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Abstract

Mo/W formate dehydrogenases catalyze the reversible reduction of CO_2 species to formate. It is thought that the substrate is CO_2 and not a hydrated species like HCO_3^-, but there is still no indisputable evidence for this, in spite of the extreme importance of the nature of the substrate for mechanistic studies. We devised a simple electrochemical method to definitely demonstrate that the substrate of formate dehydrogenases is indeed CO_2.

The reduction of carbon dioxide is an important reaction both in biology and technology, since it converts a highly stable compound into more reactive and useful organic compounds[1]. In nature, only two classes of enzymes are able to catalyze the direct reduction of CO_2 (as opposed to reductive carboxylation): formate dehydrogenases (FDHs), members of the family of molybdenum/tungsten enzymes[2], which reversibly convert CO_2 to formate (HCO_2^-)[3–5]:

$$CO_2 + H^+ + 2\,e^- \rightleftharpoons HCOO^- \tag{1}$$

and CO dehydrogenases (CODHs), which reversibly convert CO_2 to CO[6–8]:

$$CO_2 + 2\,H^+ + 2\,e^- \rightleftharpoons CO + H_2O \tag{2}$$

A great incentive in understanding their catalytic mechanism is that both these enzymes are fast (hundreds to thousands of turnovers per second) and able to work with very little driving force[9], which has so far never been achieved by synthetic catalysts[1, 10, 11]. A specific difficulty in the studies of these enzymes is to identify the actual substrate in aqueous solutions, which may either be dissolved CO_2 or hydrated species like H_2CO_3, HCO_3^- or CO_3^{2-}. In the biologically relevant pH range, the only species present to a significant extent are CO_2 and HCO_3^-, which interconvert with an apparent pK_a of 6.4:

$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+ \tag{3}$$

As CO_2 and HCO_3^- are very different molecules, one must first determine which is the substrate before trying to understand the catalytic mechanism for reactions (1) or (2). For CODH (equation (2)), there is little doubt that CO_2 is the substrate from a variety of experimental evidence, including a high resolution CO_2-bound structure[6] and the observation that COS (carbonyl sulfide) is an alternative slow substrate[12].

The case of FDH (equation (1)) is more ambiguous. Many molybdo-enzymes catalyze oxygen atom transfers, like the DMSO reductases, nitrate reductases, or arsenate oxidases[2, 13, 14]. Nap, the molybdo-enzyme that is the closest to FDH in terms of sequence and structure[15], catalyzes the reduction of nitrate to nitrite[16]. Rather than the direct reduction of CO_2, one could imagine that FDH catalyzes the abstraction of an oxygen atom from HCO_3^-. It is thus somewhat surprising that there have been very few results in the last four decades concerning the nature of the substrate of FDH.

Yu and co-workers recently concluded that CO_2 is the substrate of FDH based on the observed difference in the steady-state rate of oxidation of the co-substrate NADH, depending on

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whether carbonate or CO_2 was added to the reaction mixture[17]. However, under steady-state conditions, starting from carbonate or CO_2 should make no difference since the two equilibrate, and the observed differences could result from insufficient buffer concentration in these experiments (100 mM potassium phosphate at pH 7 for 100 mM bicarbonate).

Khangulov and co-workers in 1998[18] used mass spectrometry to characterize the product of ^{13}C-formate oxidation in ^{18}O-enriched water at pH 7. The reaction was started by adding the unlabeled enzyme to the ^{18}O-labelled solution. No information was given about the amount of CO_2 produced, but the authors detected no ^{18}O-labelled CO_2 in the first 10 s of the reaction, deducing that CO_2, rather than carbonate, is the initial product of formate oxidation. However, this strategy relies on the assumption that the water molecules in the enzyme exchange quickly with labelled water, which may not be true, as discussed by Cooper et al[19].

Other demonstrations of CO_2 being the substrate (or product) of a number of enzymes, including pyruvate carboxylase, P-enolpyruvate carboxykinase and CODH, date back to the 70s[19–23]. These experiments took advantage of the relative slowness[24] of reaction (3): for a short time after an injection of either CO_2 or carbonate, only the species that has just been added is present. Examining the transient change in catalytic rate as reaction (3) reaches equilibrium allows one to identify the substrate. These experiments required low temperatures (below 10 °C) to slow reaction (3), and fast sampling of the turnover frequency. This is difficult in solution assays, but very easy in protein film electrochemistry, as illustrated below.

