doi:10.2533/chimia.2024.13 Chimia 78 (2024) 13–21

Electron-Transferring Metalloenzymes and their Potential Biotechnological Applications

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Abstract: Modern societies rely heavily on centralized industrial processes to generate a multitude of products ranging from electrical energy to synthetic chemical building blocks to construction materials. To date, these processes have relied extensively on energy produced from fossil fuels, which has led to dramatically increased quantities of greenhouse gases (including carbon dioxide) being released into the atmosphere; the effects of the ensuing change to our climate are easily observed in day-to-day life. Some of the reactions catalyzed by these industrial processes can be catalyzed in nature by metal-containing enzymes (metalloenzymes) that have evolved over the course of up to 3.8 billion years to do so under mild physiological conditions using Earth-abundant metals. While such metalloenzymes could in principle facilitate the implementation of carbon-neutral processes around the globe, either in "bio-inspired" catalyst design or even by direct exploitation, many remaining questions surrounding their mechanisms often preclude both options. Here, our recent efforts in understanding and applying metalloenzymes that catalyze reactions such as dinitrogen reduction to ammonia or proton reduction to molecular hydrogen are discussed. In closing, an opinion on the question: "Can these types of enzymes really be used in new biotechnologies?" is offered.

Keywords: Ammonia · Biocatalysis · Hydrogenase · Metalloenzyme · Nitrogen fixation

Ross Milton began his independent career as a tenure-track Assistant Professor in Inorganic Chemistry at the University of Geneva in 2019. He completed his PhD in Chemistry at the University of Surrey (UK, 2014) where he studied enzymatic electrocatalysis under the supervision of Prof. Robert Slade and Dr. Alfred Thumser. He then conducted postdoctoral research, including a Marie Skłodowska-Curie Individual Fellowship, with Prof. Shelley Minteer (University of Utah, USA) and Prof. Dónal Leech (University of Galway, Ireland). He then performed further postdoctoral research with Prof. Alfred Spormann (Stanford University, USA). His current research programme at the University of Geneva combines the skills developed and experience acquired during his formation to investigate the mechanisms and potential biotechnological applications of metalloenzymes catalyzing reactions such as hydrogen evolution and dinitrogen fixation to ammonia.

1. Introduction

Molecular hydrogen (H_2) and ammonia (NH_3) are important commodities for energy, agricultural and chemical industries. Around 80% of the 90 Mt of H_2 that was produced in 2020 originated from fossil fuels, leading to the production of around 900 Mt of carbon dioxide (CO_2).[1] In comparison, pre-pandemic global aviation was reponsible for just over 1000 Mt of CO_2 emissions in 2019.[2] Around 44% of the H_2 produced was used in refining industries, while approximately 37% was used for the production of NH_3 by the Haber-Bosch process. Each tonne of NH_3 produced requires ~180 kg of H_2; thus, the production of 185 Mt of NH_3 in 2020 required 33 Mt of H_2 (~26 Mt of H_2 produced from fossil fuels). This, in combination with the relatively high pressures and temperatures under which Haber-Bosch plants operate, results in the consumption of around 2% of global energy and 1% of energy related and process carbon dioxide (CO_2) emissions. Effectively, 1.9 equivalents of CO_2 are released per equivalent of NH_3 produced industrially.[3]

Around 70% of produced NH_3 is used as agricultural fertilizer, which provides essential sustenance for the human population on Earth. It is thought that (i) around half of N found in the Human body today originates from the Haber-Bosch process and (ii) greater than 4 billion people born since 1908 have been supported by the Haber-Bosch process.[4] Given the target of the Paris Agreement to limit the global temperature increase to 1.5% above pre-industrial levels by 2050, the global production of both H_2 (for NH_3 production as well as H_2-powered vehicules and direct steel reduction, *etc.*) and NH_3 by Humans is linked to our future ability to produce these vital chemical commodities with reduced CO_2 emissions.

One such solution may be electrolysis, where renewable electrical energy (*i.e.,* electricity derived from solar, wind, tidal *etc.* sources) can be used to drive a bulk oxidative or reductive reaction at an electrode (*n.b.*, electrolysis is reserved here for overall non-spontaneous electrochemical reactions, although an electrochemical fuel cell which produces electrical energy while simultaneously performing a reaction of interest to Humans is conceptually feasible). This approach is also termed 'preparative bulk electrolysis' or 'electrosynthesis', since the objective is to perform electrolytic reactions with the view to obtain a bulk product, such as a chemical commodity. While the electrode of an electrolytic reactor can itself oxidize or reduce the reactant (acting as an electrocatalyst), the choice of electrode can also impact the kinetics of the conversion reaction as well as the overpotential (driving

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force) required to obtain a given rate of the reaction (related to the current passing at the electrode). For example, platinum electrodes are often considered to be the optimal electrocatalyst for H_2 production from water electrolysis, whereas carbon electrodes can require fairly large overpotentials to observe an appreciable electrocatalytic current.[5]

Indeed, electrode surfaces that exhibit poor kinetics for this reaction (*i.e.,* carbon electrodes) can be coated with catalysts which, when reduced, will subsequently catalyze the H_2 formation reaction (now acting as an electrode-confined electrocatalyst); this can include the deposition of platinum nanoparticles, for instance.[5] This has resulted in decades of research to find new electrocatalysts for H_2 production by water electrolysis, with a specific interest in precious metal-free catalysts or catalysts prepared from Earth-abundant materials. The same is true of electrocatalytic dinitrogen (N_2) reduction, or 'fixation', to NH_3. It is believed that the first reported observation of electrocatalytic N_2 reduction to NH_3 was made by Humphry Davy as early as 1807 (Humphry Davy is buried at the Cimetière des Rois (Plainpalais, 'Cemetery of Kings'), Geneva).[6,7] However, at the turn of the 20th century, the N_2-fixation reaction over iron-based catalysts first invented by Fritz Haber quickly became the preferred route for NH_3 synthesis at scale; after all, electrosynthetic NH_3 production is often plagued by parasitic H_2 evolution. Today, solid electrodes are limited to around 35% Faradaic efficiency (35% of electrons involved in the reaction are used for NH_3 production), and homogeneous catalysts that can approach the Faradaic efficiencies observed in nature (detailed below) are typically unstable and less active on a site-by-site comparison.[8]

