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Electrochemical investigations of hydrogenases and other enzymes that produce and use solar fuels

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Conspectus

Many enzymes that produce or transform small molecules such as $\text{O}_2$, $\text{H}_2$, and $\text{CO}_2$ embed inorganic cofactors based on transition metals. The active site, where the chemical reaction occurs, is buried in and protected by the protein matrix, and connected to the solvent in several ways: chains of redox cofactors mediate long range electron transfer; static or dynamic tunnels guide the substrate, product and inhibitors; amino acids and water molecules transfer protons. The catalytic mechanism of these enzymes is therefore delocalised over the protein and involves many different steps, some of which determine the response of the enzyme under conditions of stress (extreme redox conditions, presence of inhibitors, light), the catalytic rates in the two directions of the reaction, and their ratio (the “catalytic bias”). Understanding all the steps in the catalytic cycle — including those that occur on sites of the protein that are remote from the active site — requires a combination of biochemical, structural, spectroscopic, theoretical and kinetic methods. Here we argue that kinetics should be used to the
fullest extent, by extracting quantitative information from the comparison of data and kinetic models and exploring the combination of experimental kinetics and theoretical chemistry.

In studies of these catalytic mechanisms, the technique which we use and contribute to develop, direct electrochemistry, has become unescapable. It simply consists in monitoring the changes in activity of an enzyme that is wired to an electrode by recording an electric current. We have described kinetic models that can be used to make sense of these data and to learn about various aspects of the mechanism that are difficult to probe using more conventional methods: long range electron transfer, diffusion along gas channels, redox-driven (in)activations, active site chemistry and photoreactivity under conditions of turnover.

In this review, we highlight a few results that illustrate our approach. We describe how electrochemistry can be used to monitor substrate and inhibitor diffusion along the gas channels of hydrogenases and we discuss how the kinetics of intramolecular diffusion relates to global properties such as resistance to oxygen and catalytic bias. The kinetics and/or thermodynamics of intramolecular electron transfer may also affect the catalytic bias, the catalytic potentials on either side of the equilibrium potential, and the overpotentials for catalysis (defined as the difference between the catalytic potentials and the open circuit potential). This is understood by modelling the shape of the steady-state catalytic response of the enzyme. Other determinants of the catalytic rate, such as domain motions, have been probed by examining the transient catalytic response recorded at fast scan rates. Last, we show that combining electrochemical
investigations and MD, DFT and TD-DFT calculations is an original way of probing
the reactivity of the H-cluster of hydrogenase, in particular its reactions with CO, O₂
and light.

This approach contrasts with the usual strategy which aims at stabilizing species
that are presumed to be catalytic intermediates, and determining their structure using
spectroscopic or structural methods.

1 Introduction

The activation of small molecules is a key aspect of renewable energy research. The reactions
of water electrolysis and H₂ oxidation could serve for storing and returning to the grid the
energy that is intermittently produced from renewable sources such as sunlight. Recent
concerns about climate change have also boosted the research on CO₂ reduction to various
synthetically valuable organic molecules. Nature is an expert in chemical energy conversion,
and at a time when we are endangering the planet by recklessly using fossil and nuclear
energies, a rightful giving-back would be to seek inspiration from the strategies that have
proved effective since the origin of Life. The study of enzymes that use and produce small
biological fuels should be very informative in this respect.

Living organisms store energy in the form of small molecules that are continuously pro-
duced and recycled (fig. 1). Sunlight is used by photosynthetic organisms to produce energy
(in the form of ATP) and biomass. Dioxygen, the byproduct of oxygenic photosynthesis, is
reduced in the respiratory chains of plants, mammals and aerobic microorganisms in relation
to ATP synthesis. Most microorganisms also use H₂ as an energy vector: it is evolved (by
fermentation, mainly, and as a byproduct of nitrogen fixation) and re-oxidized (using dioxy-
gen, sulfate or other electron acceptors) as part of the bioenergetic metabolism of either
the organism where dihydrogen is produced or a distinct bacterium that shares the same
ecosystem. Some bacteria grow using CO as an energy source: they may use e.g. O₂ or
nitrate to oxidize the CO from natural sources into CO₂, which is fixed either by the enzyme
Rubisco (in the Calvin-Benson cycle) or reduced using H₂ as electron donor (in the reverse Krebs cycle or in the Wood–Ljungdahl pathway). And of course the biomass is re-oxidized into CO₂ in the Krebs cycle.