Here, we propose a simple, inexpensive and fast method to discriminate whether CO_2 or HCO_3^- is the substrate of any catalyst interacting with an electrode, and we demonstrate that CO_2 rather than HCO_3^- is the substrate of both FDH and CODH. The procedure is based on protein film electrochemistry (PFE)[25, 26]. It consists in immobilizing the enzyme onto a rotating disc electrode, injecting CO_2 or carbonate in the presence or absence in solution of carbonic anhydrase (CA, an enzyme that catalyzes the interconversion between CO_2 and HCO_3^-, reaction (3))[27], and following the evolution of activity over time.

Both FDH[4] and CODH[28] can be immobilized on electrodes in a configuration in which the enzymes catalyze their respective reactions, producing an electrical current proportional to the turnover frequency[25, 26]. We used chronoamperometry, which consists in applying a constant potential to this electrode and monitoring the resulting catalytic current as a function of time. We sampled the current (thus the activity) every 0.1 s, which is much shorter than the CO_2/HCO_3^- equilibration time under our conditions (around 20 s at pH 7), and also much shorter than the time for measuring activity in traditional solution assays.

We performed the measurements at pH 7 and at a potential of −660 mV vs. SHE. Under these conditions, "CO_2" reduction occurs for both CODH and FDH, since the CO_2/CO and $CO_2/HCOO^-$ couples have apparent equilibrium potentials of −530 and −430 mV, respectively, at pH 7[29, 30]. We used a highly concentrated buffer (0.2 M HEPES) to avoid pH changes following the injection of mM concentrations of carbonate or CO_2. At pH 7, once equilibrium (3) is established, we expect a 20:80 ratio of CO_2 to bicarbonate in solution, irrespective of whether CO_2 or carbonate has been injected.

Figure 1A shows the experiments performed with FDH. For the experiment plotted as a blue solid line, sodium carbonate was injected in the cell at the time $t = 0$, instantly becoming bicarbonate at pH 7. Following the injection, a reduction current appears, which slowly increases, reaches a maximum after 25 s, and then slowly decreases.

When the same carbonate-injection experiment is repeated in the presence of CA, the increase in current after the injection is instantaneous (light blue dashed line). This suggests that the initial slow increase in current observed in the absence of CA (blue solid line) is due to the slow transformation of bicarbonate into CO_2, which is the actual substrate of the enzyme.

The latter conclusion is supported by the results of experiments in which a solution containing CO_2 is injected: irrespective of the presence of CA, an instant increase in reduction current is always observed. The current of the CO_2-injection