Nature offers biological catalysts, metalloenzymes, that can also catalyze H_2 oxidation/formation and N_2 fixation, under comparatively mild conditions with comparatively large turnover numbers (the number of turnovers until the complete loss of activity). In the case of nitrogenase, a maximum of 75% of transferred electrons are consumed for the $6e^-$ reduction of N_2 to NH_3; at ambient pressure, practical electron efficiencies of ~60% are usually achieved.[9,10] While multiple versions of H_2-processing (hydrogenases) and N_2-processing (nitrogenases) enzymes are found in nature, these oxidoreductases – enzymes that catalyze the transfer of electrons between at least two reactants or substrates – do not rely on precious metals for catalysis. For these reasons, these metalloenzymes are often cited in 'bio-inspired' catalyst design, where researchers seek to create new catalysts based on their underlying catalytic mechanisms. Importantly, multiple mechanistic questions remain, which potentially inhibits the advancement of such bio-inspired catalyst design. Being electron-transferring metalloenzymes, they themselves also have the possibility to exchange electrons with electrodes and act as 'bio'electrocatalysts. This multi-disciplinary notion can, from time-to-time, introduce undue hesitation from colleagues working in abiotic research fields; under comparable conditions, these precious metal-free electrocatalysts can perform as well as the gold standard electrocatalyst (in fact, platinum) for H_2 electrooxidation.[11]

1.2 *Introduction to Enzymatic Electrocatalysis*

The Reader is directed to recently published reviews and tutorials, including those from our research group, which extensively cover different aspects of enzymatic electrochemistry and electrocatalysis.[12–17] *A simplified, brief introduction to one aspect of enzymatic electrocatalysis is discussed here.*

In some cases, oxidoreductase metalloenzymes can be directly adsorbed to electrode surfaces where electron transfer to or from the electrode can effectively replace one of the natural electron donating/accepting substrates of an enzyme (Fig. 1). If an additional redox-active/electron-transferring species (which functions to transfer electrons between the electrode and enzyme) is not included in this electrochemical reaction, then this process is typically named 'direct electron transfer' (DET). In the absence of any other substrate for the enzyme, *i.e.,* under 'non-turnover' conditions, thermodynamic properties such as apparent reduction potential(s) (E^0) of an enzyme's metal-containing cofactor(s) ('metallocofactors') can be determined.[17] Marcus electron transfer theory for nonadiabatic electron transfer by tunnelling is often invoked to describe electron transfer between an enzyme and an electrode (Eqns. 1 and 2).[18] The two following aspects are of particular interest to this article. Marcus theory predicts an 'inverted region' in which the electron transfer rate constant (k_{ET}) for a bimolecular reaction begins to decrease with a relatively large Gibbs energy for the electron transfer reaction ($\Delta_r G^0$):

$$k_{\mathrm{ET}} = \frac{2\pi}{\hbar}\frac{H_{\mathrm{DA}}^2}{\sqrt{4\pi\lambda RT}}\exp\left(\frac{-(\Delta_r G^0 + \lambda)^2}{4\lambda RT}\right) \quad (1)$$

$$H_{\mathrm{DA}}^2 = (H_{\mathrm{DA}}^0)^2 \exp\left(-\beta(r_{DA} - r_0)\right) \quad (2)$$

Where H_{DA} is the electronic coupling matrix element (whose value is proportional to wavefunction overlap between the electron donor and acceptor), H^0_{DA} is the value of H_{DA} at r_0, λ is called the 'reorganization energy' (and reflects differences in nuclear coordinates of the electron donor/acceptor as well as the surrounding medium), β is called a 'decay constant' (which depends on the medium between the electron donor and acceptor), and r reflects the distance between the donor and acceptor (r_{DA}) when not in van der Waals contact ($r_0 \neq 0$).

The inverted region is, however, not usually observed for electron transfer tunnelling reactions with (metallic) electrodes, where $\Delta_r G^0$ is now $\pm F(E - E^0)$ (E is the potential applied at the electrode), when considering the density of electronic states present (where it is assumed that H_{DA} and the density of states are the same for all states and energy) (Eqns. 3 and 4).[15,19]

$$\Delta_r G^0 = -nFE^0 \quad (3)$$

$$k_{\mathrm{ET}} = \frac{k_{\mathrm{ET\,max}}}{\sqrt{4\pi\lambda/RT}}\int_{-\infty}^{\infty}\frac{\exp\left(-\frac{1}{4\lambda RT}[\lambda \pm F(E - E^0) - RT_x]^2\right)}{1 + \exp(x)}\mathrm{d}x \quad (4)$$

where k_{ETmax} reflects the maximal value of k_{ET} at large overpotential. Essentially, large electrode overpotentials do not immediately seem to risk entering the Marcus inverted region and result in a decrease in heterogeneous k_{ET}.

A second important aspect to keep in mind when considering enzymatic electrochemistry is the distance-dependence of k_{ET}. Eqns. 3 and 4 suggest that k_{ET} usually decreases exponentially with distance.[18] For electron tunnelling from electrodes, the electronic transmission coefficient (κ_{el}, by applying transition state theory) also decays exponentially with distance.[20] Finally, the adsorption of an enzyme on an electrode surface in a range of orientations can thus complicate analyses of heterogeneous k_{ET} as well as catalytic constants of the immobilised enzyme.[21] Considering these aspects together it is then often considered favourable to immobilize enzymes on electrode surfaces in a way that minimizes electron transfer distances for optimal k_{ET}, ultimately leading to increased substrate conversion by enzymatic electrocatalytic systems.[22] This, in theory, can be achieved by site-specific conjugation with carefully placed canonical amino acids (typically cysteines by maleimide, Michael addition reactions) or, where possible, incorporated non-canonical amino acids that enable

