



HAL
open science

Transport limited adsorption experiments give a new lower estimate of the turnover frequency of *Escherichia coli* hydrogenase 1

Anna Aldinio-Colbachini, Andrea Fasano, Chloé Guendon, Aurore Jacq-Bailly, Jérémy Wozniak, Carole Baffert, Arlette Kpebe, Christophe Léger, Myriam Brugna, Vincent Fourmond

► To cite this version:

Anna Aldinio-Colbachini, Andrea Fasano, Chloé Guendon, Aurore Jacq-Bailly, Jérémy Wozniak, et al.. Transport limited adsorption experiments give a new lower estimate of the turnover frequency of *Escherichia coli* hydrogenase 1. *BBA Advances*, 2023, 3, pp.100090. 10.1016/j.bbadv.2023.100090 . hal-04221197

HAL Id: hal-04221197

<https://hal.science/hal-04221197v1>

Submitted on 28 Sep 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial - NoDerivatives 4.0 International License

Transport limited adsorption experiments give a new lower estimate of the turnover frequency of *Escherichia coli* hydrogenase 1

Anna Aldinio-Colbachini^a, Andrea Fasano^a, Chloé Guendon^a, Aurore Bailly^a, Jeremy Wozniak^a, Carole Baffert^a, Arlette Kpebe^a, Christophe Léger^a, Myriam Brugna^a, Vincent Fourmond^{a*}

^aCNRS, Aix Marseille Université, BIP, IMM, IM2B 31 Chemin J. Aiguier, 13009 Marseille (France)

*email: vincent.fourmond@imm.cnrs.fr

Abstract

Protein Film Electrochemistry is a technique in which a redox enzyme is directly wired to an electrode, which substitutes for the natural redox partner. In this technique, the electrical current flowing through the electrode is proportional to the catalytic activity of the enzyme. However, in most cases, the amount of enzyme molecules contributing to the current is unknown and the absolute turnover frequency cannot be determined. Here, we observe the formation of electrocatalytically active films of *E. coli* hydrogenase 1 by rotating an electrode in a sub-nanomolar solution of enzyme. This process is slow, and we show that it is mass-transport limited. Measuring the rate of the immobilization allows the determination of an estimation of the turnover rate of the enzyme, which appears to be much greater than that deduced from solution assays under the same conditions.

Protein Film Electrochemistry is a technique in which a redox enzyme is immobilized on an electrode in a configuration allowing direct electron transfer[1–3]. The enzyme retains its native catalytic activity on the electrode, which acts as a substitute for its redox partner, which can be either in solution or membrane-bound. The catalytic reaction generates a current whose magnitude is proportional to the enzyme's turnover rate, according to equation (1):

$$j = 2F\Gamma k_{cat} \quad (1)$$

where F is the Faraday constant, j the current density, Γ the “electroactive coverage”, the surface concentration of electrically connected enzymes, and k_{cat} the turnover rate. The electrochemical measurement can be used to monitor variations of turnover frequency under various experimental conditions (electrode potential, pH, substrate/product concentration)[1–5], or as a function of time when the enzyme inactivates or reactivates following exposure to inhibitors or changes in potential[6]. These variations can be interpreted quantitatively to yield mechanistic information[7]. However, most of the studies focus on the interpretation of relative variations of current, since it is difficult to determine the absolute value of the turnover frequency of an immobilized enzyme. Indeed, the catalytic current is also proportional to the electroactive coverage, which is most often unknown and impossible to determine. In rare cases, when the electroactive coverage is high, it is possible to measure non-catalytic signals, which result from the stoichiometric reduction/reoxidation of the redox centers present in the enzyme. These experiments must be conducted in the absence of substrate or at rates that outrun catalysis[8]. Optimisation of the interaction between the enzyme and the electrode can help increase the coverage enough to obtain non-catalytic signals[9,10]. Several strategies can be used, including some based on computational methods[11]; the reader is referred to recent reviews for further

reading[4,12,13]. These signals can be integrated to yield the electroactive coverage; combining this information with the measurement of the magnitude of the catalytic current gives the turnover frequency. This strategy has been used successfully with a number of enzymes, such as sulfite oxidase [14], formate dehydrogenase[15], fumarate reductase[16] and *Allochromatium vinosum* hydrogenase[17], *Aquifex aeolicus* hydrogenase[10]. Alternatively, it is possible to determine the enzymatic coverage in the absence of non-catalytic signals, by using strategies such as quartz microbalance electrodes[18,19], ellipsometry[20] or surface plasmon resonance[21]. These techniques allow the determination of the total coverage, including enzyme molecules that do not undergo electron transfer with the electrode and therefore do not contribute to the catalytic current. It is also sometimes possible to determine an upper limit of the electroactive coverage by measuring the amount of protein consumed by the immobilization process[10].

