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Bioelectrocatalysis **Hot Paper**

Bioelectrocatalytic CO_2 Reduction by Mo-Dependent Formylmethanofuran Dehydrogenase

Selmihan Sahin[+], Olivier N. Lemaire[+], Mélissa Belhamri, Julia M. Kurth, Cornelia U. Welte, Tristan Wagner, and Ross D. Milton**

Abstract: Massive efforts are invested in developing innovative CO_2-sequestration strategies to counter climate change and transform CO_2 into higher-value products. CO_2-capture by reduction is a chemical challenge, and attention is turned toward biological systems that selectively and efficiently catalyse this reaction under mild conditions and in aqueous solvents. While a few reports have evaluated the effectiveness of isolated bacterial formate dehydrogenases as catalysts for the reversible electrochemical reduction of CO_2, it is imperative to explore other enzymes among the natural reservoir of potential models that might exhibit higher turnover rates or preferential directionality for the reductive reaction. Here, we present electroenzymatic catalysis of formylmethanofuran dehydrogenase, a CO_2-reducing-and-fixing biomachinery isolated from a thermophilic methanogen, which was deposited on a graphite rod electrode to enable direct electron transfer for electroenzymatic CO_2 reduction. The gas is reduced with a high Faradaic efficiency ($109\pm1\,\%$), where a low affinity for formate prevents its electrochemical reoxidation and favours formate accumulation. These properties make the enzyme an excellent tool for electroenzymatic CO_2-fixation and inspiration for protein engineering that would be beneficial for biotechnological purposes to convert the greenhouse gas into stable formate that can subsequently be safely stored, transported, and used for power generation without energy loss.

Our modern society currently faces the consequences of the increase in atmospheric carbon dioxide (CO_2) of the last century. Therefore, developing planet-scale CO_2-sequestration strategies and alternatives to fossil fuels for energy production has become an urgent matter. The reduction of CO_2 constitutes an attractive solution to both problems[1,2] but faces a challenge due to the high kinetic stability of CO_2. Chemical CO_2 reduction is often achieved using rare and polluting metals, as well as extreme operating conditions, to reach significant turnovers. Electrochemical CO_2 reduction could, in theory, provide attractive operating conditions, although high overpotentials are often required (making it energetically inefficient), and metallic electrocatalysts often lack selectivity.[3–6] On the other hand, biological catalysts such as formate dehydrogenases (FDH) are great alternatives to perform CO_2-reduction as they operate under mild temperature, low pressure and with relatively low overpotential compared to electrochemical CO_2 reduction. Most enzymes have high selectivity, efficiency and exhibit turnover rates unmatched by most inorganic methods. The reaction product, formate, is stable, convenient for transport and a precursor for chemical production, energy generation, or to produce H_2,[7] making formate an excellent surrogate to H_2 for renewable energy storage.[8,9] Yet, FDHs were shown to be limited to relatively low turnover rates, substrate inhibition and relative instability, which is coherent with their physiological function of formate oxidation rather than CO_2 reduction, with rare exceptions.[10]

Hydrogenotrophic methanogens are strict anaerobic microorganisms generating methane from the reduction of CO_2, a metabolism proposed to be among the first to appear on Earth.[11,12] Their impressive short division period under chemolithotrophic conditions suggests that CO_2 reduction at high rates allows sufficient cellular energy acquisition. The entry point of carbon/energy acquisition is a functional equivalent to the FDH: the formyl-methanofuran (formyl-MFR) dehydrogenase (labelled Fwd or Fmd for the W- and

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Mo-dependent isoforms, respectively, Figure 1A).[13] Compared to FDHs, Fwd/Fmd is an all-in-one machinery capable of reversible CO_2 reduction and fixation following a two-step mechanism. In the first step, the subunits B and D, harbouring the same cofactor as FDH (a W/Mo-containing bis-pyranopterin guanosine dinucleotide, bis-PGD cofactor), perform CO_2 reduction to formate. The second step is the condensation of formate with the coenzyme MFR in subunit A catalysed by a [ZnZn] metallo-centre generating formyl-MFR and fuelling methanogenic metabolism. Structural studies suggested the sequestration of the formate in an internal cavity connecting the subunits B/D and A, facilitating the endergonic second reaction (Figure 1A). The electrons necessary for CO_2 reduction are derived from ferredoxin oxidation or direct electron transfers.[13,14] Electrons transit through extensive FeS cluster relays made of polyferredoxin subunits. The abundance of [4Fe–4S] clusters may well be advantageous for heterogeneous electron transfer in bioelectrochemical systems (Figure 1).[13–15] However, electrochemistry studies have not yet been assessed, likely due to the difficulty of growing Fwd/Fmd-producing organisms and the purification of these O_2-sensitive enzymes. Here, we describe a CO_2-reducing bioelectrocatalytic system using the Mo-dependent Fmd complex from a thermophilic methanogen, adsorbed onto a graphite rod electrode (GRE). The Fmd complex represents an opening playground for electrochemical CO_2-fixation strategies as it does not require any mediator or covalent surface modification and exhibits direct electron transfer (DET)-type bioelectrocatalysis with the appreciable property of electrocatalyzing CO_2 reduction by two orders of magnitude greater than formate oxidation.