Many of these reactions are catalyzed by large enzymes that use one or several inorganic cofactors based on transition metals. Neither the active site nor its conserved proteic environment fully determine the enzyme’s catalytic properties, and structural elements such as accessory clusters, substrate channels or residues involved in proton transfer, which are remote from the active site, play a decisive role.

![Figure 1: The biological cycling of solar fuels.](image)

More than one hundred years after Michaelis and Menten initiated the research on enzyme mechanisms by defining the methodology for the analysis of enzyme reactions, enzyme kinetics has become the poor cousin of enzymology and modern methods such as crystallography and spectroscopy, often aided by computational chemistry,¹ have taken over. However, regarding the investigation of the finest details in the catalytic cycle, these experimental methods do have a weakness: they rely on the assumption that the forms of the enzyme that are stable enough to be trapped and detected are actually catalytic intermediates. But catalytic intermediates are elusive and many metalloenzymes reversibly or irreversibly convert into stable forms that are inactive (i.e. not part of the catalytic cycle). In the particular case of hydrogenases, which catalytically evolve H₂, another level of difficulty is that the
substrate proton cannot be removed from the buffer, and therefore catalytic H₂ evolution competes with the reduction of the enzyme; this implies that the catalytically-competent reduced states of hydrogenase cannot be stabilized. At the other end of the redox scale, the same is true for the S4 state of the oxygen-evolving manganese cluster of Photosystem II. There are therefore various reasons why certain catalytic intermediates cannot be isolated and inactive species may sometimes be mistaken for catalytic intermediates. Kinetics, by focusing on activity and changes in activity, is required to identify the experimental conditions that favor the accumulation of catalytic intermediates.⁷

In Marseille, our interest has been mostly in using enzyme kinetics: we believe that designing experiments where the enzyme is actually catalyzing a reaction is a condition for learning about how this enzyme works. The method that we favor is direct electrochemistry (or “protein film voltammetry”).³⁴ ⁵ A redox enzyme is adsorbed as a submonolayer film onto the surface of a rotating disc electrode (fig 2) and electron transfer (ET) between the enzyme and the electrode is direct. In the presence of the substrate, electron transfer to/from the electrode continuously regenerates the redox state of the enzyme that is competent for catalysis. The resulting current is proportional to the turnover frequency and to the electroactive coverage. The latter is most often unknown but provided it is constant much information can be gained by analyzing the relative changes in current that are observed.

Figure 2: Typical electrochemical set-up used to measure the activity of an enzyme adsorbed onto a rotating-disk graphite “working” electrode, and to expose it to various potential steps and sweeps, substrates, inhibitors and light.
when one changes the electrode potential, the temperature, the concentration of activator, inhibitor or substrate, or shines light on the adsorbed enzyme.

This electrochemical method has disseminated from the group of Fraser Armstrong through a significant number of research groups worldwide over the last 15 years. The combination of dynamic electrochemistry and spectroscopy is a recent development. Several principles have guided our own contribution. First, we have been interested in steps in the catalytic cycles that take place far from the active site (e.g. along the ET chain or in the tunnels that guide the substrate and inhibitors, or inter-domain movement); these steps may limit the rate of catalysis and determine the global catalytic properties of the enzyme. This complexity poses a challenge to experimental and computational mechanistic studies of metalloenzymes and to the biomimetic approaches to catalysis. Second, our objective has always been to quantitatively interpret the data by developing and using various kinetic models and free softwares. In an effort to promote this approach, we shall describe in fig. S1 a few useful procedures. Last, collaborations with theoretical chemists have allowed us to establish quantitative relations between kinetic measurements and molecular mechanisms.

This approach contrasts with, and complements, traditional strategies which all aim at seeing catalytic intermediates using methods that exploit the interactions between light and matter: crystallography, spectroscopy. Here we shall describe results representative of our work, hopefully inspiring further research in this field.

2 Diffusion along gas channels: new methods and functional implications

In NiFe hydrogenase, a “tunnel” guides the substrate/product H₂ and inhibitors such as CO and O₂ in and out of the enzyme. The narrow end of this tunnel, near the active site, is shaped by the side chains of two conserved amino-acids (L122 and V74 in D. fructosovorans (Df) NiFe hydrogenase) as shown in fig. Much effort has been put in testing Volbeda’s initial
assumption that these side-chains determine O

2 access to the active site of hydrogenase and O

2 sensitivity.

