Rational Design of Artificial Ni Dependent Metalloenzymes for Solar H_2 Production and CO/CH_3 Activation

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ABSTRACT

Nickel-dependent metalloenzymes catalyze several key reactions in biology. [NiFe] hydrogenases (H_2ases) reversibly interconvert between H^+ and H_2, while acetyl-CoA synthase (ACS) assimilates CO into acetyl-CoA in the Wood-Ljungdahl pathway. Unraveling the structure-function relationships of these enzymes will significantly advance the H_2-based, carbon-neutral alternative energy production and mitigate the environmental carbon footprint by making new chemicals from CO. Here I will describe our approaches to design artificial biomolecular H_2ases (ArHs) and artificial ACSs (ArACSs) as functional analogs of native enzymes employing protein reengineering and de novo protein design methods. On the ArH side, the design of mononuclear (Ni), binuclear (Ni-Fe) and (Ni-Ni) active sites within suitable protein/peptide scaffolds will be described. Insights into H-H bond formation are obtained by detailing the timescales of electron transfer and elucidating the proton transfer pathways leading to a mechanistic understanding of photosynthetic water splitting[1-3] to produce solar H_2. Furthermore, our approaches to probe the functional properties of the Ni_p site of the ACS in de novo designed protein scaffolds and to create the A-cluster of the ACS by protein reengineering will be highlighted. Functional studies pertaining to CO and -CH_3 binding and C-C bond formation at these sites and their characterization by spectroscopic tools will be demonstrated.

KEY WORDS

De novo design, Metalloenzymes, Artificial photosynthesis, Carbonylation, C-C bond formation.

ACKNOWLEDGEMENTS

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How the multidomain flavodiiron protein from the strict anaerobe *Syntrophomonas wolfei copes with oxygen and hydrogen peroxide?*

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Flavodiiron proteins (FDPs) constitute a widespread family of metalloenzymes present in the three life kingdoms, with a crucial role in O_2/ROS detoxification and/or NO detoxification, through the reduction of these species either to H_2O or N_2O, respectively. All the FDPs have a minimal catalytic unit composed by two domains, a metallo-β-lactamase like domain, harboring the catalytic diiron site and a flavodoxin-like domain. The majority of FDPs, already characterized, have only this core, but more complex arrangements were found, namely in the genomes of Firmicutes. Recently, by *in silico* analysis, we identified nine classes of FDPs, from A to I, based on their domain's architecture. One of the most complex arrangements is represented by the class H FDP proteins, having as one of its members a putative enzyme from *Syntrophomonas wolfei subsp*. *wolfei* str. Goettingen G311 that, besides the core domains, also contains three extra C-terminal domains: two short-chain rubredoxins and a NAD(P)H:rubredoxin oxidoreductase-like domain.

Syntrophomonas wolfei is a chemoorganotrophic bacterium isolated from anaerobic environments, metabolically specialized in the β-oxidation of fatty acids in syntrophy with methanogens and other hydrogen/formate-using microorganisms. In this work, we performed the kinetic, biochemical and spectroscopic characterization of the *S. wolfei* class H FDP, aiming to contribute to the understanding of the physiological role of these complex multidomain enzymes in strict anaerobes. *S. wolfei* class H FDP was successfully overexpressed and purified with all the predicted cofactors (diiron site, two-rubredoxins, FMN and FAD). The kinetic characterization, using NADH as electron donor, shows a remarkable O_2 reduction activity with a k_{cat}= 52 ± 1.2 s^{-1} and a K_M=11 μM and an almost negligible NO reducing activity (100 times lower), i.e., it is an oxygen selective FDP. In addition, the H_2O_2 reduction activity has one of the highest turnover values (k_{cat} = 19.12 ± 2.18 s^{-1}) among FDPs. Currently we are completing the spectroscopic and kinetic studies of this FDP, in order to determine the enzymatic mechanism and the function of the extra domains, in order to understand the physiological role of this enzyme in the oxidative stress response mechanisms of the strict anaerobe *Syntrophomonas wolfei*.

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How the Anaerobic Enteropathogen *Clostridium difficile* Tolerates Low O_2 Tensions

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Clostridium difficile is the most prevalent pathogen among all healthcare-associated infections. This anaerobic bacterium can colonize the human gut, typically following agents that disrupt the normal gut microbiota, like antibiotics. In the gut, *C. difficile* is exposed to oxygen, which it must eliminate for survival. Its genome encodes for two flavodiiron proteins (FdpA and FdpF) and two reverse rubrerythrins (revRbr1 and revRbr2) likely involved in O_2 detoxification. Flavodiiron enzymes are constituted by a minimal core of two domains: a metallo-β-lactamase-like one, harboring the catalytic center, followed by a short-chain flavodoxin, as in the case of FdpA. FdpF is more complex with an extra short-type rubredoxin domain followed by an NADH:rubredoxin oxidoreductase-like one. RevRbrs are composed by a C-terminal domain harboring a non-sulfur diiron center and a rubredoxin-like [Fe(S-Cys)4] domain in the N-terminal part. The biochemical studies demonstrated that FdpF and both revRbrs harbor NADH-linked O_2- and H_2O_2-reductase activities *in vitro*, while purified FdpA mainly acts as an O_2-reductase. The reactivity of FdpF towards H_2O_2, with a non-negligible turnover ($2s^{-1}$) is a novelty in the field of Fdps. We showed that the growth of a *fdpA* mutant is affected at 0.4% O_2, while inactivation of both revRbrs leads to a growth defect above 0.1% O_2. O_2- reductase activities of these different proteins are additive since the quadruple mutant displays a stronger phenotype when exposed to low O_2 tensions compared to the triple mutants. Our results demonstrate a key role for revRbrs, FdpA, and FdpF proteins in the ability of *C. difficile* to grow in the presence of physiological O_2 tensions such as those encountered in the colon.

The conserved amino acid motif –GSSYN- is essential for the *E. coli* flavorubredoxin NO reductase its activity

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Flavodiiron proteins (FDPs) are a family of modular and soluble enzymes endowed with nitric oxide and/or oxygen reductase activities, producing N2O or H2O, respectively [1]. The FDP from Escherichia coli, which apart from the two core domains, possesses a rubredoxin-like domain at the C-terminus and therefore named flavorubredoxin, FlRd, is a bona fide NO reductase, exhibiting an O2 reducing activity that is approximately ten times lower than the one for NO [2, 3,4]. Among the flavorubredoxins, there is a strictly conserved amino acids motif, -G[S,T]SYN-, which is located close to the catalytic diiron center. To assess its role in FlRd's activity, we designed several site-directed mutants, replacing the conserved residues by hydrophobic or anionic ones [5].

While maintaining the general characteristics of the wild-type enzyme, including cofactor content (iron and FMN) and the integrity of the diiron center, the mutants revealed a significant decrease in both of their oxygen (up to 60% reduction) and NO reductase activity (up to 99%). The later, its physiological function, was almost completely abolished in some of the mutants. Molecular modelling of the mutant proteins pointed to subtle changes in the predicted structures, that result in the reduction of the hydration of the regions around the conserved residues as well as in the elimination of hydrogen bonds, which may affect proton transfer and/or product release.

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Spectroscopic Analysis of Cluster Degradation of IscR, the Master Regulator of Iron-Sulfur Biogenesis

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IscR, the iron sulfur cluster regulator, is a [2Fe-2S] cluster binding protein belonging to the Rrf2 transcriptional regulator superfamily. Other iron-sulfur cluster binding proteins within this family use their cluster as a sensing element to regulate transcription, with the cluster undergoing modifications under stress conditions[1,2]. In *Escherichia coli*, IscR can differently regulate two operons, the *isc* and *suf* operons, depending on the occupancy of the [2Fe-2S] cluster – the [2Fe-2S] form represses the transcription of the *isc* operon, and the apo form activates the *suf* operon[3]. The cluster of IscR is unusually ligated by three cysteine and one histidine residues, the impact of this ligation on the cluster is unknown but is thought to make the cluster unstable and susceptible to loss from the protein[4]. The exact mechanism of cluster loss under stress conditions is unknown. To better understand the mechanism of [2Fe-2S] cluster loss, the response of [2Fe-2S] IscR from *Yersinina enterocolotica* under different conditions was investigated, including low iron and aerobic stress. Native mass spectrometry and spectroscopy techniques were utilised to characterise the protein and monitor cluster loss over time. The data highlight the importance of the cluster oxidation state for stability of the [2Fe-2S] cluster in response to low iron stress. The effect of small molecular weight thiols on the stability of the [2Fe-2S] cluster of IscR was also investigated. The data reveal a complex picture of cluster stability, and thus regulation of transcription of the iron-sulfur cluster biogenesis machinery, to iron and oxygen levels within the cell.

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Cyanobacterial Aldehyde Deformylating Oxygenase: Biofuel Production

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Biofuels have drawn increasing attention as a renewable alternative energy source to overcome climate change, the depletion of fossil fuels, and promote energy security. Specifically, fatty acid-derived alkanes could be an ideal replacement for fossil-based fuel as a next-generation biofuel, because they are the main constituents of traditional Petro-based fuels (such as gasoline, diesel, and jet fuel), have high energy content, require minimal downstream processing, and have reduced CO_2 emissions. Cyanobacterial aldehyde deformylating oxygenase (ADO) is a metal-dependent enzyme with an a-helical structure that contains di iron at the active site. This enzyme exhibits catalytic activity toward converting C_n aldehyde to C_{n-1} alkane and formate as a co-product by using a cellular reductant like ferredoxin and oxygen molecule. These cyanobacterial enzymes are small and easy to manipulate. Engineering of the metabolic pathway in these enzymes can be done by using computational methods which give a direction to the synthetic chemists for improving ADO enzymatic activity and substrate specificity for better alkane production.

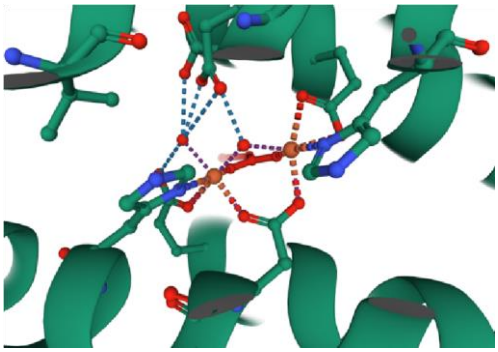

Figure: Active site cavity of ADO with PDB accessions 2OC5

By quantum mechanical study (QM) of the small active site models and QM/MM study considering the entire metalloenzymes to understand the reaction mechanism. For the QM/MM study, classical molecular dynamics can be performed using the Amber20 package followed by DFT calculations. For the DFT optimization method, our standard protocol will be the use of unrestricted hybrid functional like B3LYP along with the LACVP type of basis sets for the metal center as well as the other atoms in the QM region. Single-point energy calculations followed by solvation (SMD model) using a better basis set, i.e., def2-TZVP or def2-TZVPP will be performed for refinement of the electronic energies. Grimme's D3 dispersion correction will be incorporated. Additionally, spectroscopic parameters like EPR, Mossbauer, UV-VIS spectroscopic parameters will be calculated using ORCA with the ZORA-B3LYP/def2-TZVP method. Illuminating the electronic and structural properties, as well as the mechanism of ADO at the molecular level, is helpful in gaining insight into these biologically important systems and the knowledge that can be utilized in the design of new biomimetic hydrocarbon Production models or these systems in the laboratory.