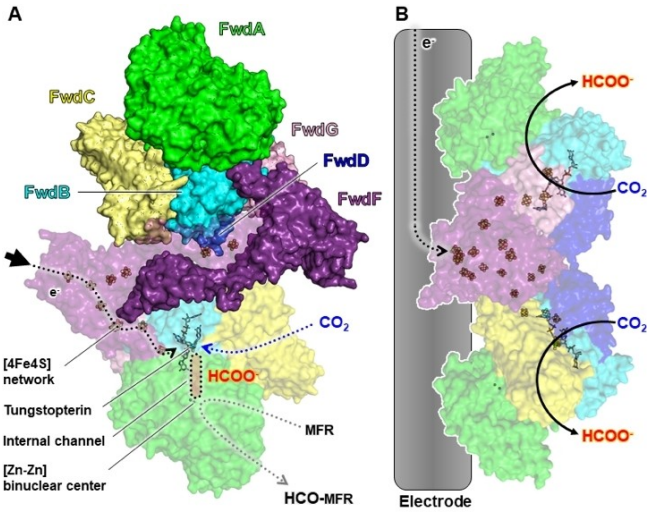

Figure 1. Structure and mechanism of formyl-MFR complex. A. Top view of the crystal structure of the Fwd dimer from the thermophilic archaeon *Methanothermobacter wolfeii* (PDB: 5T5M). The enzyme is coloured by subunit, with a monomer being represented as surface, and the other as transparent surface. A thick black arrow indicates the suspected entry point of electrons. Dashed lines highlight the different tunnels and cavity involved in reaction: electron transfer (black), hydrophobic CO_2 tunnel (blue), hydrophilic formate tunnel (red) and MFR binding site (grey). B. Bioelectrocatalytic CO_2 reduction by dimeric Fwd adsorbed to a graphite rod electrode (GRE). Cofactors are represented as balls and sticks with carbon, oxygen, nitrogen, sulphur, iron, phosphorus, tungsten and zinc atoms coloured in white, red, blue, yellow, orange, light orange, cyan and green, respectively. Despite the simplified depiction, a distribution of Fwd orientation on the electrode is expected.

The native formyl-MFR complex from the thermophilic methanogen *Methermicoccus shengliensis* was purified anaerobically by multistep chromatography (Figure S1A). Induced coupled plasma mass spectrometry (ICP-MS) demonstrated the presence of 0.9 mol of molybdenum per mol of the purified monomeric enzyme, which is therefore referred to as the Mo-dependent *Ms*Fmd complex. The complex appears to be made of five subunits by denaturing SDS-PAGE analysis, lacking the small 2[4Fe–4S] cluster-containing FwdG subunit. While native electrophoresis indicates a major and minor species compatible with an FmdABCDF monomeric and dimeric oligomer, respectively, size exclusion chromatography rather indicates a dimeric organization (Figure S1B and C). We therefore suspect that the complex is disrupted during electrophoresis and concluded that a $(FmdABCDF)_2$ species is the principal component. The purified enzyme is catalytically active, performing the viologen-dependent reduction of CO_2 and oxidation of both formate and the MFR-analogue furfurylformamide (FFA, Table 1), with kinetic parameters similar to those previously published for homologous enzymes.[16,17] Viologen-supported CO_2 reduction turnover measured in solution is around 400-fold lower than that measured with FDH in similar conditions.[18] This was expected as i) in the absence of a

Table 1: Kinetic parameters of the purified *Ms*Fmd in solution and in homologous systems. The K_{cat}^{app} was calculated based on a molecular weight of 198.50 kDa corresponding to the monomeric Fmd. All measurements were performed at least in triplicates.