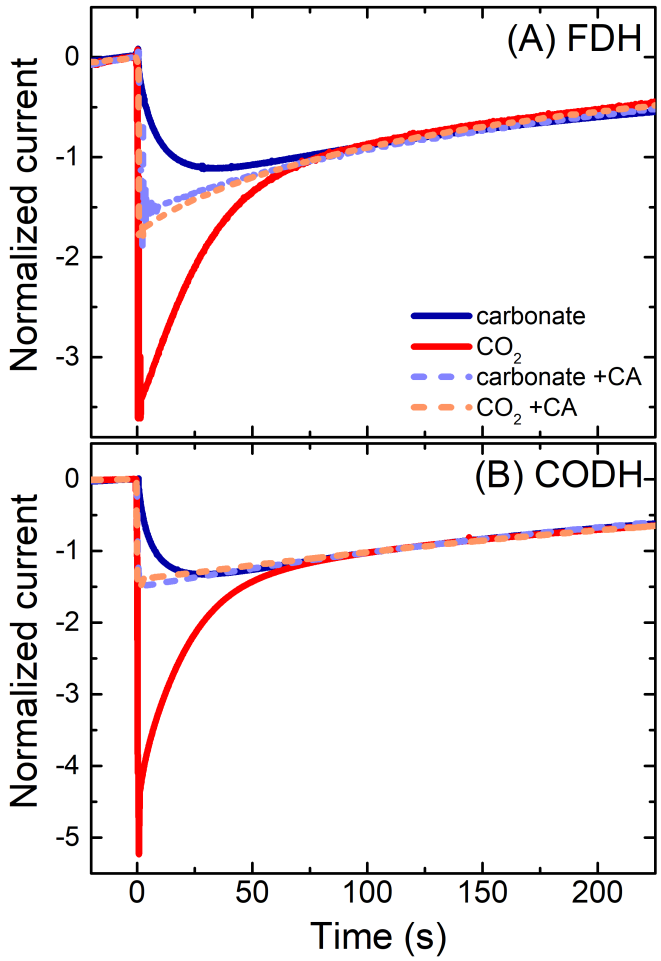

Figure 1: Chronoamperometric measurements of CO_2 reduction current following injection of carbonate or CO_2. The electrode was modified with a film of (A) W-dependent *Desulfovibrio vulgaris* FdhAB[5] (co-adsorbed with polymyxin), and (B) *Thermococcus sp. AM4* CODH 2[28]. Experimental conditions: T = 25 °C, pH 7 (0.2 M HEPES buffer), electrode rotation rate ω = 3000 rpm, E = −0.66 V vs. SHE. The measurements were performed by injecting in the cell at t = 0 s: 5 mM Na_2CO_3 (blue lines) or 3.7 mM CO_2 (red lines) in the absence (solid lines) and presence (dashed lines) of 5 µg/mL carbonic anhydrase. The current has been normalized with respect to the current at 70 s (when the CO_2/HCO_3^- equilibrium is fully established and the concentrations of CO_2 and HCO_3^- should be the same in all experiments).

experiment performed in the presence of CA (light red dashed line) is indistinguishable from that of the carbonate+CA experiment. But if CO_2 is injected in the absence of CA (red solid line), the current is initially larger than in any of the other three experiments, and then relaxes to the same value as in the other experiments: this is due to the concentration of CO_2 decaying from 100 % to 20 % of the total CO_2/HCO_3^- species as reaction (3) reaches equilibrium.

In all four cases, the final slow decrease in current, in the timescale of minutes, results from the exponential decrease in CO_2 concentration in the cell solution, which slowly equilibrates with the N_2 atmosphere of the glove box[28, 31].

The experiments above clearly demonstrate that CO_2 (not bicarbonate) is the substrate of FDH. As a confirmation, we have performed the same series of experiments with CODH adsorbed on the electrode surface, which gave the exact same results (Figure 1B), consistent with the previous knowledge that CO_2 is the substrate of CODH.

To further confirm that the relaxations on the blue and red solid curves of Figure 1 are due to reaction (3), we used carbonate injections to a CODH-modified electrode to determine the kinetics of relaxation for different pH values between 5.2 and 7.2. We systematically observed a slow initial evolution like in Figure 1 (blue solid curves), whose rate was determined by fitting a monoexponential function (Figure 2A). Figure 2B shows that these experimentally determined rates match the values calculated from the data in ref. 24 for the relaxation of equilibrium (3) (see SI section S2 for details).

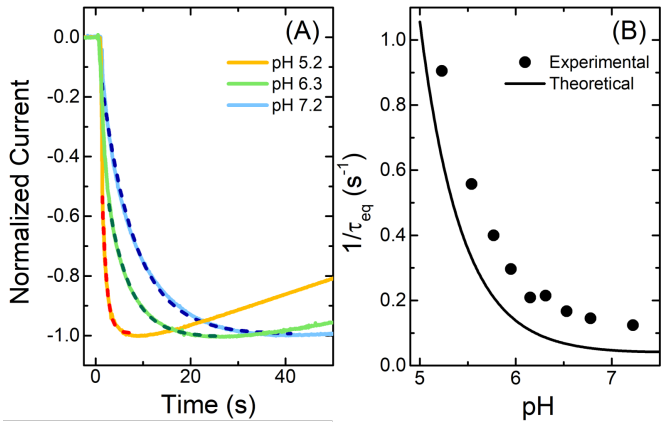

Figure 2: Chronoamperometric determination of the time constant of the CO_2/HCO_3^- equilibrium at different pH using a CODH-modified electrode. A) Example of chronoamperometric traces recorded at pH 5.2, 6.3 and 7.2 upon injecting 5 mM Na_2CO_3 at t = 0. The dashed lines represents the monoexponential fits. B) The resulting rate constant of the CO_2/HCO_3^- equilibration as a function of pH: experimental (point) and values predicted from the data in ref. 24 (line). Experimental conditions: T = 25 °C, electrode rotation rate ω = 4000 rpm, E = −0.66 V vs. SHE, buffer: 0.2 M MES.