Cu(I)-cysteine cluster formation in human metallothionein 1A

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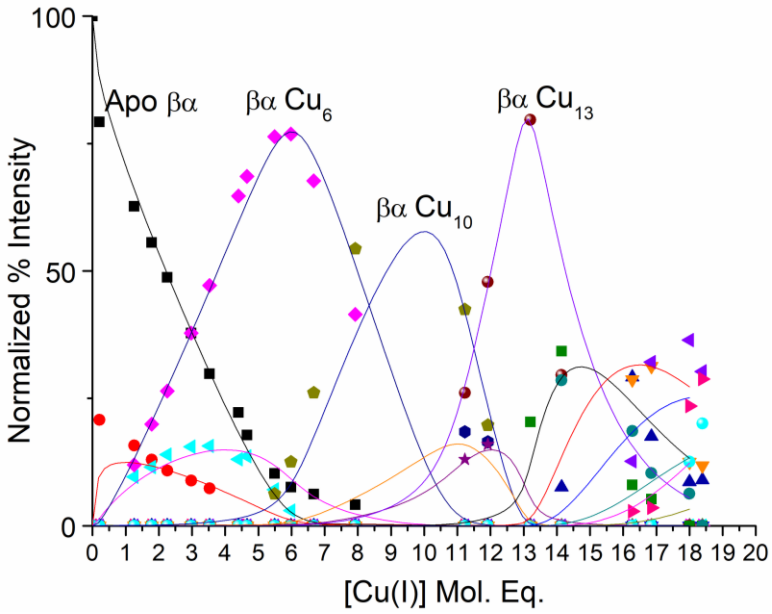
The mammalian protein metallothionein 1A (MT1A) is expressed ubiquitously throughout the body. Unusually, the amino acid sequence is approximately 30% cysteines that all exist as reduced thiols, which are used to bind soft d^{10} metals. While extensive studies of Zn(II) and Cd(II) metallation properties have been reported, much less is known about metallothionein's response to Cu(I). Copper is an essential element and is involved in central nervous function, tissue formation, as well as numerous redox reactions. This redox activity means that copper availability much be tightly controlled to avoid the formation of reactive oxygen species which are detrimental to the cell. Metallothionein provides homeostatic control of both Zn(II) and Cu(I) using its 20 cysteine residues. Native electrospray ionization mass spectrometry (ESI-MS) and emission spectroscopy studies on recombinant human MT1A protein were used to identify the stable Cu(I)-MT clusters that form at physiological pH. N-terminal β and C-terminal α domain fragments of MT1A were used to probe the stoichiometry of the Cu(I) loading in each half of the protein.[1]



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Apo-metallothionein exists primarily as a folded structure under physiological conditions

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Metallothioneins are ubiquitous proteins that regulate essential metal concentrations within the cellular environment. There are 4 isoforms of the protein, all of which perform slightly different functions. Isoforms 1 and 2 are found in all tissues and are induced by increased metal concentrations through metal responsive transcription factor 1 (MTF-1). Isoforms 3 and 4 are localized to specialized tissue and are not metal-inducible. While the metalated structure of metallothionein with cadmium and zinc is well-known from NMR and X-ray diffraction studies, the structure of the metal-free protein is more difficult to determine due to the fluxionality of the peptide[1]. In this study, we investigate the apo-form of isoforms belonging to the induced and non-induced type using electrospray ionization mass spectrometry, ion mobility mass spectrometry, and stopped-flow kinetics. Using these methods, it is clear that the apo-metallothioneins have buried cysteines under physiological conditions but these switch to a completely unfolded conformation with the lowering of pH or increase in denaturant.

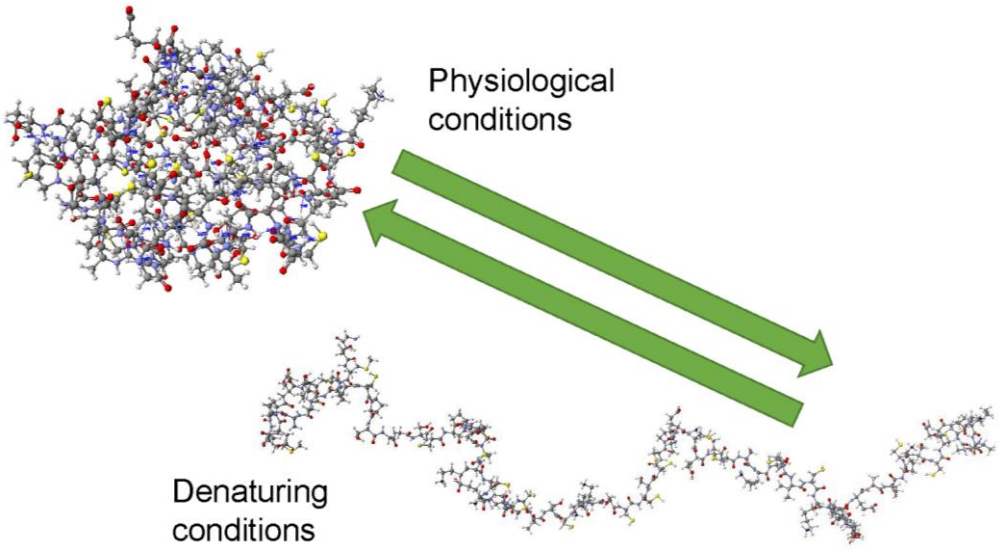

Figure 1. The folded structure of metallothionein under physiological conditions can be unfolded under denaturing or acidic conditions.

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Interaction of Cytochrome c Oxidase with Complex 1 and Cyanide

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Cyanide can be produced by different endogenous metabolisms, therefore human blood contains it in low concentrations [1]. The major route of cyanide detoxification is by its conversion to non-toxic thiocyanate. Rhodanese is the main enzyme responsible for this detoxification process [2]. While low concentrations of toxic cyanide should not be a burden for human organs, complications may occur when cyanide concentrations are increased, furthermore it may increase to lethal concentrations. As many plants, seeds and smoke contain cyanide, poisoning can often occur accidentally. Cyanide is capable of inhibiting the activity of many metalloenzymes, including hemoglobin and cytochrome *c* oxidase (CcO). Inhibition of these enzymes can lead to life-threatening conditions [2].

The sulfur donated by oxothiomolybdate, "$Mo_2O_2(\mu\text{-}S)_2(S_2)$", complexes, reacts with cyanide, forming non-toxic thiocyanate. Complex **1**, bearing a threonine and a terminal disulfide ligand, models the reactivity of the rhodanese enzyme and can "mimic" the activity of the endogenous cyanide detoxification pathway [3]. Following the isolation of CcO [4], "*in vitro"* studies with complex **1** and cyanide inhibition were performed. Both preventive and reactivation studies were performed and reactions of complex **1** as a catalytic detoxification agent were run. The results further illustrate the use of complex **1** as cyanide antidote.

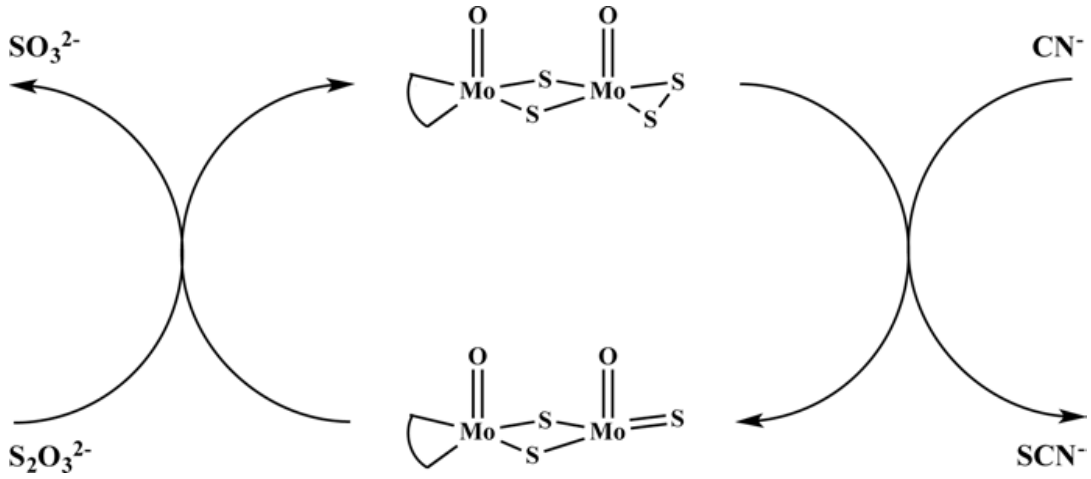

Figure 1: Catalytic cycle of molybdenum complex and cyanide [2]

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Thermodynamically Favourable States in the Reaction of Nitrogenase without Dissociation of any Sulfide Ligand

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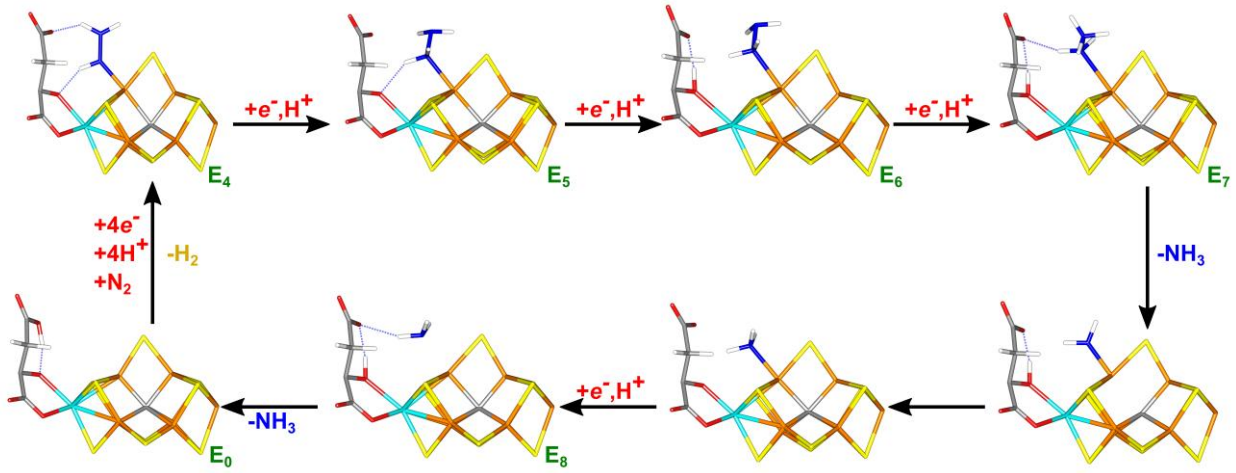
We have used combined quantum mechanical and molecular mechanical (QM/MM) calculations to study the reaction mechanism of nitrogenase, assuming that none of the sulfide ligands dissociate[1]. To avoid the problem that there is no consensus regarding the structure and protonation of the E_4 state, we start from a state where N_2 is bound to the cluster and is protonated to N_2H_2, after dissociation of H_2. We show that the reaction follows an alternating mechanism with HNNH (possibly protonated to $HNNH_2$) and H_2NNH_2 as intermediates and the two NH_3 products dissociate at the E_7 and E_8 levels. For all intermediates, coordination to Fe6 is preferred, but for the E_4 and E_8 intermediates, binding to Fe2 is competitive. For the E_4, E_5 and E_7 intermediates we find that the substrate may abstract a proton from the hydroxy group of the homocitrate ligand of the FeMo cluster, thereby forming $HNNH_2$, H_2NNH_2 and NH_3 intermediates. This may explain why homocitrate is a mandatory component of nitrogenase. All steps in the suggested reaction mechanism are thermodynamically favourable compared to protonation of the nearby His-195 group and in all cases, protonation of the NE2 atom of the latter group is preferred.