Here we focus on a NiFe hydrogenase, Hyd-1 from *Escherichia coli* (*Ec* Hyd-1), which catalyzes the oxidation of dihydrogen to protons and electrons at a bimetallic NiFe active site. This protein gives very stable and active films on graphite electrodes, which has been used to study its behavior, and, in particular, its high tolerance to inactivation by oxygen[22,23]. We show that it is possible to make electroactive films of *Ec* Hyd-1 by rotating the electrode in a sub-nanomolar solution of the enzyme, and that the adsorption is mass-transport-limited. Following the change of coverage as a function of time makes it possible to provide an estimation of the turnover frequency of *Ec* Hyd-1. This approach may be applied to other enzymes, even in the case that the amount of immobilized enzymes is undetectable via non-catalytic signals.

Figure 1 shows a series of voltammograms recorded rotating a freshly polished pyrolytic graphite edge electrode in a solution containing only 0.7 nM *Ec* Hyd-1 under 1 atm. of H₂. The voltammograms show H₂ oxidation currents at high potentials. The shape of the voltammograms is similar to that previously published by us and others[22,23]. What is new is that the magnitude of the signal increases steadily, from a current density of approximately 24 $\mu\text{A}/\text{cm}^2$ for the first scan to about 360 $\mu\text{A}/\text{cm}^2$ after about 15 minutes. Transferring the electrode to a solution devoid of enzyme stops the increase (see SI figure S5), which shows that the growth does not reflect the activation of the enzyme, but rather a slow adsorption process that is dependent on the presence of the enzyme in solution. The process slows over time, and after 16 voltammograms (at 20 mV/s, this takes about 15 minutes), the current density reaches a maximum and starts to decrease slowly. The process does not change the shape of the catalytic response (see SI figures S3 and S4), and hence does not affect the chemistry of the hydrogenase.

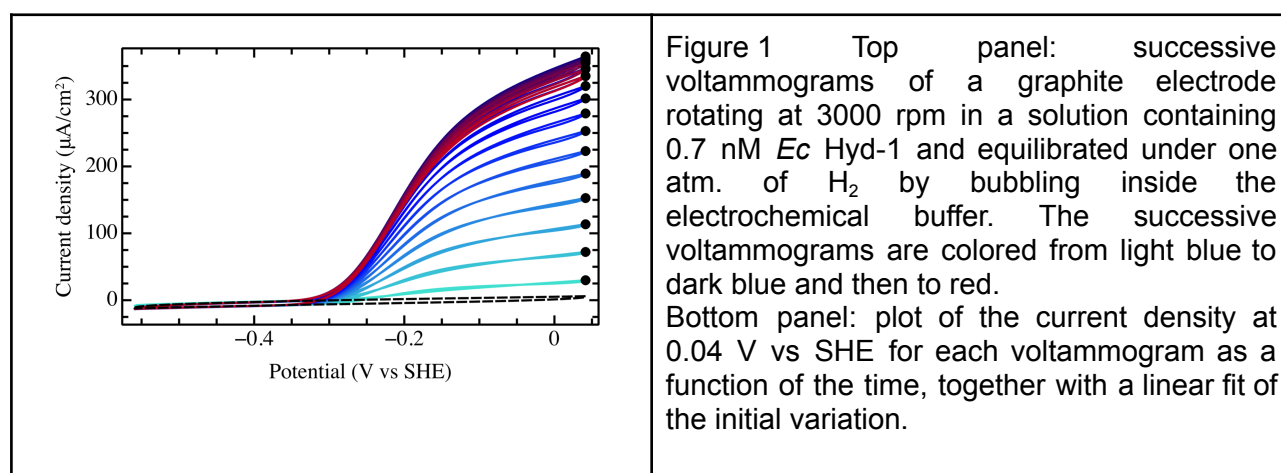
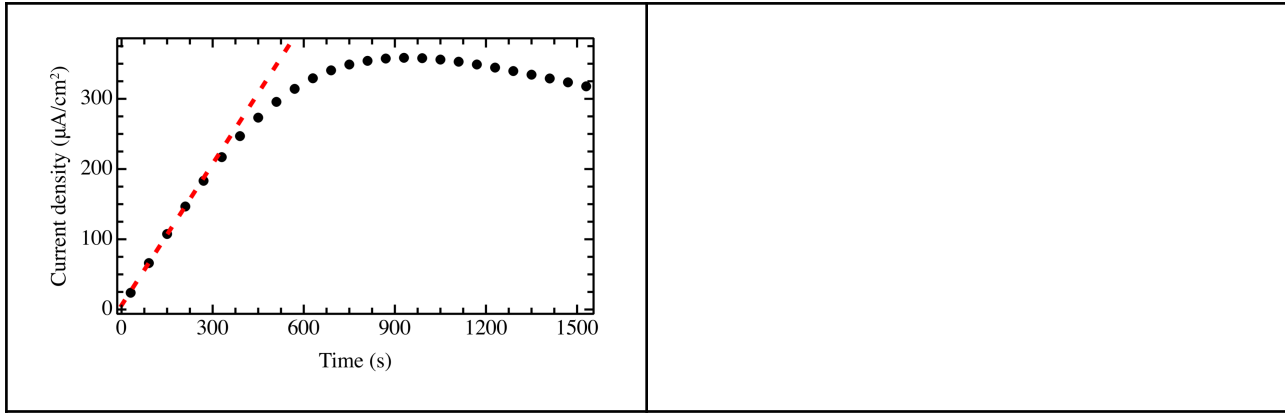