In a final confirmation, we used chronoamperometric experiments to determine the effects of the concentration of carbonic anhydrase on the rate of reaction (3). We injected carbonate to a CODH-modified electrode in a cell containing different concentrations of CA. Figure 3 shows that the rate constant of the CO_2/HCO_3^- equilibrium is proportional to the concentration of carbonic anhydrase. The slope in Figure 3 (570 mL s^{-1} mg^{-1}) gives a second order catalytic rate constant of 2×10^7 s^{-1} M^{-1} at pH 7, 25 °C, consistent with published values in the range 10^7 to 10^9 s^{-1} M^{-1}, obtained by stopped-flow measurements of the pH changes resulting from the hydration of CO_2[32, 33].

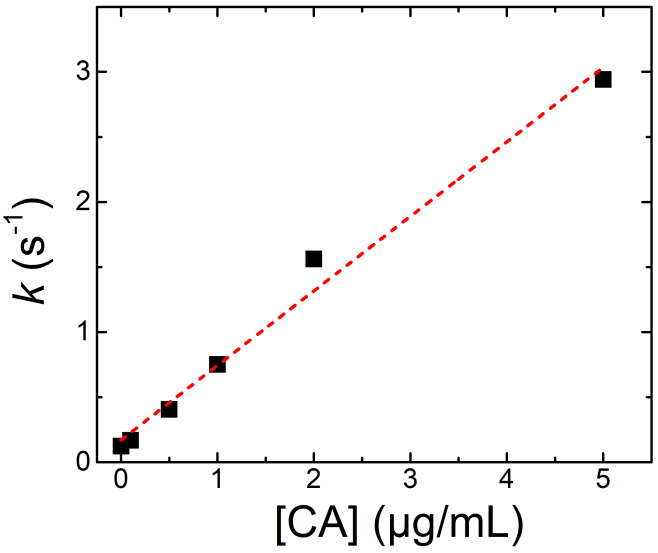

Figure 3: Determination of the rate constant (k) of the CO_2/HCO_3^- equilibrium at different concentrations of carbonic anhydrase (CA) using a CODH-modified electrode. Rate constants were determined by fitting an exponential function to the initial current increase recorded after injection of 5 mM Na_2CO_3 at $t = 0$ (like in Figure 2A). The red dashed line is a linear fit. Experimental conditions: $T = 25$ °C, electrode rotation rate $\omega = 4000$ rpm, $E = -0.66$ V vs. SHE, pH 7 (0.2 M HEPES).

In conclusion, we have devised a simple electrochemical method to discriminate between CO_2 and HCO_3^- as the substrate of the reductive reactions of FDH and CODH. This technique is very sensitive and does not require labelling of the substrates or the biocatalysts. We used it to conclusively prove that formate dehydrogenase reduces CO_2 and to confirm that NiFe CODHs also uses CO_2 as substrate. This method could also be employed to learn about synthetic catalysts, or even to measure the activity of carbonic anhydrase.

Acknowledgements

The authors acknowledge support from CNRS, Agence Nationale de la Recherche (grants ANR-14-CE05-0010, ANR-15-CE05-0020, ANR-17-CE11-002) and Region PACA. The project leading to this publication has received funding from Excellence Initiative of Aix-Marseille University - A*Midex, a French "Investissements d'Avenir" program. MM, AB, CL and VF are members of the French Bioinorganic Chemistry group (http://frenchbic.cnrs.fr). The authors also thank Fundação para a Ciência e Tecnologia (Portugal) for fellowship SFRH/BD/116515/2016, grant PTDC/BBB-EBB/2723/2014, and R&D unit MOSTMICRO-ITQB (UIDB/04612/2020 and UIDP/04612/2020). European Union's Horizon 2020 research and innovation program (Grant agreement no. 810856) is also acknowledged.

The authors have no conflicts to declare.

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