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Interaction of a Cys2His2 zinc finger protein with toxic metal ions

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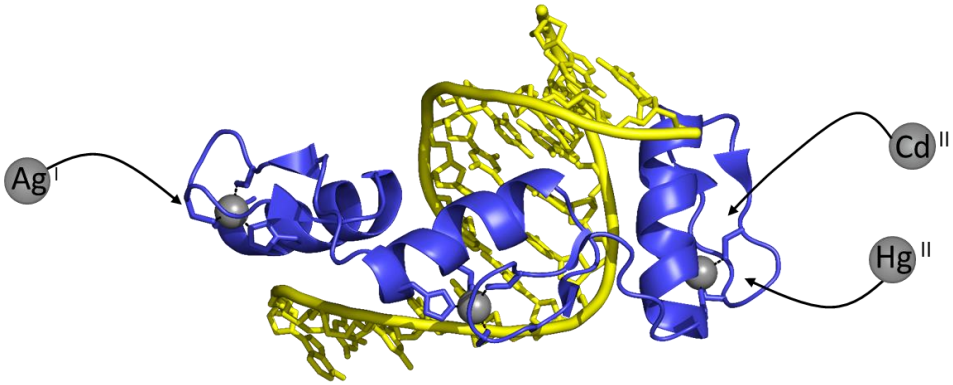
Zinc finger proteins are modular specific DNA binders that can be altered to recognize almost any selected DNA sequence[1]. In nature, they function as transcription activators, but they can be turned into specific DNA manipulating agents by attaching an otherwise nonspecific nuclease domain to a zinc finger array[2]. Zinc fingers can only function properly as specific DNA-binders, if the central Zn(II)-ion is coordinated. Therefore, it is essential to determine the zinc-binding stability of the protein. Competition reactions may also occur inside living organisms, demolishing the specific DNA binding ability, or may even cause DNA degradation. Especially toxic metal ions with soft characteristics (cadmium(II), mercury(II) and as we recently published, silver(I)) are supposed to compete with zinc(II)[3].



UV-Vis absorbance, CD spectroscopy, ESI mass spectrometry, isothermal titration calorimetry (ITC), platereader fluorimetry and electrophoretic gel-mobility shift measurements were performed to determine Zn(II)-binding of a designed zinc finger protein[4], as well as, competition with Ag(I), Cd(II) and Hg(II) in the presence or absence of DNA.

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Computational studies on the reaction mechanism of lignin biodegradation by cytochrome P450 enzymes

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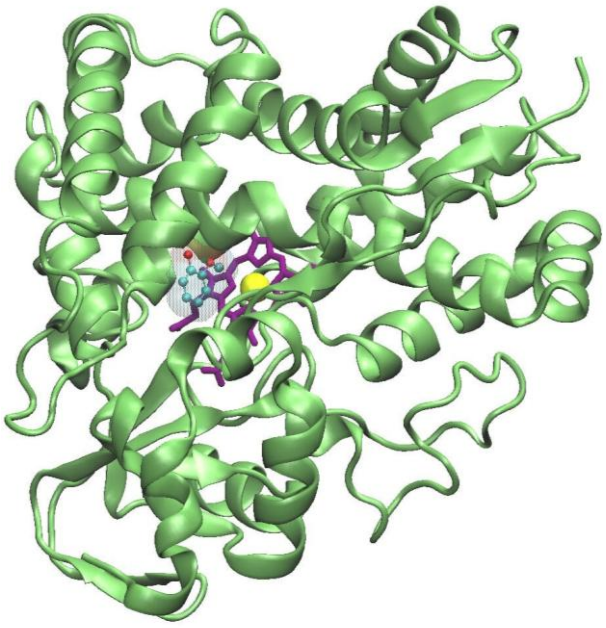
Lignin is a highly heterogeneous biopolymer present in secondary plant cell walls. Given its abundance (the second most abundant biopolymer on Earth), lignin is the major source of aromatic carbon on the planet, which highlights the opportunity of harnessing this renewable source for valuable chemicals. Although diverse and complex, the lignin structure can be depolymerized into simple and small aromatic constituents such as catechol. Since the catechol scaffold allows for chemical functionalization, this lignin monomer can be easily converted into high-value compounds – ranging from alternatives to petroleum-based and oil-based chemicals to pharmaceutical molecules – in many industrial settings, be it in the renewable synthesis of polymers or in the field of natural products. However, currently the vast amounts of lignin generated from agriculture and industry are simply wasted, due to the lack of strategies for its valorization. We, therefore, have done a computational study into the mechanisms of utilizing lignin fragments by the P450s and converting them into valuable compounds. In particular, we have done a combination of molecular dynamics and density functional theory studies on the mechanisms of products formation of lignin constituents by cytochrome P450 GcoA and engineered variants. In particular, we report the mechanisms of activation of 4-methoxybenzoate by P450 enzymes and the various possible products that can be obtained through *O*-demethylation and aromatic hydroxylation pathways. The work shows that O-demethylation is the dominant pathway due to an interaction of the carboxylate group of the substrate with an active site Arg residue that positions the substrate tightly. However, when we create an alternative variant with the position of the Arg residue shifted inside the active site, we can direct the



A P450 cytochrome (PDB ID 6YCJ) – the heme center is shown in purple with its iron core in yellow and a lignin subcomponent in the active site.

mechanism to aromatic hydroxylation instead. Our work gives insight into engineering of P450 isozymes for selective substrate activation.

Expanding enzymes' abilities with redox-dismantlable synthetic organometallic catalysts

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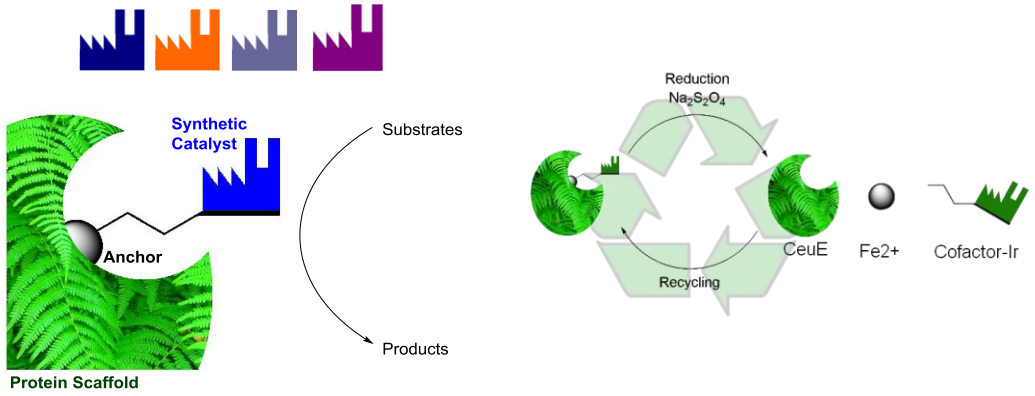
Being able to do chemistry in a more environment-friendly fashion is one of the main challenges every chemist is facing, and one of the best and the most evident way to do so is to use Nature's tools: enzymes. Enzymes are amongst the most selective and efficient catalysts we know, being able to transform large quantities of chemicals, with high TurnOver Numbers (TON) and with surgical precision. However, the high selectivity of these enzymes can also be a major disadvantage, as they are usually limited to a limited number of substrates and reactions, and their large size makes them difficult to modify. To broaden their range of action whilst taking advantage of their numerous qualities, Artificial Metalloenzymes were developed, merging natural enzymes with synthetic organometallic catalysts to obtain chimeric catalysts, able to perform unknown-to-nature reactions with the precision and the TONs of natural enzymes.[1] The work I will be presenting is about building such hybrid catalysts from a protein (CeuE) and various metallic catalysts, held together by an iron based releasable linker.[2] Such assembly allows the full electrochemical dismantling, recycling and repurposing of every part of the ArM, and could be used as a versatile platform to perform diverse chemical transformations in a multipurpose flow reactor, such as imine and ketone reductions, Diels Alder reaction and Grubbs metathesis.



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Characterization of the interaction between plutonium and calmodulin variants by spectroscopic techniques

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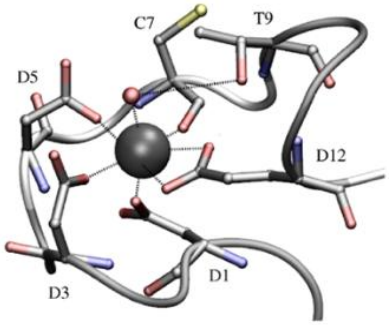
Pu is an actinide of major societal relevance due to its large stock worldwide and its key role in the cleanup challenges of legacy nuclear sites. Although its physiological impact has been widely investigated, understanding its interactions with biological molecules remains limited. The knowledge relative to actinide transportation mechanism, and in particular, the direct interaction of Pu at the molecular scale with proteins is still unclear. Recent publications have pointed that Pu interaction with transferrin or ferritin[1], an iron carrier of blood, could be at the origin of its internalization in cells[2] and that calcium-binding proteins are possible Pu targets (because as Pu, Ca is hard cation – strong Lewis acid)[3].



This work aims at investigating the interaction between actinide elements, and more specifically plutonium, and biological molecules.[4-6] Calcium-binding EF-hand protein motifs of the calmodulin N-terminal domain, which contains seven coordination sites were chosen for this study. Calmodulin is an important protein expressed in all eukaryotic cells and is involved in a large number of signal transduction pathways.

In vivo, plutonium is mainly present at oxidation state +IV, and at this oxidation state, plutonium is a very high hydrolyses propensity. In this work, we will focus on the interaction of the plutonium (IV) with two variants of the calmodulin. CaM-WT, which is the wild type calmodulin, and CaME variant in which one threonine is replaced by one glutamate, thus increasing the number of hard donor carboxylate ligands in the binding site. Different routes taking into account the constraints due to the hydrolysis of plutonium (IV) at physiological pH have been used and will be discussed.

The interactions between Pu and calmodulin ligand were then characterized using visible and X-ray absorption spectroscopies and ESI-MS and showed the formation of different type of complexes for CaM-E and CaM-WT.

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Kinetics of oxidation induced cadmium and zinc release from human metallothionein 1a

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Protein oxidation and the physiological defense mechanisms against oxidative stress are linked to aging, Alzheimer's disease, cancer, diabetes, and numerous cardiovascular diseases. The cell has many natural enzymatic and non-enzymatic antioxidants that protect against oxidative stress, including the cysteine-rich protein metallothionein (MT). Apo-MT, an intrinsically disordered protein, undergoes metal-induced folding upon binding up to 7 divalent metals (typically Zn^{2+} or Cd^{2+}) in 2 metal-thiolate clustered sites. Oxidation of MT's thiols forms disulfide bonds, resulting in the release of some of the bound metals, which has negative physiological consequences. The stepwise pathway for this metal release and its dependence on the various metal cluster structures has not yet been reported.