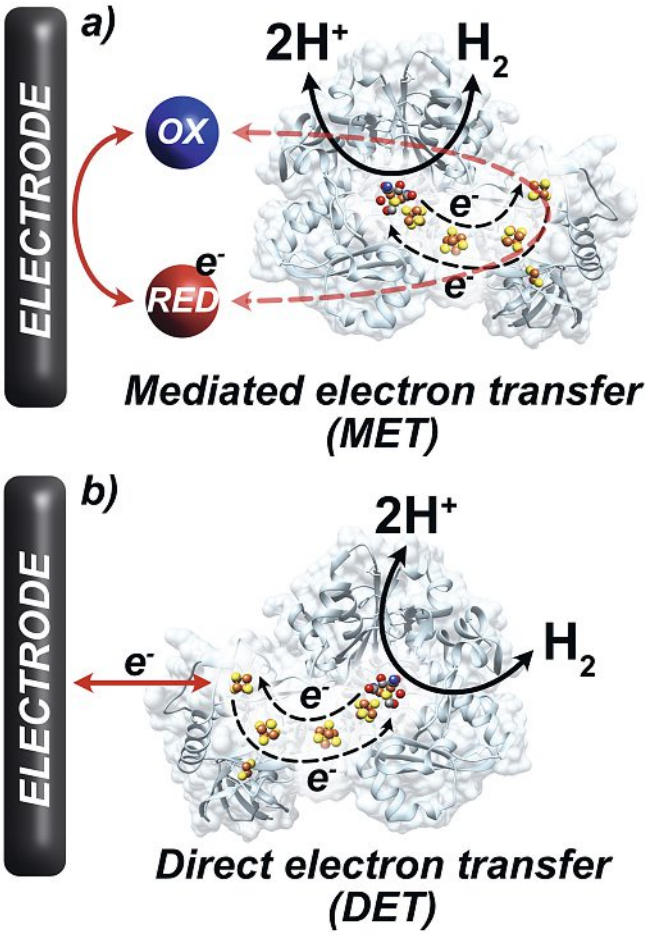

Fig. 1. Schematic of (a) mediated and (b) direct electron transfer (MET and DET) between a metalloenzyme and a solid electrode. Here, the enzyme depicted is [FeFe]-hydrogenase *Cp*I from *Clostridium pasteurianum* (PDB 6N59). Fe = rust, S = yellow, C = gray, O = red and N = blue. Adapted from ref. [12] with permission. © 2020 Wiley-VCH GmbH & Co. KGaA, Weinheim.

chemistries such as azide-alkyne cycloaddition reactions to be exploited (such as azide/alkyne-bearing amino acids).[13,22,23]

2. Nitrogenases

Note: This review introduces the Mo-dependent nitrogenase isoform. The Reader is directed to recent reviews on the two alternative V- and Fe-dependent nitrogenases.[24,25]

Nitrogenases are a class of metalloenzymes that catalyse the reduction (or 'fixation') of N_2 to NH_3 in select diazotrophic bacteria and archaea. Molecular N_2 was named 'azotic gas' (from the Greek 'azotikos') by French chemist Antoine Lavoisier, due to the asphyxiation of animals inhaling N_2 (O_2-depleted air).[26] In brief, Mo-dependent nitrogenase consists of an electron-delivering 'Fe protein' and the substrate-reducing 'MoFe protein' (Fig. 2).[25]

The homodimeric Fe protein contains two ATP-binding sites and a single [4Fe-4S] iron-sulfur cluster. In contrast, the heterotetrameric $\alpha_2\beta_2$ MoFe protein contains an [8Fe-7S] 'P' cluster and a [7Fe-9S-C-Mo]:homocitrate FeMo cofactor 'FeMoco' in each αβ half of the protein. N_2 is reduced to NH_3 in a reaction that involves the obligatory evolution of one equivalent of H_2 per N_2 fixed (optimal stoichiometry) (Eqn. 5):[9,10]

$$N_2 + 8e^- + 8H^+ + 16MgATP \rightarrow 2NH_3 + H_2 + 16MgADP + 16P_i \quad (5)$$

The association of each reduced, ATP-bound Fe protein ultimately results in the transfer of a single electron from the Fe protein's [4Fe-4S] cluster to the FeMoco, along with the hydrolysis of 2MgATP to 2MgADP and $2P_i$ (inorganic phosphate).[27] Thus, no fewer than eight transient associations between the Fe and MoFe proteins are required for the fixation of each N_2. Significant interest surrounds the idea of bypassing electron delivery from the Fe protein to the MoFe protein in a manner that yields significant TOFs; ultimately, this could lead to biological MgATP-independent N_2 fixation. Perhaps the most promising example to-date is the photoexcited delivery of electrons from CdS nanomaterials to the MoFe protein, which is believed to yield NH_3 with 63% of the specific activity of the MgATP hydrolysis-dependent activity.[28] Two of the many questions that remain surrounding how nitrogenase fixes N_2 are: (i) What is necessary to bypass the hydrolysis of MgATP for N_2 fixation, and (ii) why is nitrogenase's MoFe protein organized as an $\alpha_2\beta_2$ dimer of dimers? The two questions are linked: perhaps MgATP hydrolysis-independent ET (and N_2 fixation) requires simultaneous electron devivery on both αβ halves of the MoFe protein (or perhaps the contrary is required).

2.1 *Our Recent Research on Nitrogenases: Cooperativity*

Recent research has suggested that nitrogenase's MoFe protein (and the equivalent of the alternative nitrogenases) may indeed be capable of accepting electrons from donors, including directly from electrode surfaces, in a manner that results in substrate turnover with an appreciable rate constant.[28-35] Such ET is of course possible, otherwise reported potentiometric spectroscopic titrations of the P cluster and FeMoco would not be valid; the question is rather whether overall ET can take place with a rate constant that results in significant catalytic rate constants for the MoFe protein; a review of these titrations is reported in the literature.[27] Presumably, a conformational reorganisation(s) before/during/after Fe protein and MoFe protein transient associations accelerates substate reduction (and/or intraprotein ET) by the MoFe protein. However, it remains unclear why some approaches appear to be more effective than others at overcoming this prerequisite, *i.e.*, photoexcited electron delivery.[28,33]

In line with the enzymatic electrocatalysis aspects outlined above, it could be considered favourable to immobilize the MoFe protein on an electrode surface such that heterogeneous k_{ET} is maximized and homogeneous. We considered that this could be achieved using a MoFe protein double-mutant which was reported to accept photoexcited electrons from a tris(bipyridine)ruthenium analogue.[36] However, the question of nitrogenase's $\alpha_2\beta_2$ structure must be addressed. Specifically, is this $\alpha_2\beta_2$ structure essential for biological N_2 fixation? If activity in both αβ halves is indeed essential for N_2 fixation, then uniformly electrode-adsorbed MoFe protein may never achieve N_2 fixation with a significant rate constant. The erythrocytic Fe-dependent protein hemoglobin, present in the vast majority of vertebrates, serves as a model for the importance of such cooperative behaviour in multimeric proteins. Briefly, the organisation of four heme-containing subunits into an $\alpha_2\beta_2$ heterotetramer significantly alters the binding of molecular oxygen (O_2) to each heme cofactor relative to its monomeric counterpart protein (approximately), myoglobin.[37,38]