Figure 1 Top panel: successive voltammograms of a graphite electrode rotating at 3000 rpm in a solution containing 0.7 nM *Ec* Hyd-1 and equilibrated under one atm. of H₂ by bubbling inside the electrochemical buffer. The successive voltammograms are colored from light blue to dark blue and then to red. Bottom panel: plot of the current density at 0.04 V vs SHE for each voltammogram as a function of the time, together with a linear fit of the initial variation.



The bottom panel of figure 1 shows the evolution of the maximum current density of each voltammogram, which starts with an initial linear increase (until about 350 s), followed by a slower increase until a peak of the current (here about 800 s). The shape suggests a transition from an initial regime in which the enzyme adsorbs onto the electrode at a constant rate, to a regime in which the surface becomes saturated. We examine the two regimes one after the other below. The current density j is given by equation (1) above. As the shape of the voltammograms does not change, there is no reason to assume that k_{cat} changes during the experiments, so that all changes in j arise from variations in the surface concentration Γ :

$$\frac{dj}{dt} = 2Fk_{cat} \frac{d\Gamma}{dt} \quad (2)$$

In a first step, we hypothesize that the initial linear increase reflects an adsorption process entirely rate-limited by the transport of the enzyme to the electrode. Under this assumption, the rate of increase of the surface concentration is the flux of enzyme towards the electrode:

$$\frac{d\Gamma}{dt} = m_{enz} \times c_{bulk} \quad (3)$$

in which Γ is the surface concentration, c_{bulk} is the concentration of enzyme in the bulk (away from the electrode), and m_{enz} is the mass-transport coefficient for the transport of enzyme towards the rotating electrode (in cm/s), which is given by the Levich equation[24]:

$$m_{enz} = 0.62 \times D_{enz}^{2/3} \omega^{1/2} \nu^{1/6} \quad (4)$$

in which D_{enz} is the diffusion coefficient of the enzyme in solution (in cm²/s), ω is the angular velocity of the rotating disc electrode (in rad/s) and ν is the kinematic viscosity of water (in cm²/s). Combining equation (3) into equation (2) yields:

$$\frac{dj}{dt} = 2Fk_{cat} 0.62 \times D_{enz}^{2/3} \omega^{1/2} \nu^{1/6} \times c_{bulk} \quad (5)$$

As a consequence, under the assumption that the adsorption is fully mass-transport limited, the slope of the initial linear increase in current is proportional to the square root of the electrode rotation rate. We have therefore repeated experiments similar to that in figure 1, varying the electrode rotation rate. We have determined the slope of the initial linear variation and plotted it as a function of the square root of the electrode rotation rate in figure 2. The data confirm that the

slope is proportional to the square root of the rotation rate, as expected from equation (3), which validates the hypothesis that the initial linear increase in the current is mass-transport limited. We have also verified that the rate of initial linear increase is proportional to the concentration of enzyme in the bulk, as expected from equation (3) (see SI figure S2).