Using electrospray ionization mass spectrometry, we measured the effect of 3% H_2O_2 on the Cd_nMT and Zn_nMT clusters at physiological pH (7.4). The kinetics of displacement of Cd^{2+} and Zn^{2+} from MT due to oxidation of the cysteine residues upon addition of H_2O_2 into Cd_7MT and Zn_7MT is reported. Throughout the reaction, Cd^{2+} and Zn^{2+} were displaced in a non-cooperative manner to Cd_4MT or Zn_4MT and the remaining 4 metals were displaced semi-cooperatively. The H_2O_2 displaced Cd^{2+} and Zn^{2+}, inhibiting the metal-binding function of MT through oxidation. Our findings also suggest that each metal in the Cd_4 and Zn_4 clusters are vital for integrity of the structure. This provides insight into how MT behaves in the presence of reactive oxygen species.

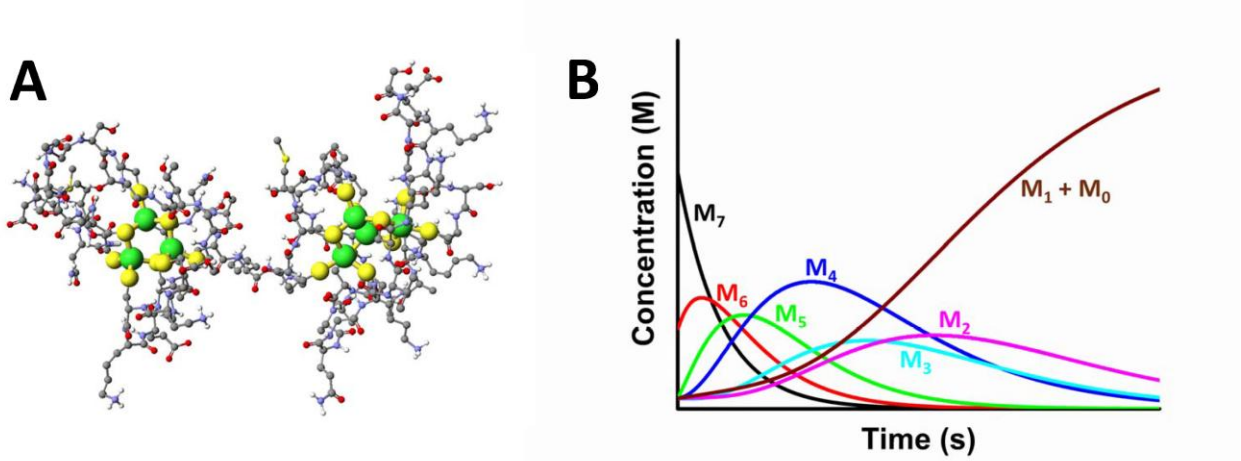

Figure 1. (A) A molecular dynamics simulation of $M(II)_7MT$ showing the 2-domain cluster structure. M(II) (green) and cysteine thiols (yellow) have been enlarged for visual clarity. (B) A graph showing the general trend of metal species concentration over time. The trend shows a weak cooperativity to the M_4 species.

In vitro shuttling of the $Fe(CN)_2(CO)$ moiety of [NiFe]-hydrogenase

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[NiFe]-hydrogenases are bipartite metalloenzymes that catalyse the reversible cleavage of molecular hydrogen into two protons and two electrons, which takes place at the inorganic $NiFe(CN)_2(CO)$ centre. Biosynthesis of the bimetallic active site requires at least six auxiliary proteins called HypA, B, C, D, E, and F. The HypCD complex harbouring a [4Fe-4S] cluster plays a pivotal role in the synthesis and delivery of the $Fe(CN)_2(CO)$ unit of the cofactor[1,2]. Exploring the function of the HypCD scaffold complex as well as the transfer of the $Fe(CN)_2(CO)$ moiety to the apo form of the hydrogenase large subunit remains a central task in elucidating the assembly process of [NiFe]-hydrogenases.

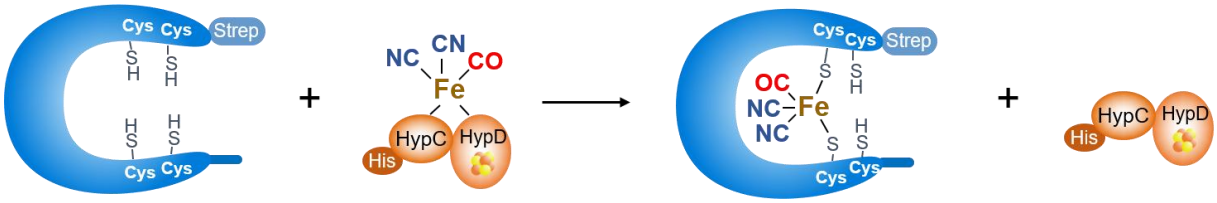

Fig. 1. Transfer of the $Fe(CN)_2(CO)$ moiety from HypCD to the hydrogenase large subunit.

Here, we investigated the transfer of the $Fe(CN)_2(CO)$ moiety from the HypCD complex isolated from *E. coli* Rosetta to apo-HoxC, the large subunit of the regulatory hydrogenase (RH) from the Knallgas bacterium *Cupriavidus necator* (formerly *Ralstonia eutropha*) (Fig. 1).

Molecular biological and biochemical in combination with infrared spectroscopy provided evidence that the isolated, cofactor-loaded HypCD complex transferred the $Fe(CN)_2(CO)$ unit to purified apo- HoxC *in vitro*. Interestingly, ATP seems to have a stimulating effect on the transfer, which is consistent with a previous study demonstrating ATPase activity of the HypCD complex[3]. The exact function of ATP in this process requires further analysis. The purified HoxC protein containing the $Fe(CN)_2(CO)$ moiety of the active site can be used to insert the nickel to obtain a catalytically competent subunit for the complete *in vitro* assembly of the regulatory hydrogenase[4].

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Insights into the multidomain Flavodiiron Protein from *Firmicutes Bacterium CAG:103*

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Flavodiiron Proteins (FDPs) are key enzymes for the detoxification of nitric oxide and /or oxygen to the innocuous N_2O and H_2O, respectively. This family of proteins has a modular nature, with a main common core constituted by a metallo-β-lactamase-like domain that harbors the catalytic diiron center, followed by a flavodoxin domain, with a flavin mononucleotide. Several FDP classes were identified *in* silico, on the basis of extra domains at the C-terminal part of the protein (thus far up to seven putative extra domains were identified) [1,2].

Here, we present the biochemical characterization of a highly complex FDP from *Firmicutes Bacterium CAG:103* (a human pathogen), identified as a Class G member, based on its domain's architecture. Besides the core domain, this enzyme was predicted to have an extra flavin reductase-like domain, followed by two short-space rubredoxin-like domains [2]. The protein and several truncated domains were successfully expressed in *E. coli* and purified to homogeneity. The predicted domains and respective redox centers were confirmed by spectroscopic analysis (UV-Visible and EPR).

The enzyme showed a preferential activity using oxygen as a substrate, with turnover rates of 8.0±0.6 s^{-1} and 12.5 ± 1.0 s^{-1} using NADH or NADPH as electron donors, respectively. These results indicate that NADPH may be the preferential electron donor. This FDP also uses hydrogen peroxide as a substrate but at lower rates (turnover values were 0.98±0.10 s^{-1} and 0.75 ±0.17s^{-1} for NADH and NADPH, respectively) when compared with oxygen for the same conditions.

The truncated version of the protein, without the rubredoxin domains, shows almost a complete abolishment of the oxygen consumption activity. This led us to consider the importance of rubredoxins for the reduction of oxygen, acting as electron donors to the catalytic domains: the data obtained indicates that the flavin reductase-like domain is the possible entrance of electrons from the electron donor. From there, electrons may flow through the two rubredoxin domains into the flavodoxin domain and finally to the diiron center where the reduction of oxygen to water occurs

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MsrQ, the membrane bound flavocytochrome component of the methionine sulfoxide reductase system MsrPQ

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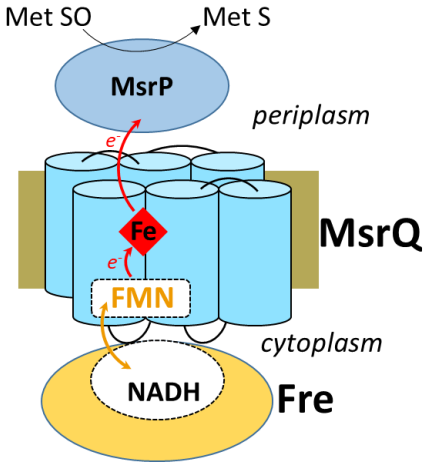
MsrPQ is a new type of methionine sulfoxide reductase (Msr) system present in bacteria. It is specifically involved in the repair of the periplasmic methionine residues that are oxidized by hypochlorous acid.[1] MsrP is a periplasmic molybdo-enzyme that carries out the Msr activity,[1] whereas MsrQ, an integral membrane bound hemoprotein, acts as the specific physiological partner of MsrP to provide electrons for catalysis.[1,2] MsrQ (previously called YedZ) belongs to the FRD protein superfamily, including the eukaryotic NADPH oxidases and STEAP proteins. Here, we have investigated the cofactors content of the *E.coli* MsrQ and its mechanism of reduction by the flavin reductase Fre.[3] We have shown by EPR spectroscopy that MsrQ contains a single HALS (highly-anisotropic low-spin) b-type heme, located on the periplasmic side of the membrane. MsrQ also holds a FMN cofactor that occupies the site where a second heme binds in other members of the FDR superfamily, on the cytosolic side of the membrane. The cytosolic flavin reductase Fre was previously reported to reduce the MsrQ heme.[2] Here we have demonstrated that Fre uses the FMN MsrQ cofactor as a substrate to catalyze the electron transfer from cytosolic NADH to the heme. Formation of a specific complex between MsrQ and Fre could favor this unprecedented reduction mechanism, potentially involving a transfer of the reduced FMN cofactor from the Fre active site to MsrQ.[3]



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On the kinetic mechanism of CO_2 reduction by Ni,Fe-CODHases

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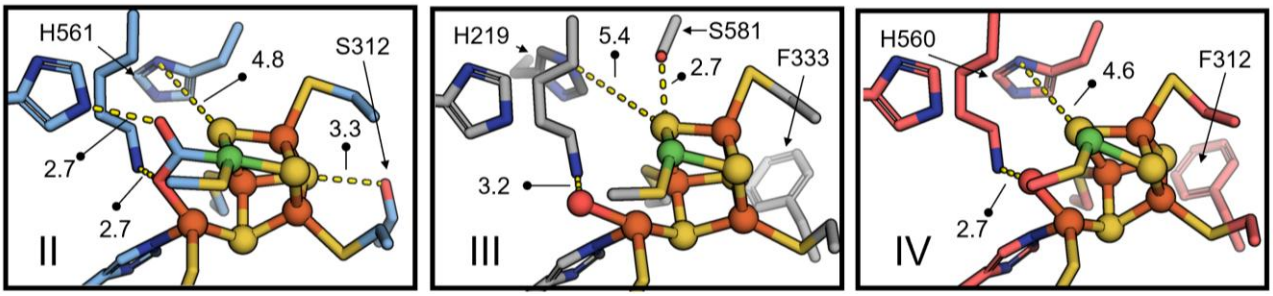

Ni,Fe-CO-dehydrogenases (CODHases) catalyze the reversible reduction of CO_2 to CO making them valuable catalysts for the generation of renewable fuels. The individual states of the active site cluster C during catalysis have been extensively studied by spectroscopic and crystallographic methods, but less so by enzyme kinetics. Here, we report the kinetic characterization of three CODHases with distinct physiological roles with two reductants and at different temperatures. For all CODHases a two-site ping-pong mechanism fits to the data for both reductants. However, the possibility of alternative mechanisms cannot be eliminated by the data. The kinetic characterization provided insights into the rate-determining steps in catalysis and allowed modeling the influence of the reduction potential of the electron transferring clusters on catalysis. Furthermore, we investigated potential clues for the different activities of the CODHases and found a point-mutation that doubled the activity of the CODH-III/ACS complex. However, the main reason for the different activities seems to be the architecture of the substrate tunnels. These findings open up new perspectives for understanding and engineering efficient CO_2 reduction catalysts.