Recent reports have begun to address this question in nitrogenase. In 2016, Danyal *et al.* reported that the negative cooperativity exists between the electron delivery cycles between the Fe and MoFe proteins on both αβ halves by pre-steady-state measurements.[39] In this case, electron delivery is suppressed in the second αβ half during electron delivery in the first αβ half. In 2021, Truscott *et al.* investigated cooperativity by inducing a non-dissociating Fe:MoFe protein complex on only one αβ half of the MoFe protein, for which cooperativity during H_2 formation was not observed.[40] Earlier research had also found that the cross-

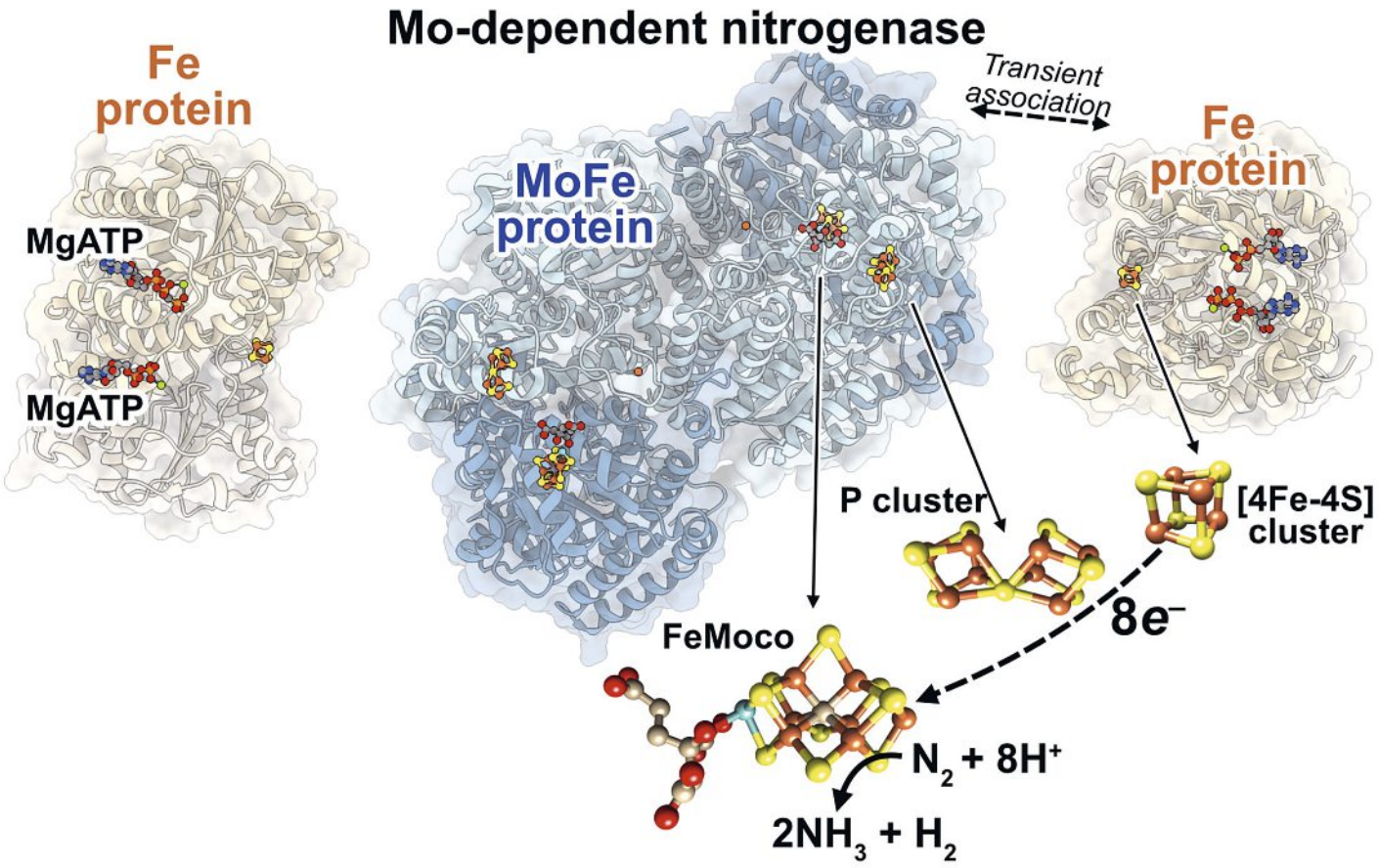

Fig. 2. Representation of nitrogenase's component proteins. The proteins in this X-ray crystal structure of the transition state complex (PDB 4WZA, formed using MgATP analogues) were manually separated in ChimeraX. Atoms are colored as in Fig. 1, except Mo = cyan, C = tan, Mg = green.

coupling of nitrogenase Fe and MoFe proteins from different organisms can lead to a 1:1 Fe:MoFe protein complex.[41] Finally, recent cryogenic electron microscopic investigation by Rutledge *et al*. during turnover conditions observed that, while particles of Fe:MoFe protein were observed, Fe:MoFe:Fe particles were not.[42] This may indeed further support the aforementioned notion of negative cooperativity: Fe protein transient association on the second αβ half is inhibited while the first αβ half is undergoing ET anda catalysis. Nevertheless the question remained: does half-active MoFe protein (while not engaged in a conformational inactivation) fix N_2 to NH_3?

To answer this question, we post-translationally modified fully active MoFe protein such that Fe protein access to one αβ half would be impeded and the second αβ half would remain in a native conformation (Fig. 3a).[43] To achieve this, we first prepared the above-mentioned α-C45A/L158C mutant MoFe protein, which carries a single solvent-accessible cysteine residue close to the P cluster.[36] We hypothesised that the modification of this cysteine residue with a steric inhibitor would inhibit the transient association of the Fe protein while not impacting the conformation of the MoFe protein (the conformation of the protein was confirmed to be unchanged by X-ray crystallography). Modification of the target cysteine residue was performed using a synthetic peptide with the sequence GGGWSHPQFEK (carrying an N-terminal maleimide functional group), usign a molar excess of the MoFe protein. This 1.4 kDa peptide served the dual purpose of (i) sterically inhibiting Fe protein transient associations while (ii) providing an affinity tag (Strep-tag) with which modified MoFe protein could be separated from unmodified MoFe protein by FPLC. Indeed, Fe protein association to the modified αβ half of the MoFe protein was found to be impacted (native-PAGE, gel filtration).