Electrochemistry can be used to measure the rates of diffusion within hydrogenase. The method consists in examining the kinetics of binding and release of the competitive inhibitor CO in a very simple electrochemical experiment where one measures the H

2-oxidation current at a constant potential and monitors the change in activity that results from a transient exposure to the inhibitor CO (fig. 3). When the experiment is performed with e.g. WT Df NiFe hydrogenase, the change in turnover rate closely follows the change in CO concentration in the cell (fig. 3DE), showing that CO diffuses very quickly in and out of the enzyme. In contrast when the side chains of the position 122 and 74 amino acids are modified (fig. 3C), the change in turnover rate occurs with a delay (fig. 3F) that results from the slow diffusion of CO along the modified tunnel. The data can be analyzed by assuming bimolecular binding and 1st order release of the ligand,

\[ E \frac{k_{\text{in}} \times [\text{CO}]}{k_{\text{out}}} \text{ECO} \]  

(1)

to measure the rates of diffusion in either direction (fig. S1 CD). The meaning of these rate constants depends on the relative rates of intramolecular diffusion and bond breaking/formation at the active site; in NiFe hydrogenase, the rate of inhibition by CO, \( k_{\text{in}} \), is a good proxy of the rate of intramolecular diffusion, \( k_1 \) (cf SI section S1).

In Df NiFe hydrogenase, certain point mutations slow the rate of binding of CO one thousand fold (fig. 4). As discussed in ref 9 the relations between the rate of CO binding and the Michaelis constant for H

2 (fig. 4b in ref 9) or the rate of inhibition by O

2 (fig. 4A) demonstrate that the mutations have the same effect on the rates of diffusion of these three molecules, which take the same path inside the enzyme. (Similar experiments could identify amino acids that determine the diffusion rates in FeFe hydrogenases.10)

Crystallography and molecular dynamics calculations had long been used to identify
Figure 3: Electrochemical estimation of the kinetics of substrate/inhibitor diffusion within \textit{Df} NiFe hydrogenase. Change in activity upon exposure to CO (panel D) of the WT enzyme (left) and the L122F-V74I mutant (right). The structures in panels ABC were drawn using the pdb files 1YQW and 3CUS. Adapted with permission from ref. \textsuperscript{8} copyright 2008 Natl. Acad. Sc. USA.
putative intramolecular diffusion pathways in hydrogenases and other enzymes. The data in fig. 4 validated new numerical methods for calculating intramolecular diffusion rates.\textsuperscript{12} Figure 4B shows the good agreement between the calculated diffusion rates ($k_1$) and the experimental CO-binding rates for several NiFe hydrogenase site-directed mutants.\textsuperscript{11}

Intramolecular diffusion partly determines the global properties of hydrogenase. For example, slowing down diffusion along the gas channel of NiFe hydrogenase slows the rate of inhibition by O$_2$ (fig. 4A and section S1), but if the reaction with O$_2$ is irreversible, the only consequence is to postpone the moment the enzyme becomes fully inactive. However, some mutations in the gas channel of NiFe hydrogenase significantly increase the resistance to O$_2$ of Df NiFe hydrogenase,\textsuperscript{13} independently of any effect on diffusion. In hydrogenases that are “naturally” O$_2$-resistant, this property is given by a particular FeS cluster in the electron transfer chain.\textsuperscript{14}

The second example is related to the reasons why certain hydrogenases are better cata-
lysts (in terms of \( v_{\text{max}} \)) in one particular direction of the reaction. Mutations that selectively slow the diffusion of \( \text{H}_2 \) in \( \text{Df} \) NiFe hydrogenase decrease the activity for \( \text{H}_2 \) production because \( \text{H}_2 \) exit is slow and determines the rate of \( \text{H}_2 \) production.\(^\text{15}\) In contrast, \( \text{H}_2 \) oxidation in solution is limited by ET to the redox partner, and indeed the mutations of the surface exposed cluster of NiFe hydrogenase that slow intermolecular ET slow down \( \text{H}_2 \)-oxidation more than \( \text{H}_2 \)-production.\(^\text{16}\) Modifying either the gas channel or the ET chain tunes the catalytic bias of a hydrogenase by slowing a step that is limiting in only one direction of the reaction.