Vibrational spectroscopic studies of an [Fe8S9]-cluster containing reductase and its ATP-dependent redox partner.

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Demanding reductive reactions in biology typically depend on ATPases harboring FeS-cluster/s. These enzymes usually constitute a two-component system where ATP hydrolysis in the donor protein is coupled to a redox reaction, providing the energy an electron needs to reduce the metal site on the acceptor protein that catalyzes the reduction.

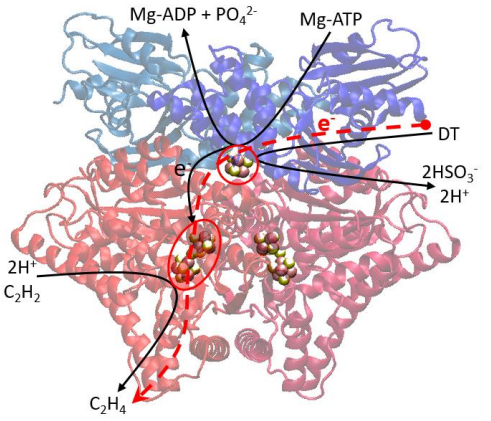

Figure 1. Crystal structure of DCCP complex and schematics of the catalytic process where ATP hydrolysis is coupled to DT oxidation and C2H2 reduction.

Herein, we present an FTIR and resonant Raman spectroscopic characterization of such a two-component system bearing a $[Fe_8S_9]$-cluster $[\{Fe_4S_4(S_{Cys})_3\}_2(\mu_2\text{-}S)]$ as active site.[1] This recently discovered double cubane cluster (DCC) is the first example in nature so far. The DCC-containing protein (DCCP) forms a complex with its specific reductase (DCCP-R) that is capable of performing efficient two-electron chemistry at very low redox potentials. Thereby, acetylene (C_2H_2) and other small molecules as azide (N_3^-) or hydrazine (N_2H_4) are reduced.

Frozen solution samples and crystals of DCCP, DCCP-R and the entire complex were characterized by resonant Raman spectroscopy after different redox treatments. Supporting calculations allowed the identification a vibrational mode involving a single sulfur bridging both cubanes, which is suggested to be the key atom for the reduction of the small molecule.

Scaffold rearrangements involved in the catalytic mechanism were studied by transmission FTIR in a thin layer cell employing caged nucleotides. Difference spectra upon ATP release revealed a well-defined band pattern in the amide I region that was tentatively assigned to specific secondary structure elements and amino acid residues. Notably, ATP binding (and not hydrolysis) triggers the conformational change and time resolved measurements suggest that this might be the gating step for the entire catalytic process.

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This ongoing vibrational spectroscopic study of the DCCP/DCCP-R complex sheds light on its catalytic mechanism and broadens our understanding of the specifics of a new Fe/S-cluster.

Structural and functional characterization of proteins involved in Iron-Sulfur cluster biogenesis in *Mycobacterium tuberculosis*: towards the discovery of antibacterial drugs ?

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Iron-sulfur (Fe-S) clusters are inorganic cofactors that are essential in various biological processes (e.g., DNA repair, respiration, photosynthesis, cofactor biosynthesis)[1]. They exist in different forms and oxidation states, the most common types being [4Fe-4S] and [2Fe-2S] clusters. In bacteria, two major pathways for Fe-S cluster biogenesis and delivery have been identified: the Isc and the Suf systems. In Escherichia *coli*, the Isc system is the housekeeping pathway for Fe-S biogenesis, whereas the Suf system is active under environmental stress and iron limitation[2]. Interestingly, *Mycobacterium tuberculosis* (*Mtb*), the causative agent of Tuberculosis (TB), contains only the Suf system as Fe-S assembly machinery[3]. Suf system is essential for in vitro growth of *Mtb* under normal conditions[4,5] and recently proved to be a point of vulnerability in *Mtb*[6]. Moreover, the Suf components of *Mtb* are induced during iron starvation, a process experienced by the pathogen in host tissues, indicating that Fe-S assembly and therefore Fe-S metabolism may be important in the establishment of latent infection[7]. Similarly, the *Mtb* Suf system is up-regulated under nitrosative and oxidative conditions, stressors of the innate immune response[8]. Therefore, there is mounting evidence that Suf sytem is essential for *Mtb* pathogenicity and targeting it might open novel avenues for the development of novel anti-TB drugs through disturbing the pathogen's Fe-S metabolism. A requisite of that consists in characterizing *Mtb* Suf proteins.

Structural and functional characterization of the Suf proteins from Mtb is currently unknown and constitutes the main goal of our research. We will present the first characterization of two proteins of the Suf system from *Mtb*.

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Iron Oxidation and Detoxification in Human Mitochondrial Ferritin

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Iron is required in many essential biological processes such as DNA synthesis and respiration, which is complicated by the bioavailability of iron and toxicity of free iron through the Haber-Weiss reaction. Part of nature's solution to this are the ferritins, a family of iron storage proteins utilising large inner cavities to provide a safe repository for and source of bioavailable iron[1]. The 24-mer structure of human cytosolic ferritins consist of a heteropolymer containing two subunit types, referred to as H- (heavy) and L- (light) chains. The two types of subunits play different functional roles, with H chain containing diiron catalytic active sites, called ferroxidase centres, which oxidise ferrous iron. Following this ferric iron is mineralised, leading to the formation of the iron oxyhydroxide mineral core in the hollow centre of the protein. The L-chain subunit has been shown to have a key role in the initiation of mineralisation[2].

Human mitochondrial ferritin (FtMt) was discovered less than 20 years ago, and is expressed only in highly metabolically active tissues, where growing evidence indicates it functions to protect against oxidative stress[3]. The protein is produced as a precursor of 242 amino acid residues, including a leader sequence that facilitates import into mitochondria where the polypeptide is cleaved to generate the 22 kDa mature homopolymer protein. The protective function of mitochondrial ferritin is clearly associated with its interaction with iron, and key to this is the diiron ferroxidase centre within each subunit. However, information about this, including both Fe^{2+} uptake and oxidation, is lagging behind knowledge of its cellular role. Here, we describe our recent studies of mitochondrial ferritin and site-directed variants using X-ray crystallography, rapid reaction kinetics, and spectroscopy, which provide provides novel insight into the mechanism of iron detoxification.

This work is funded by the UK's BBSRC, grant BB/R002363/1.

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Spectroscopic characterization of lanthanide methanol dehydrogenases cofactor

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Lanthanides (Ln) are fascinating for several reasons. First, they are mainly found in the +III oxidation state and their ionic radii are in a restricted range (for CN = 8, 0.97-1.16 Å),[1] which gives them similar chemical properties. Second, Ln(III) have different physical properties (luminescence, magnetism), making them useful tools to study metal-peptide interactions. Finally, lanthanides have recently joined the family of elements essential to living organisms with the discovery of methylotrophic bacteria capable of utilizing them.[2,3] These bacteria have a marked preference for the lighter Ln(III) (La to Nd), and cannot grow with heavier Ln(III) (Tb to Lu).

The first Ln-enzyme, a methanol dehydrogenase (MDH), has been identified in these organisms. X-ray data show that MDH contain, embedded in a hydrophobic pocket, a pyrroloquinoline quinone (PQQ) responsible for the oxidation of methanol, and a metal cation bound to the PQQ (Figure 1).[4] The latter can be a Ca(II) (Ca-MDH) or Ln(III) (Ln-MDH) ion. Recent studies demonstrated that the most active enzymes are those containing lighter Ln(III),[5] and provided a first insight into coordination of Ln(III) to PQQ.[6]

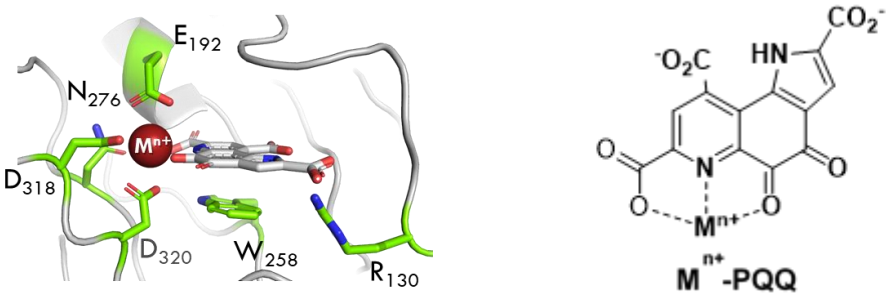

Figure 1 – *Left:* Active site of lanthanide methanol dehydrogenases; *Right:* structure of the metal-pyrroloquinoline quinone complex[4]

In this work, the spectroscopic properties of Ln(III)-PQQ complex were determined by UV-vis and luminescence spectroscopies for a series of Ln(III) (Ln = Eu, Gd, Tb).

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The secret life of phytoglobins: insights into class 1 and 3 hemoglobins from *Arabidopsis thaliana*

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Ever since their discovery in the late 1930s, phytoglobins have never stopped to amaze with their role diversity and the surprising functional networks they are part of, from the relationship between leghemoglobin and nitrogenase to plant embryogenesis control[1]. Even if angiosperm globins are the best studied[2], non-symbiotic (class 1 and class 2) and truncated (class 3) representatives still seem to hold many secrets in terms of mechanistic behaviour and physiological relevance.

In this work, some peculiar observations on class 1 and class 3 phytoglobins from *Arabidopsis thaliana*[3] are unveiled by a variety of theoretical and experimental approaches, starting from the very core of electronic make-up of a truncated plant hemoglobin, going at speed to UV-vis and electron paramagnetic resonance spectroscopy studies in the presence of small ligands, finishing at experiments on transgenic lines of plants, explored with the aid of some bioanalytical techniques. What can the architecture of a globin binding pocket teach us about tuning the spin-orbit coupling? Why do phytoglobins sometimes enjoy hydrogen peroxide? And, most intriguingly, what is the link between a well-behaved non-symbiotic hemoglobin and a two-faced chalcogen? These are just mere snapshots from the secret life of non-symbiotic and truncated phytoglobins, which awaits to be fully understood.

Acknowledgements. This work was partly supported by two grants of the Romanian Ministry of Research, Innovation and Digitization, CNCS/CCCDI—UEFISCDI, project number TE-2019-1396 within PNCDI III and project number PN-III-P2-2.1-PED-2019-2293.

