40 kJ/mol for CODH-II and 100 kJ/mol for CODH-IV. At room temperature, the activity of CODH-IV is much lower than that of CODH-II, but, because the activation energy of CODH-IV is greater, their catalytic activities become similar at high temperature. The K_m value of CODH-II is almost independent of temperature (open symbols), of the order of 10 μM . That of CODH-IV, on the contrary, increases from about 25 nM (ranging from 10 nM to 60 nM) at 25 $^\circ\text{C}$ to about 500 nM at 60 $^\circ\text{C}$. The explanation for this difference is given by the plot of the catalytic efficiency $k_{\text{eff}} = k_{\text{cat}}/K_m$ (lower panel in figure 2): the k_{eff} for CODH-IV is in the range of 3×10^8 to $10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$, which corresponds to the diffusion limit [11]; in fact, the temperature dependence of k_{cat}/K_m closely matches the temperature dependence of the diffusion coefficient of CO in water [14] (dashed line in the bottom panel in figure 2), which is consistent with the two being proportional to one another. (The theoretical “diffusion limited bimolecular rate” is determined by how fast the small substrate can diffuse towards the larger, immobile enzyme [15].) As a consequence, any change in k_{cat} must be compensated by a similar change in K_m . In contrast, the activity of CODH-II is not limited by diffusion in the temperature range that we could investigate.

We used transient exposures to CO and O_2 to compare the reactivity of CODH-II and -IV with O_2 . Using the six-injection method that we explained before [6], we exposed films of CODH-IV at 25 $^\circ\text{C}$ to O_2 spanning a large range of concentrations (from 30 nM to 20 μM) and measured the fraction of activity remaining after the departure of O_2 and following a reductive poise (an example experiment is shown in supplementary figure S1). The

results are shown in figure 3, together with the CODH-II and Dv-CODH data reproduced from ref [6]. All things being equal,



CODH-IV reactivates more after the departure of O_2 than does CODH-II (open symbols); however, unlike CODH-II and Dv-CODH, CODH-IV does not reactivate upon reduction.

Figure 2: Temperature dependence of the Michaelis constant K_m (top), the turnover number k_{cat} (middle) and $k_{\text{eff}} = k_{\text{cat}}/K_m$ (bottom) for CODH-II (open symbols) or CODH-IV (full symbols). The dashed lines in the middle panel are fits of an Arrhenius law to k_{cat} ($E_a = 40 \text{ kJ/mol}$ for CODH-II and 100 kJ/mol for CODH-IV). The dashed line in the bottom panel is an Arrhenius law with an activation energy of 24 kJ/mol deduced from the dependence of the CO diffusion coefficient in water in ref [14].

CODH-II and CODH-IV share approximately 53% (identity) of their amino acid sequence, are of very similar length (CODH-II: 636 aa; CODH-IV: 633 aa) and share conserved binding motifs for clusters B, C and D. To compare both enzymes in more detail, we determined and analyzed the crystal structure of CODH-IV.

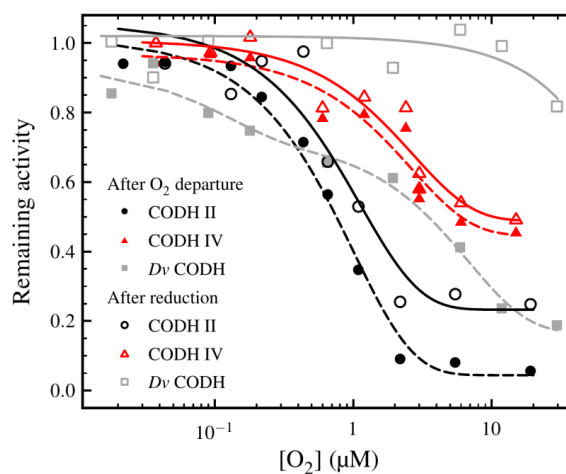


Figure 3: Activity remaining after the departure of O_2 (open symbols) and after a reductive poise (filled symbols) for CODH-II (black), CODH-IV (red) and Dv-CODH (gray). Data for CODH-II and Dv-CODH are replotted from ref [6]. The lines are just guide for the eyes.

CODH-IV crystals diffracted to a resolution of 2.52 Å and the complete structure of CODH-IV, containing residues 3–633 was located in the electron density (Table S1 in the Supporting Information). The structures of CODH-II (pdb Id: 3B53) and CODH-IV are very similar and can be superimposed with an rmsd-value for C α -atoms in the core region of 1.1 Å (Figure S2).