Substrate	K_M^{app} (mM)	V_{max}^{app} (U.mg^{-1})	k_{cat}^{app}
***Ms*Fmd**			
CO_2	ND	0.24 ± 0.03*	0.794 s^{-1}*
Formate	183.39 ± 28.7	0.92 ± 0.07	3.044 s^{-1}
Furfurylformamide	41.04 ± 12.1	6.58 ± 0.65	21.77 s^{-1}
***Mb*Fmd**[a]			
Formate	1,700	1.8	6.60 s^{-1} [b]
Furfurylformamide	200	20	73.33 s^{-1} [b]
***Mw*Fmd**[a]			
Formate	35	1.2	2.60 s^{-1} [b]
Furfurylformamide	53	0.3	0.65 s^{-1} [b]
***Mw*Fwd**[a]			
Formate	1,100	0.2	0.43 s^{-1} [b]
Furfurylformamide	1,250	0.1	0.22 s^{-1} [b]

*The kinetics parameters of CO_2 reduction were determined with a saturating concentration of 100 mM $NaHCO_3$. [a] The values for the Mo-dependent complexes from *Methanosarcina barkeri* and *Methanothermobacter wolfei* (*Mb*Fmd and *Fw*Fmd) as well as for the W-dependent enzyme from *M. wolfei* (*Mw*Fwd) are derived from Bertram *et al.*, 1994.[16] [b] The molecular weight used for calculation are derived from Wagner, Ermler and Shima (2018),[13] and corresponds to a single monomer. ND: not-determined.

formyl-carrier, formate can accumulate within the enzyme and its low diffusion would retard CO_2 reduction; ii) the redox potential of the reaction performed by *Ms*Fmd (CO_2/formyl-MFR couple, potential estimated to be $E^{0\prime} = -0.530$ V[13]) is considerably lower than that of FDH (CO_2/$HCOO^-$ couple, $E^{0\prime} = -0.420$ V[10,19]) and therefore the cofactors in *Ms*Fmd are expected to exhibit lower potentials than that of FDH, limiting electron transfer from methyl viologen ($E^{0\prime} = -0.446$ V[20]). The formate oxidation activity of *Ms*Fmd is also lower than that of FDHs, as the enzyme exhibits poor affinity for formate (Table 1). This is coherent with the proposed reaction mechanism of these enzymes, in which formate is an intermediate trapped in the internal cavity. This intrinsic property would be advantageous for electrochemical CO_2 reduction applications as formate oxidation rates would not compete with CO_2 reduction.

The *Ms*Fmd enzyme was immobilised on to the electrode by simple adsorption and no external redox mediator was included. Cyclic voltammograms (CV) measured before and after the addition of 100 mM $NaHCO_3$ as a source of CO_2 show a HCO_3-dependent reduction current, attributed to CO_2 reduction to formate under these working conditions, as no formyl carriers are available (Figure 2A). The data indicate an approximate onset potential of −0.45 V (vs. SHE), beyond which a significant negative (reductive) current for substrate reduction is evident. A more positive onset potential would normally be expected for CO_2 reduction in the absence of formate in the bulk ($E^{0\prime} = -0.420$ V[19]). We hypothesize that this reflects the relatively negative $E^{0\prime}$ values of FeS clusters of Fmd compared to those of FDHs. CV experiments carried out with 100 mM sodium formate instead of $NaHCO_3$ yielded unreproducible results, except if the protein adsorption promoter neomycin[21] was included in the electrolyte (Figure S2). In the presence of neomycin, formate addition led to a weak oxidative activity, several fold lower than that measured with $NaHCO_3$ (Figure 2B). Furthermore, formate oxidation could not be monitored in the presence of both 100 mM $NaHCO_3$ and 100 mM formate (Figure 2C). The weak formate electrooxidation activity can be explained by the apparent low affinity of the enzyme for formate. The reasons for the apparent competition between CO_2 and formate might come from the access to the active site, which remains to be experimentally verified. No activity could be detected for both reactions using an oxygen-inactivated enzyme (Figure 2D).