Intramolecular mass transport is an important aspect of the reactivity of CO-dehydrogenases (CODHs), the enzymes that convert CO and \( \text{CO}_2 \). In the bifunctional enzyme where the CODH subunit is associated with an acetyl-coA synthase (ACS) subunit, a dynamically formed gas channel transports CO from the production site in the CODH subunit to the utilization site in the ACS subunit.\(^\text{17}\) The pathways used by CO in the CODH dimer are still elusive, but intramolecular transport is likely to be very fast, based on the observations that these enzymes react with \( \text{O}_2 \) and CO very quickly (\( > 10^7 \text{s}^{-1} \text{M}^{-1} \))\(^\text{18,19}\) and some CODHs have a Michaelis constant in the nM range\(^\text{19}\) (fig S1 EF).

### 3 Interpreting catalytic wave shapes

Understanding the dependence of turnover rate on electrode potential is the most important goal in direct electrochemistry. Different situations must be distinguished, depending on whether or not the response reaches steady-state (fig. 5). If the potential is swept, the scan rate \( \nu \) defines the time scale \( \tau = RT/F\nu \) of the experiment. A departure from steady-state occurs when this time scale matches that of a redox transformation of the enzyme. Redox driven (in)activations are often slow (time constants in the range 5–500 s), and are therefore manifested in a hysteresis in the voltammograms recorded at slow scan rates (\( 10^{-4}–10^{-2} \text{ V/s} \)). Redox transformations between catalytic intermediates occur on the time scale of
turnover ($10^{-4}$ s–$10^{-2}$ s), and making the system depart from catalytic steady-state requires scan rates in the range 0.5-50 V/s. At even faster scan rates catalysis does not proceed during the potential sweep and the catalytic signal vanishes. By changing the scan rate, one can therefore turn on and off (and learn about) some of the redox reactions that affect the catalytic response.

We have discussed how slow redox transformations between active and inactive forms result in a strong hysteresis in the voltammetric wave-shape. Here we focus on two other aspects of the interpretation of voltammetric signals.

Figure 5: How scan rate ($\nu$) affects the catalytic response. Actual (solid lines) or simulated (dotted lines) voltammograms of a film of *Aquifex aeolicus* hydrogenase at scan rates ranging from very slow (for which both catalysis and the anaerobic inactivation are at steady-state) to very fast (for which they are both outrun).

### 3.1 How the kinetics of intramolecular ET affects steady-state catalytic wave shapes

To describe a voltammetric response, an important phenomenological parameter is the “catalytic potential”, i.e. the mid-point potential of the sigmoidal catalytic signal, whose value defines the catalytic “overpotential” (and thus, the driving force required to trigger catalysis) and the “catalytic bias” (the relative magnitude of the current in the two directions). A simple model of catalytic cycle (fig. 6), which depicts a *two-electron* active site “A” wired to the electrode by a relay “R,” shows how the catalytic potentials depend on the properties of the enzyme (redox potentials, intramolecular ET rate constants).
Figure 6: A reversible catalytic cycle that takes into account two-electron redox chemistry at the active site "A" in three redox states, oxidized O, intermediate I, and reduced R (potentials $E^0_{O/I}$ and $E^0_{I/R}$) and intramolecular ET via an oxidized or reduced relay "R" (potential $E^0_R$). Steps 2 and $-2$ include substrate binding and release, and $k_2$ and/or $k_{-2}$ may depend on substrate concentrations. Reproduced with permission from ref. 22 copyright 2013 ACS.

Figure 7: The shapes of the steady-state voltammograms predicted from the scheme in fig. 6 (left panels and circled numbers) and the changes in the values of the two one-electron catalytic potentials versus the potential of the relay (also indicated by vertical lines on top of the voltammograms) as a function of the parameter $\lambda$, which is large when intramolecular ET is fast (panels abcd); the dotted lines show the position of the observed wave(s) (SI section S2). The left (ac) and right (bd) panels are for $k_2/k_{-2} = 0.5$ and $\infty$ (hence bi- or uni-directional catalysis, respectively), and the top (ab) and bottom (cd) panels show the situations where the active site potentials are uncrossed, or crossed over, respectively.
The model assumes that the active site exists in three redox states (oxidized, intermediate and reduced) characterized by the two potentials $E^0_{\text{OI}}$ and $E^0_{\text{IR}}$. $E^0_R$ is the potential of the relay. The ratio $k_2/k_{-2}$ defines whether the active site works one-way or both ways. Intramolecular ET is defined by the rate constants $k_{\pm 1}$ and $k'_{\pm 1}$, and the parameter $\lambda = k_1/k_2$ describes whether intramolecular ET is fast or slow compared to active site chemistry.