In equation (2), the only unknown, besides the catalytic turnover rate k_{cat} , is the diffusion coefficient of the enzyme. We estimated a value of $D_{enz} = 10^{-6} \text{ cm}^2 \cdot \text{s}^{-1}$ based on the Stokes-Einstein relationship, assuming a hydrodynamic radius of 3.5 nm (see SI section 5). With this value, we deduce a value of k_{cat} of $2700 \pm 300 \text{ s}^{-1}$ from the linear fit to the data of figure 2. This value is a lower bound because it is likely that only a fraction of the enzyme molecules adsorbed are immobilized in a configuration that actually allows electron transfer. Indeed, it is very common that the enzymes are immobilized with a dispersion of distances [25], and indeed the voltammograms of figure 1 show the typical linear increase at high potentials that is indicative of a uniform distribution of the distance between the redox active centers and the electrode [26,27].

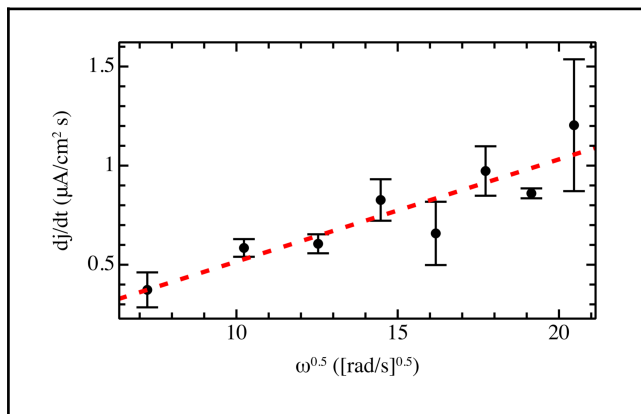


Figure 2: slope of the initial linear increase in current density (the slope of the red dotted line in figure 1) as a function of the square root of the electrode rotation rate. Conditions as in figure 1. The error bars represent the standard deviation across two experiments except for 3000 rpm (3).

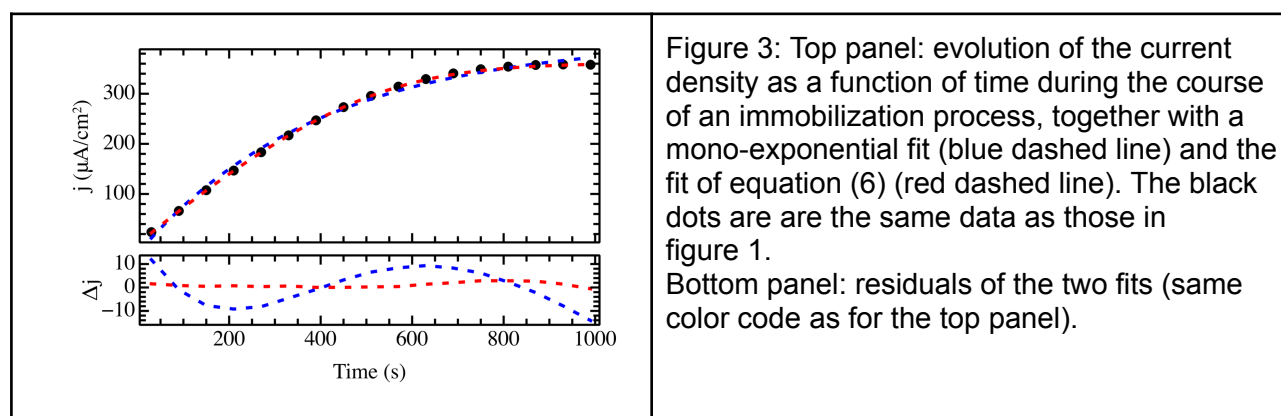
It is also possible to analyze the complete dependence over time of the increase in current, and in particular to reproduce the transition to a plateau after some time. We hypothesized that the immobilization of the enzyme follows a Langmuir adsorption isotherm, and that the plateau reflects that the surface sites are in equilibrium with the bulk concentration of the enzyme. Under this assumption, we derived equation (6), a differential equation predicting the evolution over time of the surface concentration and, hence, of the current density (see SI section 6):

$$\frac{d\Gamma}{dt} = m_{enz} \times \left(c_{bulk} - \frac{\Gamma}{K_{ad} \times (\Sigma_0 - \Gamma)} \right) \quad (6)$$

This equation can be integrated numerically to fit the experimental traces, using the free software QSoas[28]. The parameters of the fit are m_{enz} , which was calculated from equation (2), the solution concentration of enzyme $c_{bulk} = 0.7 \text{ nM}$, and three free parameters: the catalytic rate constant k_{cat} , the surface concentration of sites Σ_0 and the equilibrium constant K_{ad} of the reaction of a molecule of enzyme in solution with a free surface site to form a surface-bound enzyme molecule. Equation (6) predicts that the adsorption process gradually slows down until the surface concentration of enzymes corresponds to the value in equilibrium with the concentration in the bulk.