We first evaluated the activity of this partially inhibited MoFe protein in comparison to uninhibited MoFe protein. Interestingly, the MoFe protein's H_2 evolution activity in the absence of N_2 (under an Ar atmosphere) was found to be 79% of that of the uninhibited enzyme. This is in agreement with a negative cooperativity mechanism; a MoFe protein sample with only a single αβ half available for activity would yield an activity of 50% in the absence of any cooperativity effects. This H_2 evolution activity in the absence of N_2 fixation serves as a simple standard activity assay. The consensus mechanism of metal-hydride accumulation at the FeMoco means that nitrogenase is always liable to metal-hydride protonolysis (H_2 evolution).[9,10] Since the rate-limiting step of nitrogenase catalysis is currently thought to be the release of P_i by the Fe protein, H_2 evolution in the absence of N_2 fixation therefore takes place with full activity.[44]

We next evaluated N_2 fixation and concomitant H_2 production by this partially inhibited MoFe protein under one atmosphere of N_2. Importantly, we confirmed that partially inhibited MoFe protein does indeed maintain its N_2 fixing capability. Further, the selectivity of nitrogenase for NH_3 production over parasitic H_2 evolution remains unchanged. Interestingly, NH_3 formation was found to take place at 53% activity of the uninhibited MoFe protein. The determination of a cooperativity mechanism from this value alone is delicate; the 'total' electron flux passing through nitrogenase (in other words, the total product yield of H_2 and NH_3 combined) decreases in the presence of N_2.[45,46] The total electron flux of inhibited MoFe protein was therefore evaluated between 0-1 atm of N_2 and found to be on average 75% of that obtained from the uninhibited protein. Further, Michealis-Menten analysis indicated an unchanged Michaelis constant (affinity) for N_2 upon partial inhibition, as well as an increase in catalytic efficiency of 70% per active αβ half. A Hill coefficient was not

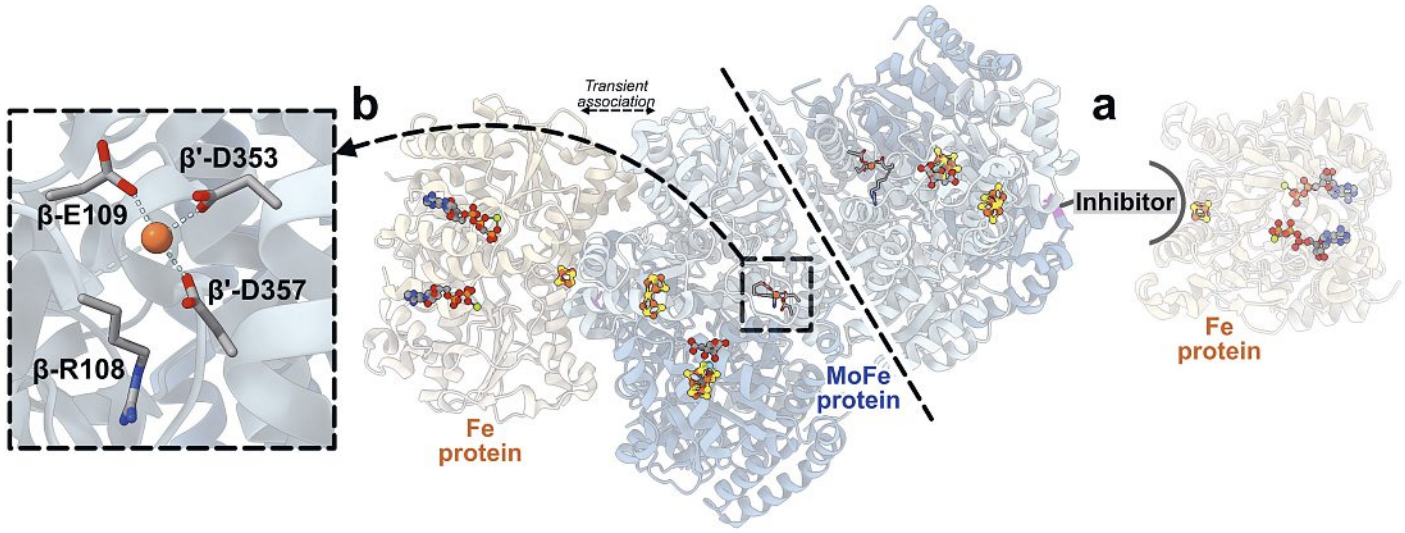

Fig. 3. Two approaches to evaluate cooperativity in Mo-nitrogenase (PDB = 4WZA). (a) A peptide steric-inhibitor was introduced to Fe protein-docking region of the MoFe protein. The α-L158 residue highlighted in magenta was mutated to a cysteine residue (α-L158C), which was then covalently modified with a 1.4 kDa peptide (GGGWSHPQFEK) carrying an N-terminal maleimide functional group. (b) Mo-nitrogenase's mononuclear metal-binding site (MMB) is highlighted, occupied by a Fe atom in this model; two coordinated water molecules were omitted for clarity. Two mutations were made to disrupt metal coordination at this MMB site: β-D353G/D357G. The atom coloring of Fig. 1 and model manipulation described in Fig. 2 apply.

determined, since cooperativity was not found to impact product selectivity.

Thus, we conclude that (i) cooperativity is not essential for N_2 fixation and that (ii) negative cooperativity is employed by nitrogenase regardless of N_2 fixation taking place or not, which is likely a result of negative cooperativity during electron delivery by the Fe protein. Importantly this suggests that, while protein conformational dynamics likely play an important role in N_2 fixation by nitrogenase, conformational dynamics induced by the opposite half of the MoFe protein are not strictly necessary for N_2 fixation.

Two caveats remain: (i) The approach to achieve 'half'-inhibited MoFe protein has some flaws (some doubly inhibited MoFe protein is expected to be present), and (ii) these *in vitro* observations do not necessarily reflect the nature of a crowded and dynamic cytoplasmic environment in which nitrogenases function. The following question then remains unanswered: Why is the MoFe protein organized as an $\alpha_2\beta_2$ heterotetramer (dimer of dimers)?