Depending on the parameters of the model, four distinct wave-shapes are predicted (circled numbers in fig. 7) and two one-electron catalytic potentials are defined (marked by blue and red vertical lines): the signal may consist of two broad (one-electron) waves each centered on one of the two catalytic potentials, (1); or a single, sharp (two-electron) wave centered on their average value, (2) and (4); or a single one-electron wave centered on one of the two catalytic potentials, (3). These catalytic potentials depart from $E^0_{\text{OI}}$ and $E^0_{\text{IR}}$ (just like Michaelis constants are not true thermodynamic parameters).

Figure 7 shows the changes in apparent catalytic potentials against $\lambda = k_1/k_2$ (fast intramolecular ET being on the right of each X-axis), for irreversible or reversible catalysis (left or right panels, respectively), assuming that the active site potentials are crossed or uncrossed ($E^0_{\text{OI}}$ greater or smaller than $E^0_{\text{IR}}$, top and bottom panels, respectively). Each panel shows the continuous change from the situation intramolecular ET is slow in both directions of the reaction ($\lambda$ small) and one of the two catalytic potential equates $E^0_R$, to the situation where intramolecular ET is fast (rightmost) and the catalytic potentials match the active site potentials. In contrast to a common assumption, the situation where the signal is a single wave at $E^0_R$ can occur only when catalysis is irreversible (wave (3)).

### 3.2 Fast scan catalytic voltammetry

Jones et al. have shown that fast scan rates can outrun catalysis by fumarate reductase: the fast-scan voltammetric response consists of pairs of peaks, as observed in the absence of substrate. Slower scan rates result in a transient catalytic response which can also be interpreted.
The enzyme human sulfite oxidase consists of a catalytic domain, containing the mononuclear molybdenum active site, and an ET domain, with a heme cofactor. The two are linked by a flexible hinge. The enzyme can adopt an open conformation, in which the heme domain can interact with redox partners but not with the molybdenum site, and a closed conformation, in which intermolecular ET is blocked but electrons can be exchanged between the heme and the molybdenum. By recording catalytic voltammograms at various scan rates, we could observe and interpret the deviation from steady-state catalysis, up until very fast scan rates where catalysis is outrun. Only a model which takes into account conformational changes can reproduce the data, showing that conformational changes still occur when the enzyme is adsorbed onto the electrode. This conclusion could not be reached from the examination of data recorded under steady state conditions. Interpreting this type of signals is difficult because it must rely on numerical simulations but we believe that this effort will eventually prove as significant as the historical step from steady-state enzyme kinetics to stopped flow.

In an alternative approach, Parkin and coworkers have also recently taken advantage of the possibility to mute the catalytic response by modulating the potential at high frequency, and recording concomitantly the steady-state catalytic response and the high frequency harmonics. The latter embed the information about non-catalytic ETs. They used this strategy to explore the relation between the properties of the ET chain and the catalytic bias in *E. coli* hyd-1 WT and various mutants.

4 Combining kinetics and theory to understand the reaction of the H-cluster with small ligands

To illustrate the approach that combines electrochemical kinetics and theoretical calculations, we discuss a few reactions of the active site H-cluster of FeFe hydrogenase (fig. 8).

The inhibition of the enzyme by CO is fully reversible only at high potential. When the electrode potential is slightly above or lower than the open circuit potential, CO release
competes with an irreversible transformation of the H-cluster into an inactive dead-end (fig S1 CD):

\[
E \xrightleftharpoons[k_{\text{out}}][k_{\text{in}} \times [CO]] \text{ECO} \xrightarrow{k_3} \text{dead end}
\]  