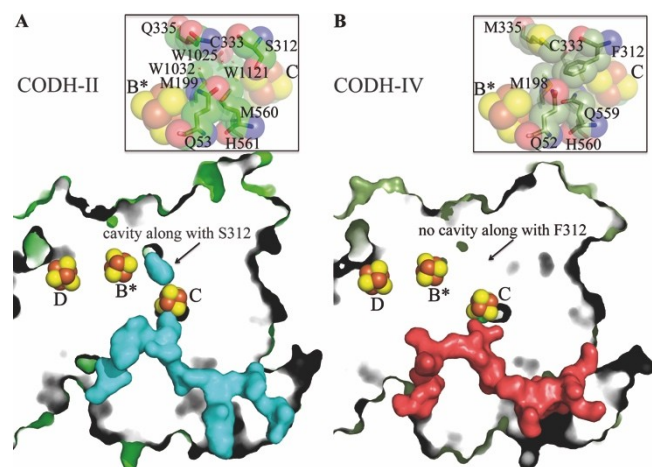


Figure 4. Comparison of channels in (A) CODH-II and (B) CODH-IV. Channels in CODHs were calculated using HOLLOW 1.1^[16] and are shown as surfaces. Upper panels represent van der Waals-radii (spheres) of residues around S312 in CODH-II and F312 in CODH-IV.

As reactivity and O₂-sensitivity of CODH depends on the state of the metal clusters, we compared the direct environment of the Fe/S-clusters in CODH-II and CODH-IV. The homodimeric structure of CODH-IV harbors five iron-sulfur clusters, whose position and coordination is the same as that of CODH-II. The subunit-bridging cluster D as well as cluster B have not only identical locations but also indistinguishable second coordination environments. Earlier crystallographic studies hinted at cluster C, the active site cluster, as the most O₂- and CO-sensitive metal site, whose alteration/destruction is reflected in loss of activity.^[17] This was recently supported by the observation that the competitive inhibitor and alternative substrate *n*-butylisocyanide, which binds to cluster C,^[18] protects *Dv*-CODH from O₂.^[6] Residues around the CO₂ binding site and the accessibility of Ni and the *exo*-Fe (H92, Q332, K562, I566) are structurally conserved, showing the same conformations in both CODH structures. In contrast, the Fe₃S₄ cores of cluster C have different environments and accessibilities in the two CODHs (Figure 4). In CODH-IV, a cluster of residues, F312, Q559, M198, C333 and H560 are in van der Waals contact, effectively shielding cluster C at S4 and Fe2. In contrast, in CODH-II F312 is replaced with S312, Q559 by M560, which interact weakly with one another, leaving enough space for water molecules (W1121, W1025 in the structure of CODH-II, pdb-Id: 3B53) to reach cluster C (Figure 4). Given the otherwise strict conservation of the cluster environments, the dense packing around cluster C in CODH-IV compared to the loose packing in CODH-II is surprising. Assuming that positions that may be reached by water may also be approached by molecules of similar size, the backside of cluster C could either react directly with O₂ in CODH-II or could be more easily reached by reactive

O₂ species generated at another position of cluster C, e.g. at the Ni/Fe1 site where CO and CO₂ bind. This approach to the backside of cluster C is hindered in CODH-IV, which appears to be the most obvious structural difference between both CODHs.

Tight gas channels as a means to protect an active site from the destructive power of O₂ have been discussed for O₂-tolerant Ni,Fe-hydrogenases.^[19–21] CODHs have remarkably long channels running through the entire dimer, reaching the substrate-binding site of the Ni ion of cluster C with a side arm, in which Xenon and *n*-butylisocyanide have been detected.^[18, 22–23] CODH-IV features similar channels as found in CODH-II (Figure 4). Given the similar extensions of CO and O₂, discrimination by size-exclusion appears as an unlikely means to protect a site from O₂, which has to be reached by the substrate molecules CO and CO₂. Accordingly, the diameter and extensions of the calculated channels in CODH-II and CODH-IV are similar. However, again the cavities at the backside of cluster C appears to be the most obvious difference between the two CODHs (Figure 4).