Figure 3 shows the evolution of the current over time for one of the adsorption experiments, together with the fit of an exponential decay to the data (blue dashes) and the fit of equation (6) (red dashes). The latter fits the experimental trace better than the former (with a corresponding five-fold reduction of the residuals from $7.5 \mu\text{A}/\text{cm}^2$ to $1.4 \mu\text{A}/\text{cm}^2$ average error), although both

equations depend on the same number of free parameters (3). This confirms that the adsorption follows a Langmuir isotherm. The parameters determined from the fit are $k_{cat} = 2220 \pm 40 \text{ s}^{-1}$ (in which the error corresponds to the 95% confidence interval of the fit), consistent with the value deduced from the slope of figure 2 and equation (3), a density of sites of $\Sigma_0 = 1.2 \text{ pmol/cm}^2$ and a dissociation constant $1/K_{ad} = 0.26 \text{ nM}$, which suggests that the surface sites are about 75 % saturated when the plateau of the current density is reached. The density of sites corresponds to an intersite distance of 12 nm, i.e. slightly less dense than in a fully packed monolayer, considering that the size of the enzyme is about 8 nm, and an atomically flat electrode surface. However the latter assumption greatly underestimates the actual surface available for adsorbing the enzyme. Similar parameters were obtained from the experiments carried out at other rotation rates (see supplementary figure S6).



In parallel to the determination of the catalytic activity from electrochemistry experiments, we performed solution assays to determine the catalytic activity of *Ec Hyd-1* in various H_2 -saturated buffers, using 10 mM of benzyl viologen as electron acceptor. The results are presented in table 1.

	Tris HCl 0.1 M pH 8	Tris HCl 0.1 M pH 7	Electrochemical buffer (pH 7)
Specific activity ($\mu\text{mol H}_2/\text{min}/\text{mg}$)	88 ± 7	50 ± 8	92 ± 10
Catalytic rate (s^{-1})	139 ± 11	79 ± 12	146 ± 15

Table 1: results of the solution assays of *Ec Hyd-1*, using various buffers as indicated, saturated with 1 atm. H_2 and containing 10 mM benzyl-viologen. Temperature: 40°C.

Since the beginning of Protein Film Electrochemistry, a range of techniques to prepare the electroactive films were employed, from simply drop-casting, sometimes with a co-adsorbant to help the adhesion to the electrode[1], to the chemical modification of electrodes and proteins to allow covalent grafting[29–32]. Films have also been made by slowly rotating an electrode in a buffer containing micromolar concentrations of enzyme under catalytic conditions, leading to a gradual increase over time of the catalytic current[17]. Here, we observed that in the case of *Ec Hyd-1*, it is possible to apply this strategy from solutions containing sub-nanomolar concentrations of enzyme, and that under these conditions, the adsorption process is limited by the transport of the enzyme towards the electrode, which is induced by the rotation of the electrode. We showed

that, by following the increase in current over time due to very diluted enzyme solutions adsorbing under mass-transport control, it is possible to determine a higher boundary estimate of the amount of enzyme immobilized on the electrode, by quantifying the amount of enzyme that actually reaches the electrode per unit of time. Like the other methods that quantify the total amount of enzyme immobilized on the electrode, based on surface plasmon resonance or on quartz microbalance, this method only provides a higher boundary of the number of connected enzyme molecules – however the approach we propose here does not require specifically engineered electrodes. In all of these approaches, the number of electrically connected enzyme molecules can be significantly lower than the enzyme loading – for instance, in the case of macroporous carbon felt electrode modified by carbon nanotubes, Mazurenko and coworkers were able to determine both the amount of electroactive enzymes (from non-catalytic signals) and the total amount of immobilized enzymes (by comparing the activity of the solution before and after immobilization); they found that under their conditions, only 14% of the enzymes were immobilized in a configuration that allowed direct connection.