2.1 *Our Recent Research on Nitrogenases: The Mononuclear Metal-Binding Site*

Elemental analyses, vibrational/optical/EPR/Mössbauer/X-ray spectroscopies and structural analyses (X-ray crystallography and cryogenic electron microscopy) have greatly advanced our understanding of nitrogenase, but more specifically, the metallocofactors at the core of the enzyme's mechanism.[47] While the P cluster and the FeMoco have been thoroughly characterized, a third metal-binding site only recently gained interest. In 2013, Zhang *et al*. reported on the '16th Fe of nitrogenase', drawing attention to a mononuclear metal-binding (MMB) site that is well-conserved in nitrogenases (as well as evolutionarily related metalloenzymes) and located only 15 Å away from the homocitrate residue of the FeMoco (Fig. 3b).[48] In fact, the 16th Fe was a nickname given due to the presence of additional Fe content for nitrogenase that is often detected during elemental analysis, although this site appears to be relatively labile and X-ray crystal structures often depict this coordination site to be occupied with Mg/Na/Ca.[48]

The assignment of Fe yields an approximately octahedral metal center that is tetracoordinated by two of the MoFe protein's β (NifB) subunits; interestingly, two of the four coordinating amino acids are provided by the second β subunit. Thus, this MMB site caught our attention due to its possible importance in transmitting cooperativity across the two αβ halves of the MoFe protein. To address the question of cooperativity, we performed site-directed mutagenesis to replace the highly conserved metal-coordinating aspartic acids with glycines, where it was hypothesized that the absence of the carboxylate groups could (i) disrupt a coordination bond-mediated cooperativity mechanism and/or (ii) result in complete loss of the additional metal.[49]

Following purification, this β-D353G/D357G mutant MoFe protein was crystallized under anoxic conditions resulting in an X-ray crystal structure refined to 1.55 Å showing typical MoFe protein organization with no significant change in conformation. Importantly, the MMB site was found to be vacant of metals and filled with water molecules. Thus, we concluded that the MMB site does not play a vital role in assembly of the MoFe protein.

The resulting *Azotobacter vinelandii* mutant strain retained its diazotrophic capability, thus, we initally hypothesized that the MMB site is also not essential to N_2 fixation. Indeed, *in vitro* activity assays for H_2 evolution as well as N_2 fixation confirmed that the β-D353G/D357G MoFe protein lacking the MMB site (i) is equally as active as the wild-type MoFe protein, (ii) does not exhibit a dramatic difference in affinity or selectivity towards N_2 fixation, and (iii) exhibits similar thermostability (determined by circular dichroism).

One interesting observation was made when considering one of nitrogenase's protection mechanisms against O_2 deactivation in the obligate aerobic *A. vinelandii*. In the presence of O_2, the Fe and MoFe proteins engage in the formation of a catalytically inactive tripartite complex with a small ferredoxin-like protein ('Shethna', or FeSII protein), which offers short-term protection of nitrogenase's FeS clusters from O_2.[50-52] 'Protection' assays performed using the wild-type MoFe protein in the presence of O_2 confirmed that 66% of nitrogenase's H_2-formation activity in the absence of N_2 could be retained after the exposure of nitrogenase to 2% O_2 for 10 minutes. Importantly, the β-D353G/D357G MoFe protein retained only 16% of its activity under the same conditions. We thus concluded that the MMB may indeed play an important and as-of-yet poorly understood role in long-range conformational regulation of the nitrogenase complex. Importantly, however, this difference in protection mechanism

was not observed in the presence of N_2. This could be explained by a different reaction mechanism used for N_2 fixation, although this may be complicated by the aforementioned difference in total electron flux passing through nitrogenase when fixing N_2. Further research is required to uncover the potential role of this MMB in cooperavity. In addition, it is of course possible that *in vitro* activity assays are not highly representative of *in vivo* turnover conditions.

3. [FeFe]-hydrogenases

Metal-dependent hydrogenases catalyze the reduction and oxidation of H^+ and H_2. We are primarily interested in the [FeFe]-hydrogenases because their H_2 evolution rates are typically the greatest, reaching up to 10,000 H_2 evolved per second.[53] Fig. 4a presents the X-ray crystal structure of [FeFe]-hydrogenase I from *Clostridium pasteurianum* ('*Cp*I' – only the [FeFe]-hydrogenase will be discussed further). *Cp*I contains three [4Fe-4S] clusters that are implicated in electron transfer to/from the active site, as well as a [2Fe-2S] cluster. The active site houses the 'H cluster', which is comprised of a [4Fe-4S] cluster that is coordinated to a [2Fe] site (*via* a μ-S ligand provided by a cysteine residue). This [2Fe] active site contains multiple CO and CN^- ligands as well as a 2-azapropane-1,3-dithiolate (adt) ligand that plays a role in H^+ insertion/abstraction on the [2Fe] site during turnover.[53] The location of ET FeS clusters close to the surface of *Cp*I results in it being amenable to DET, as well as to 'mediated ET (MET)'.[54–56] We recently employed *Cp*I on rotating disk electrodes where a mediator covalently tethered to a polymeric backbone, yielding a 'redox polymer', was used to immobilize *Cp*I while facilitating MET.[57]

3.1 *Our Recent Research on [FeFe]-hydrogenases*

There are various aspects that need to be addressed in order to eventually utilize enzymes in new biotechnologies. In the case of [FeFe]-hydrogenases, electrode architectures that simplify enzyme incorporation must be designed. This includes the simplicity of fabricating electrodes, while also considering how enzymes are prepared at scale for enzymatic electrocatalysis (addressed in Section 5). Further, the ET mechanism between the electrode and enzyme must be carefully considered. For example, MET presumably requires a redox polymer to be designed at the cost of overpotential.[58] As discussed above, DET could require specific surface chemistries for orientation-optimized ET. Finally, the quantity of enzyme immobilized at an electrode surface in a stable manner (adsorbed mass as well as longevity) should also be augmented in order to maximize the quantity of product generated at each electrode. In this regard, the Reisner group has pioneered the use of porous hierarchical electrodes for enzyme immobilization.[59-63]

As discussed above, we have an ongoing interest in immobilizing the MoFe protein of nitrogenase onto electrode surfaces for electroenzymatic catalysis. With the specific aim of addressing product distribution of nitrogenase (*i.e.,* H_2 evolution *vs.* N_2 fixation) we envisaged the use of rotating ring disk electrochemistry, where any H_2 generated at enzyme-modified disks electrodes would be detected in close proximity by a Pt ring electrode. To prove this concept, we initially immobilized *Cp*I onto the disk electrode using a cobaltocene-containing redox polymer, given the significantly improved turnover rates expected for *Cp*I *vs.* nitrogenase (Fig. 4b).[57] Indeed, effective H_2 quantification could be achieved. We also coupled MET-based H_2 production to mass spectrometry to confirm that electroenzymatically generated isotopes of H_2 (*i.e.,* HD and D_2) could be quantified at this scale. This has particular importance in the case of nitrogenase, where HD production could, in principle, be used as a diagnostic of having successfully generated the E_4 state (N_2-binding) of the MoFe protein's FeMoco.[9,64]