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Fully Synthetic Iron(IV)-oxido Complex Achieves TET Reactivity

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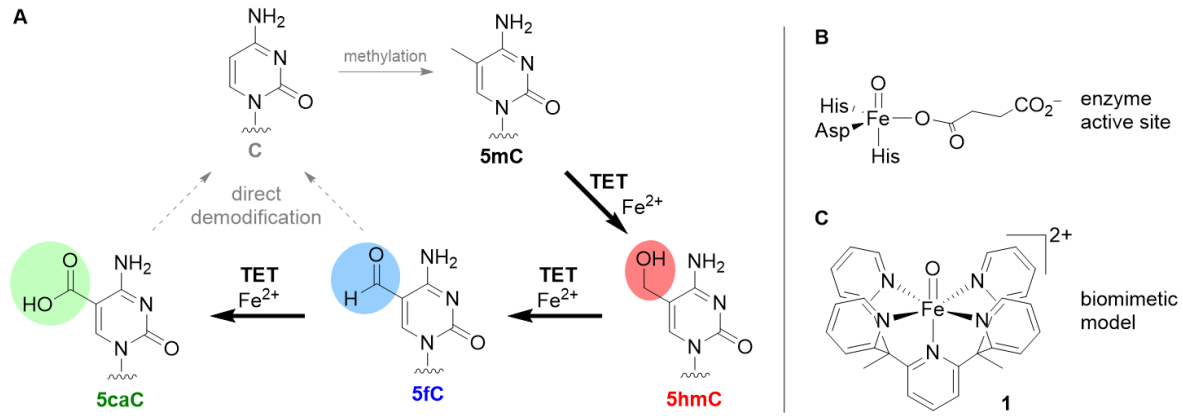
Ten-eleven translocation (TET) 5-methyl cytosine (**5mC**) dioxygenase enzymes are part of the iron(II)/α-ketoglutarate enzyme superfamily and play a significant role in molecular epigenetics by oxidizing (**A**) the epigenetic marker **5mC** sequentially to 5-hydroxymethyl cytosine (**5hmC**), 5-formylcytosine (**5fC**), and 5-carboxycytosine (**5caC**).[1,2] Understanding the mechanism of TET enzymes has been a challenge for (bio)chemists for years, as the bond dissociation energies (BDEs) of the substrates do not correlate with the observed k_{cat} values.[2] Furthermore, achieving the transformation from **5mC** to **5hmC**, **5fC**, and **5caC** by synthetic means is highly desirable for epigenetic sequencing applications as the current gold standard, oxidative bisulfite sequencing,[3] is damaging to the DNA sample and inherently incapable of discriminating between **5mC**/**5hmC** as well as **C**/**5fC**/**5caC**.[4,5]



We found that the fully synthetic iron(IV)-oxido complex $[Fe^{IV}(O)(Py_5Me_2H)]^{2+}$ (**C**, **1**) is capable of mimicking this reaction on a nucleobase,[6] nucleoside, and even oligonucleotide[7] level: in reactions of **1** and a **5mC**-containing substrate we identified all of the expected TET-like products using GC-MS, HPLC, or triple-quad MS. On the nucleobase level no side reactions occur, whereas oxidation of the C1' carbon atom at the deoxyribose unit in nucleosides and oligonucleotides was observed in small amounts. We did not find any evidence for the formation of 8-oxo-guanine, a common oxidative DNA lesion, neither were any internal strand breaks detected when oligonucleotides were used as substrates.[6]

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Engineering [FeFe] hydrogenases: Step-wise Truncation of the Iron-Sulfur Cluster Motifs in the F-domain

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[FeFe] hydrogenases are the most active H_2-converting catalysts in nature, requiring essentially no overpotential (i.e. they operate very close to the thermodynamic $2H^+/H_2$ potential).[1] As such, they are a source of inspiration to design and develop synthetic catalysts. Within the [FeFe] hydrogenase family, the M3 type possess an F-domain containing four accessory iron−sulfur (FeS) clusters, two all Cys-ligated [4Fe−4S] clusters, one His-ligated [4Fe−4S] cluster, and one [2Fe−2S] cluster, in addition to the H-domain that harbors the active site H-cluster.[2] The clusters in the F-domain (F-clusters) act as an electron shuttle between the buried active site and the protein surface. The complexity of these enzymes has limited their use in spectroscopic investigations, however, they are particularly interesting because of their catalytic bias toward H_2 production and their lower sensitivity to O_2 compared to other [FeFe] hydrogenases'. In this work, the M3 type [FeFe] hydrogenase from *Clostridium acetobutilicum* (*Ca*HydA) was produced in *E. coli* as an apo-enzyme (lacking part of the active site H-cluster). This system allows the preparation of semi-semisynthetic enzymes with modified H-clusters, as well as the preparation of truncated variants lacking FeS motifs from the F-cluster domain, both of which can facilitate investigation of the electron relay clusters. Functional and spectroscopic characterisation of semi-synthetic enzymes showed clear long-range effects of the FeS cluster relay, as all the truncated variants yielded very different spectroscopic properties of the H-cluster and O_2 sensitive compared with the wild-type (WT) enzyme, without their catalytic behavior being particularly perturbed. Overall, this work provides important insights into the catalytic mechanism of [FeFe] hydrogenases, and shows how they can be engineered to produce semi-synthetic systems with modified electron relays.

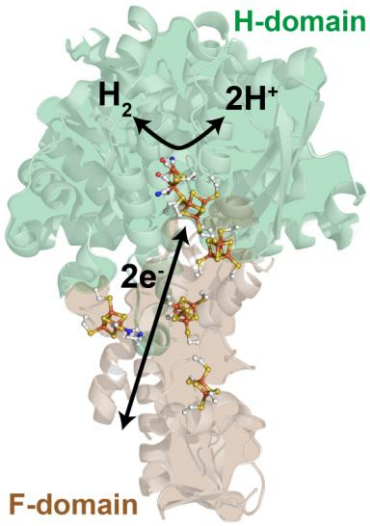

Fig. 1: Structure of the M3 type *C. pasteurianum* HydA1 hydrogenase (PDB ID 4XDC), which is predicted to be similar to *Ca*HydA.

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X-ray free-electron laser (XFEL) crystallographic study of E. coli NiFe hydrogenase under electrochemical control

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Nickel iron hydrogenase 1 (Hyd1) from Escherichia coli is a well-known example for the class of "O2-tolerant" NiFe hydrogenases, which catalyse the interconversion of hydrogen gas and water. We describe the generation of slurries of microcrystals for Hyd1 for X-ray free-electron laser (XFEL) crystallography at different redox levels of the [NiFe] active site. Unlike conventional synchrotron X-ray diffraction, instead of exposing the crystal sample to a continuous X-ray beam, the XFEL source provides very short and intense flashes of X-rays. These allow radiation damage-free structures whereas continuous illumination of a crystalline sample can generate photoelectrons causing reduction of the metal centres. Importantly, XFEL work also opens up opportunities for time-resolved crystallography. We have previously demonstrated electrochemical control over protein single crystals, coupled with infrared (IR) microspectroscopy for evaluating the redox state of hydrogenase active site.[1,2] IR signals for the microslurries of crystals are detectable, making it possible to confirm the IR signature, providing that the crystals are sufficiently concentrated. Here we combine xfel data collection with the electrochemical control over redox state for hydrogenase crystals.

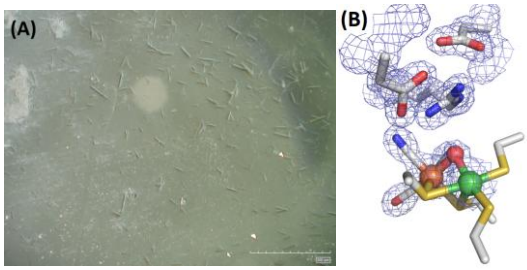

Fig. 1. Microcrystals of E. coli Hyd1 were prepared and manipulated under electrochemical control for XFEL structural measurements on defined redox states.

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LmrR: A hydrophobic protein pore for natural and non-natural metal complexes

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The multidrug resistance regulator LmrR is a PadR-related transcriptional repressor from *Lactococcus lactis*. In the center of its homo-dimeric interface, which is formed by four α-helices, is a large hydrophobic pore (Figure 1, left). This pore has been shown to be a privileged scaffold for the construction of artificial metalloenzymes (ArMs), which are rationally designed hybrids consisting of proteins and catalytically active transition-metal complexes.[1] Initially, copper-based Lewis acid catalysis has been demonstrated. More recently, the catalytic repertoire has been expanded towards organocatalytic reactions.[2]

The construction of artificial metalloenzymes is a promising strategy to merge chemo- and biocatalysis for new-to-nature reactions.[3] Therefore, metal ions are manipulated in a biological context achieving remarkable rate accelerations and selectivities. Here, we present our recent advances in chiral carbon-carbon bond formations through carbene transfer reactions catalyzed by FePPIX or CoPPIX complexes incorporated into LmrR and reactions catalyzed by platinum group metals.

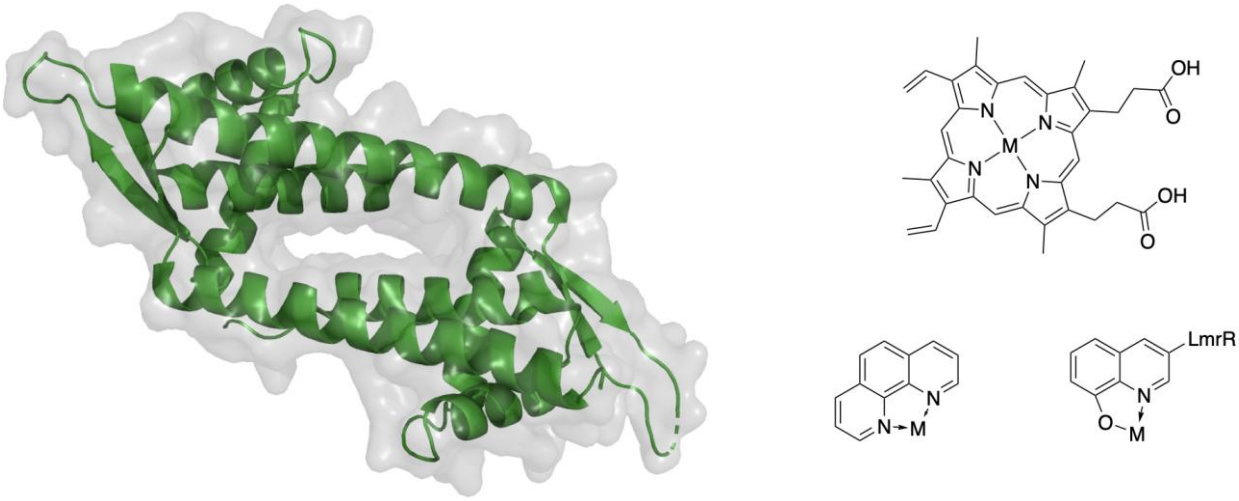

Figure 1. Left: X-ray structure of LmrR with its hydrophobic pore in the middle (PDB: 3F8B). Right: Metal cofactors based on protoporphyrin IX (PPIX) or phenanthroline can be bound supramolecuarly into LmrR; hydroxiquinoline-based non-canonocial amino acids for metal binding can be genetically incorporated into LmrR through stop-codon suppression.

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Why does sulfite reductase employ siroheme?