There is often an important discrepancy between the value of the catalytic rate determined by solution assays and that determined by electrochemical methods, be it a true value or a lower boundary estimate. In the case of human sulfite oxidase, it was remarked early on that the catalytic rate determined from catalytic voltammogram was 20 times lower than the rate determined in solution assays under similar conditions, leading to the initial conclusion that only a small subset of the immobilized enzymes was in an active conformation[14]. However, it was later demonstrated that the reason for the decreased activity is that a conformational change necessary for the catalytic activity[33] is slowed down on the electrode to the point of becoming rate-limiting[34]. In some cases, the activity deduced from solution assays matches the value determined from catalytic voltammograms, as was the case for *E. coli* fumarate reductase FrdAB on pyrolytic graphite edge electrodes[16], or bilirubin oxidase immobilized on carbon felt modified with carbon-nanotubes[10]. Sometimes, the catalytic rate measured in solution was much smaller than that deduced from protein film electrochemistry experiments; this is the case of *Allochromatium vinosum* hydrogenase, for which values of the turnover rates in the 1500 s⁻¹ – 9000 s⁻¹ range were extrapolated from Koutecky–Levich plots[17], with solution assays in similar conditions giving values up to 900 s⁻¹. It should be noted however that enzyme-modified electrodes are not expected to obey Koutecky-Levich relationships[35], therefore, the catalytic rates deduced by these extrapolations are likely to be overestimations.

Concerning *Ec Hyd-1*, solution assays conducted under the same conditions as those of figure 1, yielded values of turnover rates of $146 \pm 15 \text{ s}^{-1}$, more than 15 times lower than the lower values deduced from experiments such as that of figure 1 (it should be noted that, as the same determination of the enzyme concentration is used for both computations, errors in the determination of the concentration would have no impact on the final ratio). This suggests that solution assays greatly underestimate the actual catalytic activity of *Ec Hyd-1*. This may arise from non-optimal conditions being used in the solution assays, in particular in terms of choice of artificial redox partner. Our conclusion is consistent with the fact that early electrochemical studies of *Ec Hyd-1* yielded very large current densities in spite of solution assays giving very small activities (1.5 s⁻¹)[22], for which it is usually considered that catalytic currents should be very hard to detect. Our results are also consistent with the relatively high values of the catalytic rate (around 600 s⁻¹) measured using Fourier-transformed AC voltammetry with the same enzyme[36].

Using a complete model taking into account Langmuir adsorption isotherms, we could also determine the adsorption equilibrium constant (3.8 nM⁻¹, corresponding to a half-saturated layer at 0.26 nM), which is comparable to those determined for the hydrogenase from *Aquifex aeolicus* (0.16 nM⁻¹, corresponding to a half-saturated layer at 6.3 nM)[10]. These low values also suggest that very dilute solutions may be used to form films using the usual drop-casting methods. The decrease observed at high times cannot be explained by the Langmuir adsorption process, which

predicts that the system reaches an equilibrium between the surface-immobilized enzyme and the one in solution. It is more likely attributable to irreversible damage, like enzyme inactivation on the electrode.

Acknowledgments

The authors acknowledge support from CNRS, Agence Nationale de la Recherche (grants ANR-14-CE05-0010, ANR-17-CE11-0027, ANR-18-CE05-0029), 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. The authors are members of the French Bioinorganic Chemistry group (<http://frenchbic.cnrs.fr>).