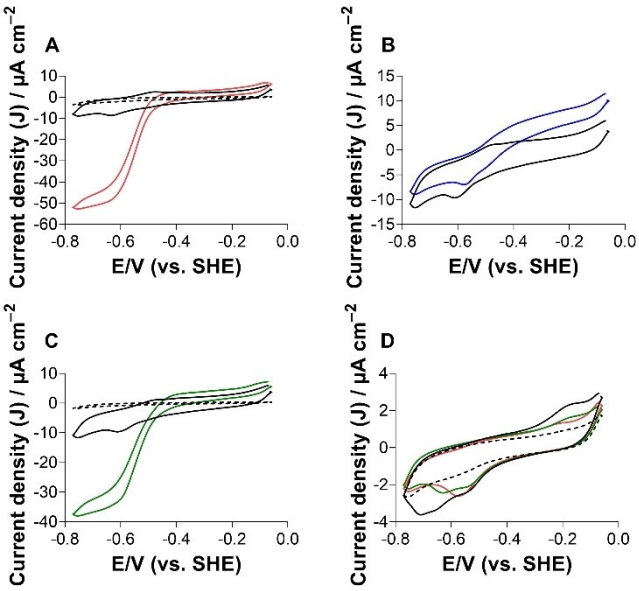

Figure 2. CVs of CO_2 reduction/formate oxidation catalysed by *Ms*Fmd on GRE. A. Representative CV of a *Ms*Fmd-bound GRE in the presence of 100 mM $NaHCO_3$ (red) in 0.1 M MOPS buffer (pH 7.0). B. Representative CV of *Ms*Fmd-bound GRE in the presence of 100 mM formate (blue) in 0.1 M MOPS buffer (pH 7.0) and 4 mM neomycin. C. Representative CV of a *Ms*Fmd-bound GRE in the presence of 100 mM $NaHCO_3$ and 100 mM formate (green) in 0.1 M MOPS buffer (pH 7.0) and 4 mM neomycin. D. Representative CV of an O_2-inactivated *Ms*Fmd-bound GRE in the absence (black) and presence (red) of 100 mM $NaHCO_3$ and 100 mM formate (green). The recorded voltammograms with bare electrodes are shown as dashed traces; background cycles recorded in the absence of any substrate are also shown (black solid line). All experiments were performed by stirring at 22 °C and a scan rate of 5 mV s^{-1}.

Non-turnover redox peaks are present in the CVs of *Ms*Fmd in the absence of substrate, although here we refrain from directly assigning values of $E^{0\prime}$ of the enzyme (where we assume $E^{0\prime} = E_m$) since (i) there are many FeS clusters present in *Ms*Fmd (which may undergo direct electron transfer with the electrode) and (ii) the reductant dithiothreitol (DTT) is present in the preparation of *Ms*Fmd. To address this, we performed an additional wash of *Ms*Fmd immediately prior to electrochemical analysis (Figure S3) to remove residual DTT. After washing, these redox peaks virtually disappear. We conclude that these redox peaks may have then been due to DTT present in the sample, or that the absence of DTT results in the rapid degradation of the complex (explaining the comparatively lower electrocatalytic currents that are observed after washing, (Figure S3)). We therefore sought to estimate the $E^{0\prime}/E_m$ of *Ms*Fmd using the catalytic response in the presence of $NaHCO_3$. The potential at $i_{cat/2}$ was used to estimate the standard enzyme potential for catalysis ($E_{cat/2} = E^{0\prime}{}_{app} \approx -0.510$ V vs. SHE, Figure S4). The calculated $E^{0\prime}{}_{app}$ value is similar to the estimated $E^{0\prime}$ of the CO_2/Formyl-MFR couple (−0.530 V vs. SHE).[13,22]

The steady-state catalytic currents were measured to determine the kinetic parameters of GRE-bound *Ms*Fmd (Figure 3A–C, Table S1). The apparent Michaelis constant ($K_M{}^{app}$) and the apparent maximum current density ($J_{max}{}^{app}$) were determined by nonlinear regression to be 3.8 ± 0.3 mM CO_2 and 124 ± 17 µA cm^{-2}, respectively (Table 2). This current density is within the range obtained with FDHs (5 to 200 µA cm^{-2}),[6,23–25] showing that simple adsorption of *Ms*Fmd on GRE yields a potent electrochemical CO_2 reduction system. This is particularly impressive considering the difference in viologen-based CO_2 reduction rates measured in solution.

GRE-bound *Ms*Fmd exhibits an affinity for CO_2 on the same order of magnitude as that determined in the Fmd complex from *Methanosarcina barkeri* (0.7 mM)[26] and FDHs (0.420–2.7 mM).[18,19,25] However, a drastic difference