In summary, CODH-IV differs remarkable from so far characterized CODHs: (i) CODH-IV has a very high affinity for CO (Figure 1); (ii) it oxidizes CO over a large temperature range at a rate that is limited by CO diffusion (Figure 2); (iii) it withstands transient exposures to higher concentrations of O₂ than CODH-II or *Dv*-CODH (Figure 3) and (iv) its Arrhenius activation energy for CO oxidation is more than twice that of CODH-II. We believe that these properties are to some extent connected.

Arrhenius-type activation energies for enzymatic CO oxidation have been determined for NiFe-CODHs^[24–25] and for the unrelated CuMo-containing CODHs^[26–27], all of which are in the range between 40 and 60 kJ/mol. Thus, the E_a = 40 kJ/mol of CODH-II is within the expected range, but the activation energy determined for CODH-IV (E_a = 100 kJ/mol) is remarkably larger. On the other hand, CODH-IV operates at the limit of diffusion in CO oxidation, whereas other CODHs show k_{cat}/K_m values between 10⁶–10⁸ M⁻¹ s⁻¹. Surprisingly, although CuSMo-CODHs only catalyze the oxidation of CO and not also the reduction of CO₂, they have with $k_{cat}/K_m = 8.7 \times 10^6$ M⁻¹ s⁻¹ a comparably small catalytic specificity,^[26, 28] whereas the bifunctional CODH from *M. thermoacetica* reaches 2 × 10⁷ M⁻¹ s⁻¹, the monofunctional CODH from *R. rubrum* has a k_{cat}/K_m of 2.5 × 10⁸ M⁻¹ s⁻¹ and CODH-II from *C. hydrogeniformans* reaches 1.7 × 10⁹ M⁻¹ s⁻¹ at 70 °C, but is below the diffusion limit in the temperature range 20–60 °C, reported here.^[9, 29–30]

We can only speculate on the physiological relevance of the properties of CODH-IV. As only very few enzymes are able to operate at the diffusion limit,^[11] the ability of CODH-IV to oxidize CO at the diffusion limit over a large range of temperature is likely the result of intense selective pressure. Its role is probably that of a scavenger, to extract the reducing power of minute amounts of CO, at the lowest cost possible for the cell. Therefore, it appears likely that when *C. hydrogeniformans*, living as a free-swimming bacterium in hot CO-containing springs, faces periods of oxidative stress in colder, CO-poor conditions, e.g. near the oxygenated top layer of the hot spring, CODH-IV would allow it to generate electrons from traces of CO to supply the electrons for reduction of reactive oxygen species by detoxifying enzymes such as rubrerythrin. This high efficiency

may benefit the cell in two ways, first by limiting the amount of protein produced, and, second, by hastening the response, since fewer enzymes have to be synthesized for a fully operational response to oxidative stress. The larger resistance to O₂ compared to CODH-II further supports this role. However, more importantly, the unusual catalytic properties of CODH-IV show that Ni,Fe-CODHs, despite sharing high sequence similarities, are far more diverse in reactivity with CO and stability in the presence of dioxygen, than previously anticipated.

Acknowledgements

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H.D. acknowledges the cluster of excellence "Unifying concepts of catalysis – UniCat" and DFG DO 785/6-1 for funding of the project. M.M, C.L, S.D, and V.F acknowledge financial support from CNRS, Aix Marseille Université, Agence Nationale de la Recherche (ANR-12-BS08-0014, ANR-14-CE05-0010, ANR-15-CE05-0020) and the A*MIDEX grant (ANR-11-IDEX-0001-02) funded by the French Government "Investissements d'Avenir" program. M.M, C.L, S.D, and V.F are members of the FrenchBIC (<http://frenchbic.cnrs.fr>)

Keywords: Carbon dioxide reduction • O₂ tolerance • electrochemistry • activation energy • keyword 5

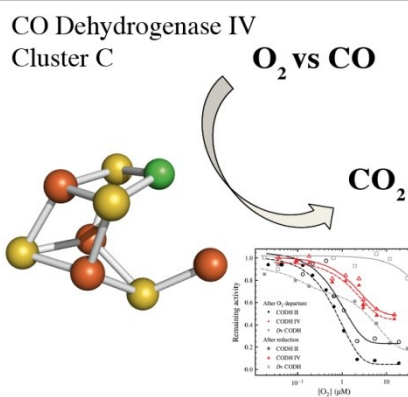
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