References

- [1] C. Léger, P. Bertrand, Direct electrochemistry of redox enzymes as a tool for mechanistic studies, *Chem. Rev.* 108 (2008) 2379–2438.
- [2] M. Del Barrio, M. Sensi, C. Orain, C. Baffert, S. Dementin, V. Fourmond, C. Léger, Electrochemical Investigations of Hydrogenases and Other Enzymes That Produce and Use Solar Fuels, *Acc. Chem. Res.* 51 (2018) 769–777.
- [3] F.A. Armstrong, R.M. Evans, C.F. Megarity, Protein Film Electrochemistry of Iron-Sulfur Enzymes, *Methods Enzymol.* 599 (2018) 387–407.
- [4] N.D.J. Yates, M.A. Fascione, A. Parkin, Methodologies for “Wiring” Redox Proteins/Enzymes to Electrode Surfaces, *Chemistry.* 24 (2018) 12164–12182.
- [5] C. Cadoux, R.D. Milton, Recent enzymatic electrochemistry for reductive reactions, *ChemElectroChem.* 7 (2020) 1974–1986.
- [6] M. Barrio, V. Fourmond, Redox (in)activations of metalloenzymes: A protein film voltammetry approach, *ChemElectroChem.* 6 (2019) 4949–4962.
- [7] M. Meneghello, A. Uzel, M. Broc, R.R. Manuel, A. Magalon, C. Léger, I.A.C. Pereira, A. Walburger, V. Fourmond, Electrochemical Kinetics Support a Second Coordination Sphere Mechanism in Metal-Based Formate Dehydrogenase, *Angew. Chem. Int. Ed Engl.* 62 (2023) e202212224.
- [8] A.K. Jones, R. Camba, G.A. Reid, S.K. Chapman, F.A. Armstrong, Interruption and time-resolution of catalysis by a flavoenzyme using fast scan protein film voltammetry, *J. Am. Chem. Soc.* 122 (2000) 6494–6495.
- [9] S. Frasca, O. Rojas, J. Salewski, B. Neumann, K. Stiba, I.M. Weidinger, B. Tiersch, S. Leimkühler, J. Koetz, U. Wollenberger, Human sulfite oxidase electrochemistry on gold nanoparticles modified electrode, *Bioelectrochemistry.* 87 (2012) 33–41.
- [10] I. Mazurenko, K. Monsalve, P. Infossi, M.-T. Giudici-Ortoni, F. Topin, N. Mano, E. Lojou, Impact of substrate diffusion and enzyme distribution in 3D-porous electrodes: a combined electrochemical and modelling study of a thermostable H₂/O₂ enzymatic fuel cell, *Energy Environ. Sci.* 10 (2017) 1966–1982.
- [11] M.A. Ruiz-Rodríguez, C.D. Cooper, W. Rocchia, M. Casalegno, Y. López de Los Santos, G. Raos, Modeling of the Electrostatic Interaction and Catalytic Activity of [NiFe] Hydrogenases on a Planar Electrode, *J. Phys. Chem. B.* 126 (2022) 8777–8790.
- [12] L.J.C. Jeuken, Structure and Modification of Electrode Materials for Protein Electrochemistry, *Adv. Biochem. Eng. Biotechnol.* 158 (2016) 43–73.
- [13] I. Mazurenko, V.P. Hitaishi, E. Lojou, Recent advances in surface chemistry of electrodes to promote direct enzymatic bioelectrocatalysis, *Curr. Opin. Electrochem.* 19 (2020) 113–121.
- [14] S.J. Elliott, A.E. McElhaney, C. Feng, J.H. Enemark, F.A. Armstrong, A voltammetric study of interdomain electron transfer within sulfite oxidase, *J. Am. Chem. Soc.* 124 (2002) 11612–11613.
- [15] L.M. Walker, B. Li, D. Nicks, R. Hille, S.J. Elliott, Deconvolution of reduction potentials of formate dehydrogenase from *Cupriavidus necator*, *J. Biol. Inorg. Chem.* 24 (2019) 889–898.