Motivated by the need to prepare a simple electrode surface for efficient enzymatic electrocatalysis and biotechnological exploitation (ideally in a DET system), we evaluated and adapted a previous report demonstrating the use of indium:tin oxide (ITO) nanoparticles with enzymes. Typically, hierarchical electrodes modified with metal oxides require high-temperature annealing in order to yield adequately conductive surfaces. In order to create a conductive and transparent electrode surface, Hoertz *et al.* reported the simple deposition of an ITO nanoparticle suspension (using a commercial source of ITO nanoparticles).[65] Later, Kornienko *et al.* adapted this reported approach for the study of photosystem II at rotating ring disk electrodes, employing a low-temperature drying step (80 ºC) and ozone pretreatment.[66] We omitted the ozone treatment step to yield an approach that requires a single electrode-preparation step prior to the adsorption of *Cp*I hydrogenase.[67]

Following ITO nanoparticle electrode (nanoITO) preparation, approximately 25 μg of *Cp*I was deposited directly onto the nanoITO surface; no further measures were taken to ensure the stable adsorption of *Cp*I to the electrode surface (Fig. 4c). Cyclic voltammetry in pH 7 buffer revealed electroenzymatic H_2 evolution reaching large current densities of over 8 mA cm^{-2}, with *Cp*I acting as a reversible electrocatalyst (appreciable bidirectional catalysis either side of E^0).[68] Potentiostatic and galvanostatic gas chromatographic measurements performed in collaboration with the Prof. Broekmann (University of Bern) confirmed near-ideal Faradaic efficiency. Further, the *Cp*I nanoITO electrodes exhibited remarkably enhanced operational stability, retaining approximately 94% of their initial H_2 evolution activities after 120h of continuous potentiostatic operation. Significant *Cp*I desorption

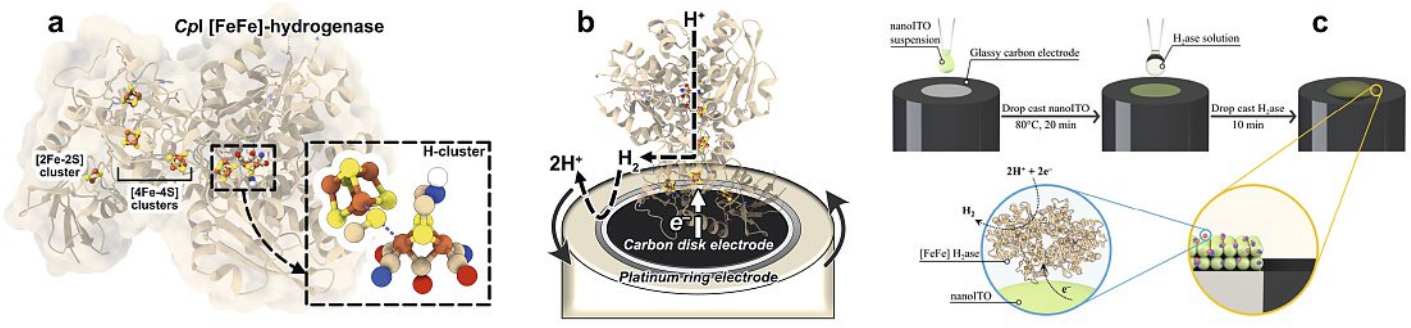

Fig. 4. (a) Representation of the X-ray crystal structure of *Cp*I [FeFe]-hydrogenase from *Clostridium pasteurianum* (PDB = 6N59). Atom coloring as per Fig. 1. The inset highlights the cysteine residue that μ-coordinates the [4Fe-4S] cluster and [2Fe] cofactor of the H-cluster. (b) Schematic of the immobilization of *Cp*I on the carbon disk electrode of a rotating ring disk electrode, where electroenzymatically generated H_2 is directly quantified at the neighboring platinum ring electrode. (c) Scheme for the preparation of simplified ITO nanoparticle-electrodes ('nanoITO') using *Cp*I, reproduced without modifications from ref. [67].

from the nanoITO electrode was not observed, beyond the initial desorption of excess *Cp*I (<10% of the loaded enzyme). Research is underway to understand precisely how *Cp*I appears to be stabilized on these nanoITO electrodes, in a way that enables comparatively larger quantities of H_2 to be evolved with significantly improved operational stability.

4. Our Research on Related Biotechnologically Relevant Metalloenzymes

One of the major challenges surrounding nitrogenase research is the ability to observe intermediates, or even to accurately quantify NH_3 electrochemically produced by nitrogenases.[10,47,69,70] An additional challenge concerns the production of active nitrogenase enzymes, and of single-point mutants in particular, which is related to the complex biosynthesis pathway of nitrogenase's cofactors.[71] Thankfully, related enzymatic systems can offer insight into biological N_2 fixation. Of specific interest in dark-operative protochlorophyllide oxidoreductase, or DPOR, an evolutionarily related enzyme involved in chlorophyll biosynthesis (Fig. 5).[72] While catalyzing a very different reaction, DPOR employs a similar ET mechanism to that of nitrogenase. DPOR's BchL protein undergoes repeated transient associations to the substrate-reducing BchNB protein, where each ET event is also coupled to the hydrolysis of ATP. In contrast to nitrogenase, DPOR: (i) does not evolve H_2 at a significant rate, (ii) only contains 'simple' [4Fe-4S] FeS clusters (permitting its recombinant production in the malleable organism *Escherichia coli)*, (iii) the conversion of substrate to product only requires $2e^-$ to be transferred and (iv) substrate reduction (product formation) can easily be quantified by UV/visible spectroscopic methods.[73-76] We have recently reported on DPOR (electro)catalysis, where we are able to initiate enzymatic turnover using alternative electron donors to commonly utilized dithionite.[77] In particular, we are able to separate ET from substrate association to DPOR, as well as to initiate ET using electrochemical methods *in situ* (spectroelectrochemistry). Research in this area is ongoing.