(At very low potential, the affinity of the H-cluster for CO is so low that neither binding nor damage occurs.) The dependence of \(k_{\text{in}}\) and \(k_3\) on the electrode potential (fig. 2A in ref [24]) shows that the species that reacts irreversibly with CO is one-electron more reduced than the resting state “Hox” ([4Fe4S]^{2+}Fe^{II}Fe^{I}). According to DFT, Hred has the electronic structure [4Fe4S]^{2+}Fe^{I}Fe^{I}, in which the iron atoms already fulfill the 18-electron rule. Upon CO binding to Hred, a bond must be cleaved: either the exogenous CO ligand is released or the bond between the proximal Fe and the cysteine that bridges the [4Fe4S] subcluster breaks, giving the inactive, inert compound \([\mu-\text{dtma})\text{Fe}_2(\text{CO})_4(\text{CN})_2]^{2–}\). Not all FeFe hydrogenases inactivate under these conditions.\(^{25}\) A recent FTIR investigation of the enzyme from \(D.\ desulfuricans\) showed that the binuclear cluster is indeed released upon reduction of the HoxCO state,\(^{26}\) but the spectroscopists believe that this follows the reduction to [4Fe4S]^{1+}Fe^{I}Fe^{I}. When the disruption of the H-cluster occurs in a closed FTIR cell, the binuclear cluster can rebind under oxidizing conditions.\(^{26}\) All this is relevant to the biological maturation of the H-cluster.\(^{27}\)

That CO binding to the H-cluster is weakened by irradiation can be observed in electrochemical experiments where the enzyme is adsorbed on an electrode and simultaneously irradiated by the light beam of e.g. a laser diode (fig 2). Under these conditions, the larger the power and the shorter the wavelength, the lower the magnitude of the inhibition. Fig. 9 CD clearly reveals that the illumination has an effect only on \(k_{\text{out}}\), which increases with the power density of the incident light. From the value of the slope (155 cm\(^2\)/s/W at 450 nm, converted into \(\epsilon \approx 18\text{mM}^{-1}\text{cm}^{-1}\)) and the molar absorption coefficient of the H-cluster, we concluded that most of the absorbed blue photons lead to photo-dissociation of the apical CO. This is consistent with the results of TDDFT calculations, which show that the excitations that involve the dinuclear subcluster occur in the high energy part of the visible
Figure 8: The active site H-cluster of FeFe hydrogenase and its reactions with H$_2$, CO, O$_2$, and light.

Figure 9: The effect of CO (left) and O$_2$ (right) on the H$_2$-oxidation activity of FeFe hydrogenase. The enzyme was exposed to CO and to the light of a 450 nm laser beam as shown in panel A. The catalytic response (panel B) was used to measure the CO in/out rate constants plotted against light power in panels CD. Panel F shows the response of *C. acetobutylicum* and *C. reinhardtii* FeFe hydrogenases exposed to O$_2$ as indicated in panel E. The rate constants of reactivation ($k_a$ in eq. [3]) are plotted against electrode potential in panel G. Adapted with permission from ref. 28 copyright 2016 ACS and ref. 10 copyright 2017 NPG.
spectrum and identify a barrierless relaxation pathway that dissociates the exogenous, apical CO\(^{28}\).

TDDFT calculations also helped us understand the molecular basis of the photoinhibition of FeFe hydrogenase\(^{30}\). The FeFe hydrogenases from \(C.\) acetobutylicum and \(C.\) reinhardtii are irreversibly damaged by UVB; this results from the photodissociation of one of the intrinsic CO ligands, followed by rearrangement of the binuclear cluster. This photoinhibition reaction prevents the use of the full solar spectrum to drive artificial photocatalytic dyads where either an enzyme or a bacterium receives electrons from a photosensitizer. Some FeFe hydrogenases are destroyed even by visible light\(^{31}\) and the possibility of irreversible photoinhibition will have to be considered to optimize \(H_2\) photoproduction systems where a particular FeFe hydrogenase is wired to a light-harvesting systems.