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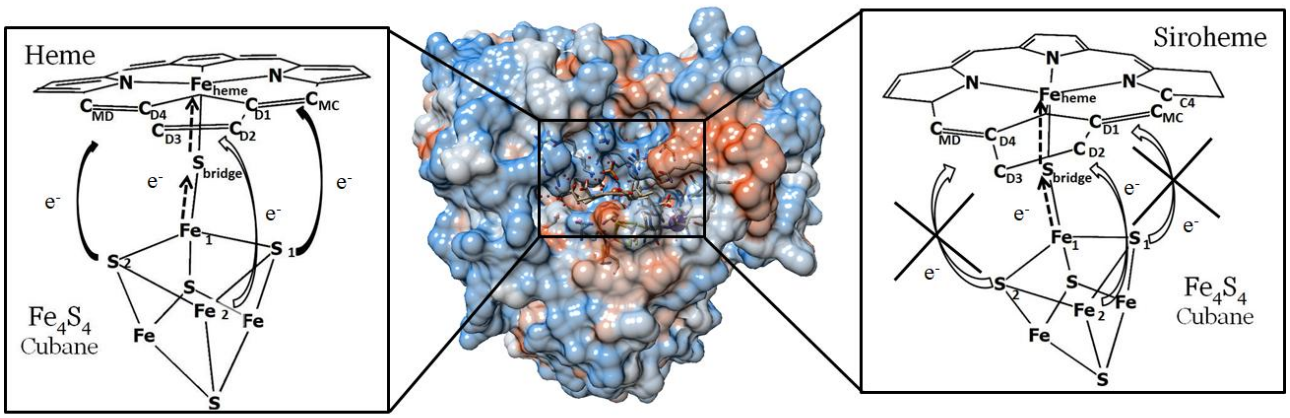
Sulfite reductase (SiR) contains in the active site a unique assembly of siroheme and a [4Fe4S] cluster, linked by a cysteine residue. Siroheme is a doubly reduced variant of heme that is not used for a catalytic function in any other enzyme. We have used[1] non-equilibrium Green's function methods coupled with density functional theory computations to explain why SiR employs siroheme rather than heme. The results show that direct, through vacuum, charge-transfer routes are inhibited when heme is replaced by siroheme. This ensures more efficient channelling of the electrons to the catalytic iron during the six-electron reduction of sulfite to sulfide, limiting potential side reactions that could occur if the incoming electrons were delocalized onto the macrocyclic ring.



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Design of a miniature protein in a fully artificial electron transport chain triggered by visible light

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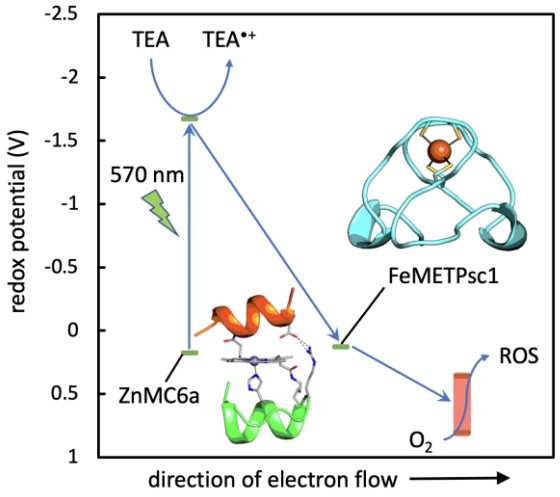
The design of *de novo* metal-binding proteins tailored for specific applications is continuously improving[1]. The identification of the least set of primary and secondary sphere interactions around the metal able to delineate the observed function still challenges the bioinorganic chemistry community[2]. Here, we benchmarked our understanding with a very well-studied[3], but at the same time insidious, case. We report the design of a single-chain linear protein able to recover all the requirements needed to fold and function as a natural Rubredoxin ($FeCys_4$ site) in only 28 residues[4]. Despite the simplicity of this metal site, de novo proteins featuring tetrathiolate metal clusters have never been characterized before by single-crystal X-ray diffraction. In this study, we show, for the first time in *de novo* protein design, the crystal structure of a tetra-thiolate metal-binding protein within sub-Å agreement with the intended design. Despite sharing strikingly similar structural arrangement, this miniaturized protein does not hold any sequence correlation to the known Rds. As a further achievement, we purposely programmed a high reduction potential compared to natural and designed $FeCys_4$-containing proteins, and we exploited it as terminal electron acceptor of a fully artificial electron transport chain triggered by visible light.



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Reversible or irreversible catalysis of H+/H2 conversion by FeFe hydrogenases

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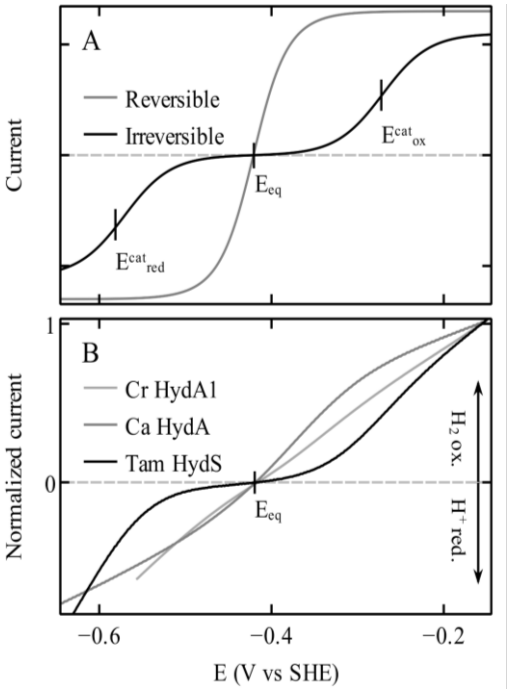
FeFe Hydrogenases are metalloenzymes that catalyse the oxidation and evolution of molecular hydrogen, an important energy vector in the metabolisms of most microorganisms.



Different hydrogenases can have very different catalytic properties. One of them is catalytic reversibility, which is defined in relation to the thermodynamic driving force (overpotential in the case of electrochemical experiments) that is needed for the enzyme to start catalyzing the reaction in one direction or the other[1]. Prototypical hydrogenases (such as HydA1 from *Chlamydomonas reinhardt*ii and *Clostridium acetobutylicum*, gray in panel B) are reversible catalysts: a small difference between the electrode potential and the equilibrium potential of the H^+/H_2 couple leads to a significant hydrogen oxidation or proton reduction current. Reversibility is a desirable property that could only recently be engineered in artificial catalysts of hydrogen oxidation/evolution[2,3]. Understanding how hydrogenases catalyze this reaction so efficiently is needed to improve biomimetic catalysts.

The recently identified sensory hydrogenase from *Thermoanaerobacter mathranii* (*Tam*) shows a very unusual catalytic response (black in panel B), as it starts catalysing the reaction in either direction only if a large overpotential is applied[4]. We characterized this irreversible catalytic response using Protein Film Electrochemistry (PFE)[3], demonstrating that: (1) unlike often assumed in the inorganic chemistry literature, an "ordered" reaction mechanism can lead to an irreversible catalytic response; (2) slow electron transfer between the electrode and the enzyme is not the reason catalysis is irreversible in this particular case[5]. We conclude that irreversibility is an intrinsic property of *Tam* HydS. This finding opens new questions about the molecular determinants of (ir)reversible catalysis, which we are currently addressing by combining the information from PFE and Fourier-transform infrared spectroscopy (FTIR).

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Inertness of superoxide dismutase mimics Mn(II) complexes based on an open-chain ligand is a key feature for bioactivity and detection in intestinal epithelial cells

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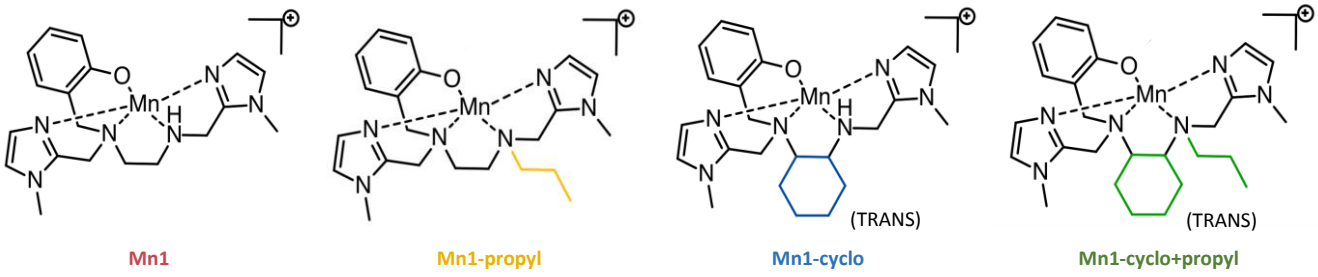
Superoxide Dismutases (SODs) are metalloenzymes involved in the cellular antioxidant defenses. They regulate the concentration of the superoxide anion, a reactive oxygen species (ROS).[1] It has been shown that SOD defenses are weakened in intestinal epithelial cells of patients suffering from inflammatory bowel diseases (IBDs).[2] The resulting increase in ROS amount, leading to oxidative stress, may contribute to the pathogenesis in IBDs. Low-molecular weight complexes, mimicking SOD activity may be promising antioxidant metallodrugs for the treatment of IBDs. The research conducted in Policar's group has led to the development of the manganese complex Mn1, based on an open-chain ligand, that has shown anti-oxidant and anti-inflammatory activities in intestinal LPS-stressed epithelial cells, an inflammation model mediated by oxidative stress.[3] However, Mn1 is very flexible compared to the native SOD and is prone to metal-assisted dissociation in cells. Indeed, metal exchanges might occur between the manganese center and metal ions present in the biological environment. Aiming at improving the bioactivity of this SOD mimic, three new MnSOD mimics derived from Mn1 have been designed. Their structure include additional cyclohexyl and propyl groups. In one hand, by rigidifying the ligand structure, the cyclohexyl group may provide a compact and preorganized coordination cavity to encapsulate the manganese ion and may improve the inertness of the complexes. In the other hand, the propyl group may prevent any deprotonation issue during the subsequent speciation studies and



increases the lipophilicity of the complexes.

We have assessed the potential of new SOD mimics derived from Mn1 to demonstrate higher intrinsic SOD activity, higher lipophilicity and improved resistance to metal exchanges.[4] Very interestingly, the new Mn1 derivatives were shown to provide anti-inflammatory and antioxidant effects in intestinal LPS- stressed intestinal epithelial cells at lower doses than Mn1, which could be correlated to their higher stability in the cellular environment.[4] The anti-inflammatory activities of the four SOD mimics were also evaluated on a murine model of colitis chemically-induced by DNBS.The rationalization of the SOD mimics bioactivities can be boosted by the direct detection and quantification of the active species inside the cells. The detection of open-chain manganese complexes in biological environments is not straightforward due to their high lability and thus possible metal exchanges. Thanks to its higher kinetic inertness, Mn1-cyclopropyl was detected inside cell lysates by mass spectrometry and its intracellular concentration was estimated.[4]

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Novel insights into the structure and reactivity of the molybdenum active site of sulfite-oxidizing enzymes

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Sulfite-oxidizing enzymes (SOEs) are molybdenum enzymes that exist in almost all forms of life where they carry out important functions in protecting cells and organisms against sulfite-induced damages[1,2]. Those SOEs which are able to directly convert sulfite into the less toxic sulfate ion are mononuclear molybdenum enzymes called sulfite oxidases in eukaryotes or sulfite dehydrogenases (SDHs) in most prokaryotes. Although these enzymes have been studied for decades[1,2], the details of their catalytic mechanism remain to elucidate. Using the sulfite dehydrogenase from the hyperthermophilic bacterium *Thermus thermophilus* as model enzyme, we succeeded to trap and characterize at the atomic level the structure of a molybdenum-phosphate adduct mimicking the still experimentally uncharacterized Mo(IV)-O-SO_3 reaction intermediate proposed to arise during the catalytic cycle of SOEs following substrate binding. Indeed, by combining X-ray crystallography, continuous wave and pulsed EPR (HYSCORE) spectroscopies and DFT modelling, we provide clear evidence for direct coordination of the product analogue HPO_4^{2-} to the Mo atom at the active site of *Thermus thermophilus* SDH (*Tt*SDH), and for its stabilization through an intricate H-bond network[3]. Moreover, we show for the first time the possibility to generate several pH-dependent Mo(V) species in the wild-type *Tt*SDH[4], a property that was only reported in eukaryotic sulfite oxidases or in a SDH mutant[1,2]. These results are discussed in the light of the crystallographic structure of the enzyme that we have also solved in the absence of HPO_4^{2-}, allowing us to address the role of the protein environment in fine-tuning the reactivity of the molybdenum active site of SDHs and more generally of sulfite-oxidizing enzymes[4].