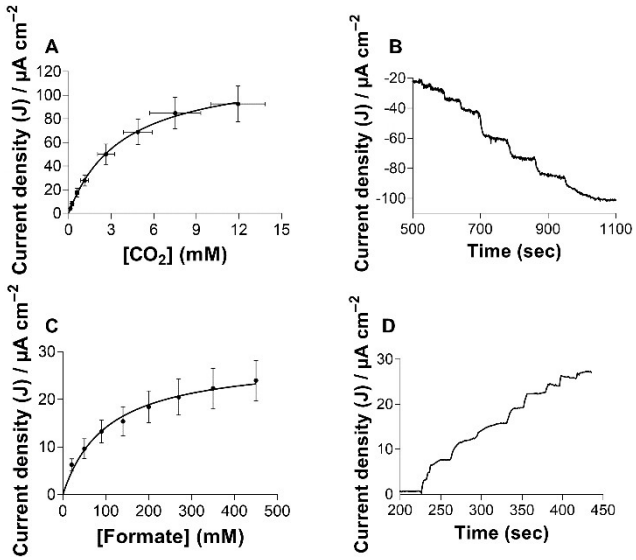

Figure 3. Steady-state kinetic study of *Ms*Fmd-bound GREs. Kinetic parameters of CO_2 reduction (A and B) and formate oxidation (C and D). A potential of −0.63 V vs. SHE was applied to the GREs for CO_2 reduction, whereas a potential of −0.2 V vs. SHE was applied to the GREs for formate oxidation. All experiments were performed in a stirred MOPS buffer solution (pH 7.0) at 50 °C, and successive injections of either $NaHCO_3$ or formate were made. Current densities (*J*) are plotted as the current density magnitudes in the case of CO_2 reduction since a reductive reaction yields an increasingly negative current density. Panels B and D show representative *i*-t curves for the two electroenzymatic reactions (data used to plot the K_M fits). Standard error (SE) was calculated based on three replicates of each sample concentration.

Table 2: Kinetic parameters of *Ms*Fmd bound on electrode and calculated Faradaic efficiency for CO_2 reduction.

Kinetic parameters			
Substrate		K_M^{app} (mM)	J_{max}^{app} (μA.cm^{-2})
CO_2		3.8 ± 1.3	124 ± 17
Formate		103 ± 45	29 ± 4
Faradaic efficiency			
Formate calculated (nmol)	**Formate detected (nmol)**	**Charged passed (mC)**	**Formate efficiency (%)**
375 ± 77	408 ± 79	723 ± 18	109 ± 1

Potentiostatic bulk electrolysis was performed for 180 min at −0.63 V *vs.* SHE in 0.1 M MOPS (pH 7.0) containing 100 mM $NaHCO_3$ at 50 °C (balance Ar). All measurement were performed in triplicates.

exists for formate: in characterized FDHs,[18,19,27] the affinity for formate is superior or in the range of that for CO_2, while Fmds/Fwds generally exhibit comparatively high K_M for formate as the molecule is not the substrate.[16] Accordingly, the K_M^{app} for formate of GRE-bound *Ms*Fmd was calculated to be 103 ± 45 mM formate, similar to that estimated with the enzyme in solution, with a maximal current density estimated to be 29 ± 4 μA cm^{-2} (Figure 3C–D and Table 2). Enzymatic quantification of formate after bulk bioelectrocatalytic CO_2 reduction confirmed its production with high efficiency (109 ± 1 % of CO_2 converted into formate, Figure S5, Table 2). Hence, no significant amount of alternative product is synthetized in the electrode working conditions. This efficiency of over 100 % likely originates from the secondary enzymatic assay for resulting formate oxidation (detailed in the Supporting Information). While the activity decreases with time, possibly due to enzyme inactivation of the release of the electrode, it was nevertheless possible to monitor CO_2 reduction after 3 hours at 50 °C (Figure S5).

Our study revealed for the first time how formyl-MFR dehydrogenase complexes from methanogens can be employed as biocatalysts for CO_2 reduction to formate when bound on graphite electrodes. Unlike characterised FDHs, the enzyme presents weak formate oxidation activity, which is disabled in the presence of CO_2, leading to formate accumulation during electrocatalysis. The efficiency of the system is in the same range as that obtained with FDHs considering current density and formate accumulation.[6,23,24] The non-physiological generation of formate should be impaired by several structural features of the enzyme (Figure 1A), and bioengineering will be necessary to unleash its full formate-production potential. Modification of the CO_2-diffusion tunnel, the [Zn–Zn] binuclear formate condensation site, the MFR binding interface (or even complete removal of the FmdA subunit) could, in theory, considerably enhance the formate generation rates, as they are superfluous for CO_2 reduction applications. Before undertaking such modifications, more insights into the mechanism of Fmds are imperative, and future works must capture new states of the complex under turnover conditions to unveil how this impressive multi-enzymatic machine operates.

Supporting Information

The authors have cited additional references within the Supporting Information.[18,22,28–32] Raw data are freely available on Zenodo (DOI: 10.5281/zenodo.8250713).

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are openly available in Zenodo at https://doi.org/10.5281/zenodo.8250713, reference number 8250713.

Keywords: Bioelectrocatalysis · CO_2-Sequestration · Direct Electron Transfer · Formylmethanofuran Dehydrogenase · Molybdopterin Cofactor

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