Enzyme electrocatalysis is also of interest to CO_2 reduction and upgrade. Research has demonstrated that electrons can be delivered to metal-dependent formate dehydrogenase (Fdh) for the $2e^-$-reduction of CO_2 to formate with near-perfect Faradaic efficiencies (Fdhs do not evolve H_2).[78-85] While Fdhs catalyze CO_2 reduction at significant rates, they typically perform better in the reverse reaction (formate oxidation). To this end, we very recently reported a large enzymatic complex isolated from a thermophillic methanogen, formylmethanofuran dehydrogenase (Fwd).[86] While Fdh's physiological role is to oxidize formate, Fwds are often the CO_2 entry enzyme for methanogenesis in methanogens, a metabolic pathway that yields ATP synthesis as well as the fixation of CO_2 to methane. In our electrochemical study of Fwd, we observed preferential CO_2 reduction activity over formate oxidation, once again achieving complete Faradaic efficiency for CO_2 reduction in aqueous solutions at neutral pH. In fact, formate oxidation by Fwd-modified electrodes was comparatively challenging to achieve. This preference towards CO_2 reduction over formate oxidation renders Fwd a promising candidate for future CO_2-reducing biotechnologies.

5. Outlook

Can These Metalloenzymes Really Be Used in New Biotechnologies?

Before considering electrochemical biotechnologies below, an obvious and highly impactful potential future use of nitrogenase must be mentioned. As outlined above, nitrogenases are found in select bacteria and archaea. Some plants (eukaryotes) are able to undergo symbiosis with nitrogenase-producing bacteria (*i.e. Rhizobia*) whereby the plants encapsulate these bacteria in O_2-depleted root nodules and benefit directly from N_2 fixed locally by these bacteria.[87] Understanding how to form adequate root nodules (and symbiotic N_2 fixation) on demand could effectively remove the need for modern agriculture to introduce N fertilizer to crop fields. The production and turnover of nitrogenases in bacteria and archaea can require as little as 6 and as many as 35 genes.[71] Naturally, this can complicate the production of nitrogenases by non-diazotrophic organisms. The possibility of nitrogenase production by eukaryotic systems themselves (not in symbiosis) represents another grand challenge which could, as above, rid the need for synthetic N fertilizer in modern agriculture.[88,89] Both of these challenges heavily rely on the information fed from ongoing *in vivo* and *in vitro* nitrogenase studies.

One recurring concern with respect to the direct use of isolated enzymes in electrochemical systems is the presumed need to purify enzymes from microbial cell cultures. In my opinion, the applied enzymatic electrocatalysis field currently remains largely centered around questions of how to develop and optimize the enzyme:electrode interface for eventual deployment, which has probably limited the true evaluation of their actual deployment. Nevertheless, factors such as long-term stability and sensitivity to O_2 must still be addressed (even if engineering solutions can tackle the latter, as is the case for O_2 exclusion in the Haber-Bosch process). The field of microbial electrocatalysis offers a way in which enzymes can be exploited without the need to rupture cells.[90,91] To this end, the methanogenic archaeon *Methanococcus maripaludis* gained attention due to its high apparent rates of direct electron uptake (electron transfer from electrodes directly to the organism). In 2015, Deutzmann *et al*. demonstrated that the spontaneous lysis (autolysis) of *M. maripaludis* releases hydrog-

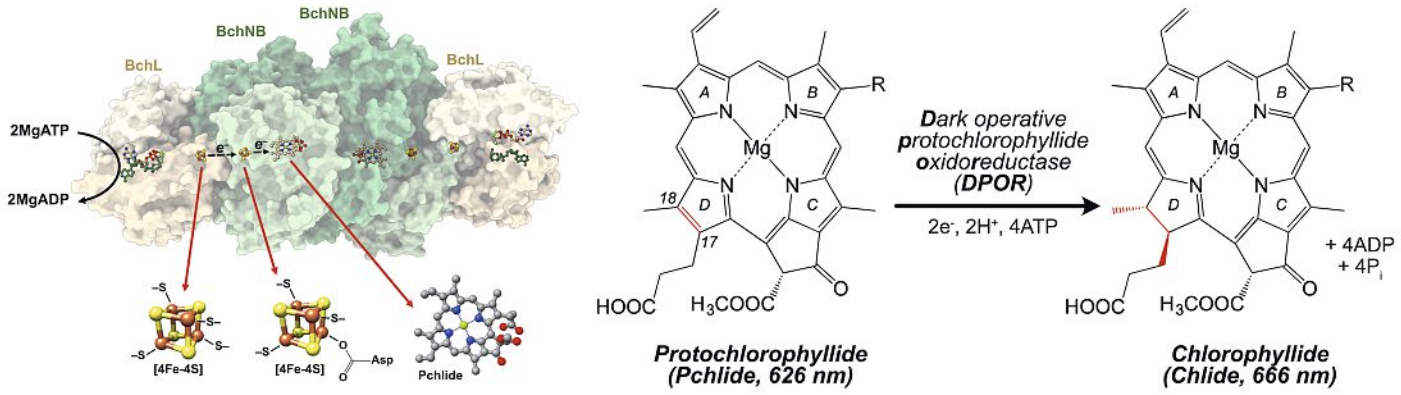

Fig. 5. Representation of the X-ray crystal structure of dark operative protochlorophyllide oxidoreductase (DPOR) from *Prochlorococcus marinus* (PDB = 2YNM), crystallized in the transition-like state using non-hydrolyzable ATP analogues. Atom coloring as per Fig. 1 and Mg = green. The inset highlights the cysteine residue that μ-coordinates the [4Fe-4S] cluster and [2Fe] cofactor of the H-cluster. Adapted from Ref [77].

enases and formate dehydrogenases into the surrounding electrolyte, which can subsequently adsorb to electrode surfaces for H_2 formation and CO_2 reduction by DET (direct electron uptake is therefore not the predominant mechanism and electroenzymatically generated H_2 is consumed by *M. maripaludis* for metabolism).[92] In this case, enzyme purification is not necessary. Thus, future electrode designs tailored for the adsorption of specific enzymes may well entirely negate the need to purify enzymes, facilitating their deployment at scale.

Acknowledgements

I am grateful to all the scholars (my research group members in particular) that I've had the pleasure of working with to date, as well as to all previous and ongoing collaborations and contributors. I thank my direct and indirect supervisors and mentors whose guidance has been invaluable, particularly (alphabetical): Dónal Leech, Shelley D. Minteer, Robert C.T. Slade, Alfred M. Spormann and Alfred E. Thumser. I thank the Swiss National Science Foundation (SNSF) for their support, both through a project grant (200021_191985) and the NCCR Catalysis (grant number 180544, a National Center of Competence in Research). I also thank the Ernest Boninchi foundation, the Academic Society of Geneva, and the Ernst & Lucie Schmidheiny foundation for support.

Received: November 28, 2023

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