An obstacle to using FeFe hydrogenases is also their sensitivity to \(O_2\). Figure 9F shows that their activity decreases following each transient exposure to \(O_2\), after which a partial reactivation is observed. The change in activity over time is again consistent with eq 3, where the active enzyme reacts with \(O_2\) to form an inactive \(O_2\)-adduct that can either give back the active form (rate constant \(k_a\)) or be irreversible transformed into a dead-end.

\[
E \xrightarrow{k_{in} \times [O_2]} E:O_2 \xrightarrow{k_3} \text{dead end}
\]  

The rate of inhibition involves the diffusion of \(O_2\) through the protein matrix (forward and backward rate constants \(k_1\) and \(k_{-1}\)) followed by the formation of a Fe-\(O_2\) bond (rate constants \(k_2\) and \(k_{-2}\)). The measured inhibition rate \(k_{in}\) is a combination of all the corresponding rate constants (section S1):

\[
k_{in} = \frac{k_1 k_2}{k_{-1} + k_2}
\]  

Molecular dynamics calculations could be used to elucidate the intramolecular diffusion pathways and to predict the rates of diffusion to and from the active site: \(k_1 = 3 \times 10^3 \text{ s}^{-1}/\text{mM}\)
and \( k_{-1} = 8 \times 10^7 \text{ s}^{-1} \). The rate of \( \text{O}_2 \) binding to the H-cluster has been calculated by DFT.\(^{29}\) The values of the rate constants (\( k_2 = 2 \times 10^3 \text{ s}^{-1} \) and \( k_{-2} = 1.4 \times 10^{-2} \text{ s}^{-1} \), \( k_2 \ll k_{-1} \)) suggest that most \( \text{O}_2 \) molecules leave the enzyme without binding to the H-cluster. Combining all calculated rate constants gives \( k_{\text{in}} = 9 \times 10^{-2} \text{ s}^{-1}/\text{mM} \), which is only one order of magnitude smaller than the experimental value (\( k_{\text{in}} = 1 \text{ s}^{-1}/\text{mM} \)). The agreement is fair, considering that each of the contributing calculated rate constants includes systematic errors.

The reactivation that is seen in fig. 9F when \( \text{O}_2 \) is flushed away from the electrochemical cell is not just the unbinding of \( \text{O}_2 \). Indeed, \( k_a \) is very dependent on pH and isotope-exchange and increases exponentially as the electrode potential decreases: the reactivation is actually the reduction of the \( \text{O}_2 \)-bound adduct (this reduction is a negligible contribution to the faradaic current). According to DFT calculations, the reduction product is \( \text{H}_2\text{O} \).

The calculations also suggest that under certain conditions (high pH, high \( E \)) a hydroxyl ligand formed at the H-cluster en route to \( \text{H}_2\text{O} \) may react with a nearby cysteine; indeed, this cysteine was found oxidized in a structure of the enzyme\(^{32}\) and its replacement with aspartate decreases \( k_3 \) ten-fold.\(^{10}\) This reaction may explain that the inhibition by \( \text{O}_2 \) is not fully reversible.

**Biographical Information**

Melisa del Barrio  obtained her Ph.D. from the University of Zaragoza in 2014, then held a post doctoral position in Toulouse. She is now a post-doctoral researcher in Marseille. Her current research is focused on kinetic studies of hydrogenases.

Matteo Sensi  obtained his Ph.D. from the Universities of Aix Marseille and Milano Bicocca in 2017. He studied the photoreactivity of FeFe hydrogenases.

Christophe Orain  obtained his Ph.D. from the Universities of Brest in 2012, and held a post doctoral position in Marseille.
Carole Baffert obtained her Ph.D. from the University of Grenoble in 2003 and was a post-doctor in the group of A. Bond. She is associate professor at Aix Marseille Univ. Her interests is in redox metalloenzymes, in particular the mechanisms and physiological functions of hydrogenases.

Sébastien Dementin obtained his Ph.D. from the University of Paris-Sud in 2001. He is “Chargé de Recherche” at CNRS. He studies mechanisms (including catalysis and biosynthesis) and structure-function relationships in metalloenzymes such as hydrogenases and CODH.

Vincent Fourmond received his Ph.D. from Universite Paris VII in 2007 and held two post-doctoral positions, first in Marseille and then with Vincent Artero in Grenoble, before coming back to Marseille as a permanent “Chargé de Recherche” at CNRS. His research interests include kinetic and mechanistic studies of complex metalloenzymes, with a focus on quantitative analysis.

Christophe Léger obtained his Ph.D. from the University of Bordeaux and was a post-doctor in the group of Fraser Armstrong from 1999 to 2002. He is “Directeur de Recherche” at CNRS. His interests lie in kinetic and mechanistic studies of complex metalloenzymes. Visit the group’s web site at bip06.fr.

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Supporting Information Available: the meaning of the experimental binding/release rate constants; the apparent potentials plotted in fig. 7; a few examples of quantitative data analysis.

References