- [16] C. Léger, K. Heffron, H.R. Pershad, E. Maklashina, C. Luna-Chavez, G. Cecchini, B.A. Ackrell, F.A. Armstrong, Enzyme electrokinetics: energetics of succinate oxidation by fumarate reductase and succinate dehydrogenase, *Biochemistry*. 40 (2001) 11234–11245.
- [17] H.R. Pershad, J.L. Duff, H.A. Heering, E.C. Duin, S.P. Albracht, F.A. Armstrong, Catalytic electron transport in *Chromatium vinosum* [NiFe]-hydrogenase: application of voltammetry in detecting redox-active centers and establishing that hydrogen oxidation is very fast even at potentials close to the reversible H⁺/H₂ value, *Biochemistry*. 38 (1999) 8992–8999.
- [18] K. Singh, T. McArdle, P.R. Sullivan, C.F. Blanford, Sources of activity loss in the fuel cell enzyme bilirubin oxidase, *Energy Environ. Sci.* 6 (2013) 2460.
- [19] V.M. Badiani, S.J. Cobb, A. Wagner, A.R. Oliveira, S. Zacarias, I.A.C. Pereira, E. Reisner, Elucidating Film Loss and the Role of Hydrogen Bonding of Adsorbed Redox Enzymes by Electrochemical Quartz Crystal Microbalance Analysis, *ACS Catal.* 12 (2022) 1886–1897.
- [20] D. Pankratov, J. Sotres, A. Barrantes, T. Arnebrant, S. Shleev, Interfacial behavior and activity of laccase and bilirubin oxidase on bare gold surfaces, *Langmuir*. 30 (2014) 2943–2951.
- [21] C. Gutierrez-Sanchez, A. Ciaccafava, P.Y. Blanchard, K. Monsalve, M.T. Giudici-Ortoni, S. Lecomte, E. Lojou, Efficiency of enzymatic O₂ reduction by *Myrothecium verrucaria* bilirubin oxidase probed by surface plasmon resonance, PMIRRAS, and electrochemistry, *ACS Catal.* 6 (2016) 5482–5492.
- [22] M.J. Lukey, A. Parkin, M.M. Roessler, B.J. Murphy, J. Harmer, T. Palmer, F. Sargent, F.A. Armstrong, How *Escherichia coli* is equipped to oxidize hydrogen under different redox conditions, *J. Biol. Chem.* 285 (2010) 3928–3938.
- [23] M. del Barrio, C. Guendon, A. Kpebe, C. Baffert, V. Fourmond, M. Brugna, C. Léger, Valine-to-cysteine mutation further increases the oxygen tolerance of *Escherichia coli* NiFe hydrogenase hyd-1, *ACS Catal.* 9 (2019) 4084–4088.
- [24] A.J. Bard, L.R. Faulkner, H.S. White, *Electrochemical Methods: Fundamentals and Applications*, John Wiley & Sons, 2022.
- [25] H.O. Lloyd-Laney, M.J. Robinson, A.M. Bond, A. Parkin, D.J. Gavaghan, A Spotter's guide to dispersion in non-catalytic surface-confined voltammetry experiments, *J. Electroanal. Chem.* . 894 (2021) 115204.
- [26] C. Léger, A.K. Jones, S.P.J. Albracht, F.A. Armstrong, Effect of a dispersion of interfacial electron transfer rates on steady state catalytic electron transport in [NiFe]-hydrogenase and other enzymes, *J. Phys. Chem. B.* 106 (2002) 13058–13063.
- [27] V. Fourmond, C. Léger, Modelling the voltammetry of adsorbed enzymes and molecular catalysts, *Curr. Opin. Electrochem.* 1 (2017) 110–120.
- [28] V. Fourmond, QSoas: A Versatile Software for Data Analysis, *Anal. Chem.* 88 (2016) 5050–5052.
- [29] O. Rüdiger, J.M. Abad, E.C. Hatchikian, V.M. Fernandez, A.L. De Lacey, Oriented immobilization of *Desulfovibrio gigas* hydrogenase onto carbon electrodes by covalent bonds for nonmediated oxidation of H₂, *J. Am. Chem. Soc.* 127 (2005) 16008–16009.
- [30] E. Lojou, X. Luo, M. Brugna, N. Candoni, S. Dementin, M.T. Giudici-Ortoni, Biocatalysts for fuel cells: efficient hydrogenase orientation for H₂ oxidation at electrodes modified with carbon nanotubes, *J. Biol. Inorg. Chem.* 13 (2008) 1157–1167.
- [31] F.A. Al-Lolage, M. Meneghello, S. Ma, R. Ludwig, P.N. Bartlett, A flexible method for the stable, covalent immobilization of enzymes at electrode surfaces, *ChemElectroChem.* 4 (2017) 1528–1534.
- [32] F. Tasca, W. Harreither, R. Ludwig, J.J. Gooding, L. Gorton, Cellobiose dehydrogenase aryl diazonium modified single walled carbon nanotubes: enhanced direct electron transfer through a positively charged surface, *Anal. Chem.* 83 (2011) 3042–3049.
- [33] C. Feng, R.V. Kedia, J.T. Hazzard, J.K. Hurley, G. Tollin, J.H. Enemark, Effect of solution viscosity on intramolecular electron transfer in sulfite oxidase, *Biochemistry*. 41 (2002) 5816–5821.
- [34] T. Zeng, S. Leimkühler, U. Wollenberger, V. Fourmond, Transient Catalytic Voltammetry of Sulfite Oxidase Reveals Rate Limiting Conformational Changes, *J. Am. Chem. Soc.* 139 (2017) 11559–11567.
- [35] M. Merrouch, J. Hadj-Saïd, C. Léger, S. Dementin, V. Fourmond, Reliable estimation of the kinetic parameters of redox enzymes by taking into account mass transport towards rotating electrodes in protein film voltammetry experiments, *Electrochim. Acta.* 245 (2017) 1059–1064.

[36] H. Adamson, M. Robinson, J.J. Wright, L.A. Flanagan, J. Walton, D. Elton, D.J. Gavaghan, A.M. Bond, M.M. Roessler, A. Parkin, Retuning the Catalytic Bias and Overpotential of a [NiFe]-Hydrogenase via a Single Amino Acid Exchange at the Electron Entry/Exit Site, *J. Am. Chem. Soc.* 139 (2017) 10677–10686.