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Toward Novel Reactivities: Screening Protein Scaffolds and Active-site Cofactors in Semi-synthetic [FeFe] Hydrogenases

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Hydrogenases are metalloenzymes that catalyse the reversible conversion of hydrogen. Among all hydrogenases, [FeFe] hydrogenases are particularly interesting as they show the highest activity and reversibility. Their active site consists of a [4Fe-4S] cluster ($[4Fe\text{-}4S]_H$) covalently attached via S-Cys to a di-iron site ($[2Fe]_H$). In the $[2Fe]_H$, the two iron ions are linked together via a dithiolate bridge and a bridging CO, and each iron is further coordinated by one terminal CO and CN^- ligand, which are biologically unusual but critical to stabilising the iron ions in low oxidation and spin states.[1]

Recently, an artificial maturation process, that combines an apo-hydrogenase (containing the [4Fe-4S] clusters but lacking the binuclear $[2Fe]_H$ site) produced recombinantly in *E. coli*, and a chemically synthesised active-site cofactor, was developed.[2] In this work, we harnessed this strategy by independently modifying both the protein scaffold and the active-site cofactor and then screening different combinations, to produce a set of semi-synthetic enzymes to explore novel reactivities.

Herein, different hydrogenase families are being explored, working with protein scaffolds from organisms including the sulphate reducing bacteria *Desulfovibrio desulfuricans,* the well-studied green algae *Chlamydomonas reinhardtii* and the thermophilic bacteria *Thermotoga maritima* (a particularly interesting organism for biotechnological applications, as it is the most thermophilic bacterium currently known which grows up to 90^{o}C by a fermentative metabolism)[3]. Additionally, synthetic active-site cofactors with different combinations and substitutions of the strong-field ligands CO and CN^- are being explored to ascertain whether they are able to maturate the apo-enzyme. All the semi-synthetic systems produced are characterised using infrared (IR) spectroscopy to assess the cofactor's ability to specifically bind to the active site pocket within the apo-protein, while their catalytic activity is investigated via solution activity assays and protein film electrochemistry.

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Using Spectroscopy to Study the Electron Relay and Modified Active Sites in Semi-Synthetic [FeFe] Hydrogenases

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Metalloenzymes catalysing H_2 conversion -hydrogenases- have drawn much attention due to their impressive catalytic efficiency. [FeFe] hydrogenases have the highest turnover frequency of all the hydrogenases.[1] Their active site, the H-cluster, is shown in Figure 1. [FeFe] hydrogenases can now be produced in high yield and purity by using a semi-synthetic approach where the apo-enzyme (i.e. the enzyme lacking the active site) is recombinantly produced in *E. coli* and subsequently reconstituted *in vitro* with chemically synthesised active-site cofactors. In this work, we show how the semi-synthetic approach to produce [FeFe] hydrogenases can be applied to study mechanistic aspects of these fascinating enzymes, as well as to produce semi-synthetic enzymes with modified active sites to explore novel reactivities. We have used the dimeric [FeFe] hydrogenase from *Desulfovibrio desulfuricans* (*Dd*HydAB). *Dd*HydAB is composed of two subunits: the large subunit, HydA, holds the active site and two additional [4Fe4S] clusters (the F-clusters) acting as electron relay. The small subunit, HydB, serves a structural function.[2]

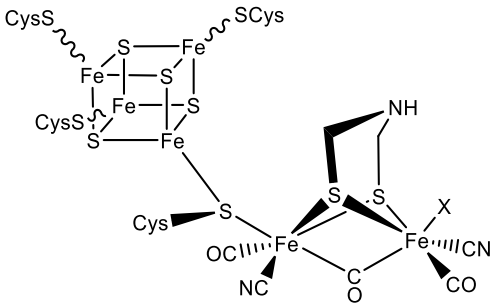

Figure 1: Structure of the active site H-cluster in [FeFe] hydrogenases.

Here, we tackle question of electron loading in the [4Fe4S] cluster relay in *Dd*HydAB using an infrared (IR) spectroscopic approach and incorporating an unnatural amino acid with an IR marker, para-cyano-L-phenylalanine (*p*CNF), close to the proximal [4Fe4S] cluster in the electron relay. The vibrational frequency of the CN group of *p*CNF responds subtly to nearby redox changes, thus acting as a reporter for the proximal cluster redox state.

Additionally, we have prepared a semi-synthetic enzyme featuring a synthetic asymmetric mono-cyanide cofactor, and its structure and reactivity has been investigated to help elucidate the role of the biologically-unique coordination environment of the H-cluster in hydrogenases.

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Redox properties of pyrroloquinoline quinone cofactor in fangal quinohemoprotein with direct electron transfer

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Pyrroloquinoline quinone (PQQ) is an important cofactor alongside nicotinamide adenine dinucleotide (NAD^+) and flavin adenine dinucleotide (FAD), which are all two-electron carriers. Although several studies of direct electron transfer (DET)-type bioelectrocatalysis have been reported for PQQ-dependent enzymes, there are few analyses about a redox reaction coupled to electron transfer between PQQ in the enzyme and the electrode. We suggested this could be due to an unestablished suitable DET reaction for Protein film voltammetry analysis. Previous work already made progress on understanding DET reaction in a fungal PQQ-dependent pyranose dehydrogenase (*Cc*PDH).[1] The PQQ domain separated from the full-length enzyme (containing only the PQQ cofactor) was also able to engage the DET-type bioelectrocatalysis without its built-in mediator cytochrome domain.[2,3] In the present work, we demonstrated the non-catalytic redox signal by DET reaction and performed electrochemical analyses together with spectroscopic studies on PQQ in the active site of *Cc*PDH.

Under the non-turnover conditions, redox peak currents of PQQ bound to the enzyme were successfully observed with a gold nanoparticle modified electrode having a large specific surface area. Redox peaks were quantified with two pairs of fully reversible reduction and oxidation peaks at E_1 = -44 mV and E_2 = -216 mV, pH 6.0, which were surface adsorbed redox species. Electron paramagnetic resonance (EPR) spectroscopy showed the formation of a PQQ semiquinone radical at an applied potential between E_1 and E_2, while the EPR signals disappeared outside of this potential range. These results are the first indicators that two-step one-electron transfer takes place via a semiquinone intermediate in the DET reaction. The redox potential of $E_{sem/red}$, E_2, is close to the onset potential of the catalytic current for L-fucose oxidation in DET-type bioelectrocatalysis of the PQQ domain of *Cc*PDH. Redox reactions of quinones are classic examples of proton-coupled electron transfer (PCET) processes. Because an acid-basic equilibrium for PQQ should be involved, its redox potential can be expected to change in dependence on pH. The redox peak potentials of $E_{ox/sem}$ and $E_{sem/red}$ were dependent on pH with each different slope. $E_{ox/sem}$ linearly decreases by -63 mV/pH, which corresponds to the Nernstian slope for the apparent potential of a pH-dependent $1e^-/1H^+$ process. On the other hand, the shift of $E_{sem/red}$ is -40 mV / pH from pH 5.5 to 7.0, and no change at pH 7.5. The results indicated that at neutral pH the reduced form is predominantly deprotonated $PQQH^-$ and 1e- transfer without proton transfer would occur between $PQQH^-$ and neutral semiquinone radical (PQQH•).

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Formate dehydrogenases reduce CO_2 rather than HCO_3^-: an electrochemical demonstration

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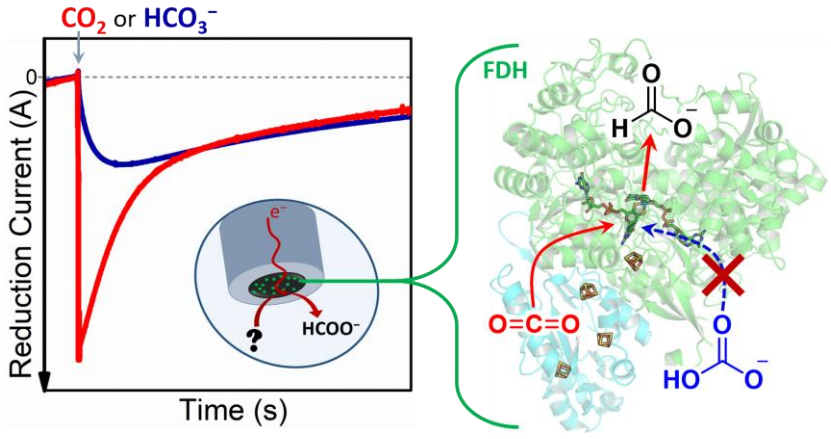
The reduction of carbon dioxide is an important reaction both in biology and in technology, since it converts a highly stable compound into more reactive and useful organic compounds. In nature, only two classes of enzymes catalyse the direct reduction of CO_2: formate dehydrogenases (FDHs) and CO dehydrogenases (CODHs). Both enzymes are fast and able to work with very little driving force. However, a specific difficulty to study these enzymes is to identify their actual substrate in aqueous solutions, which may be either dissolved CO_2 or hydrated species, like HCO_3^-.

For CODH, there is little doubt that CO_2 is the reduction substrate. The case of FDH is more ambiguous since many enzymes of the same family (the Mo/W enzymes) catalyse oxygen atom transfers. Therefore, rather than the direct reduction of CO_2, one could imagine that FDH catalyses the abstraction of an oxygen atom from HCO_3^- to give formate (HCO_2^-). As CO_2 and HCO_3^- are very different molecules, we should first determine which one is the substrate in order to investigate the catalytic mechanism of FDH.

Even though some work tried to address this issue, we believe that there is still no indisputable evidence for this. Therefore, we devised a simple electrochemical method to definitively demonstrate that the substrate of formate dehydrogenases is indeed CO_2.[1]



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Vibrational spectroscopic study on the first and second coordination sphere of Mo-containing enzymes

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In light of the impact of carbon dioxide on the climate, catalysts capable of converting CO_2 into fuels are of great interest. Metal-containing CO_2 reductases, e.g. formate dehydrogenases (FDH), perform the reversible interconversion of formate to CO_2, one proton, and two electrons under physiological conditions and have been already extensively studied in the field of (photo)electro-chemical generation of carbon-based fuels with respect to potential biotechnological applications. [1,2,3] However, mechanistic studies on this class of enzymes are still rare and the catalytic process is not fully understood, yet.[4,5] To shed more light on the underlying mechanism of *Rhodobacter capsulatus* (*Rc)* FDH, a combined approach comprising infrared (IR) and Resonance Raman spectroscopy was accomplished in combination with density functional theory (DFT) calculation. The *Rc*FDH is a NAD^+ dependent Mo-containing enzyme, consisting of a $(\alpha\beta\gamma\delta)_2$ dimer of hetero tetramers. The bis-MGD cofactor constitutes the active site for CO_2 / formate interconversion which is harbored in the α-subunit. The involved electrons can be transferred via a FMN prosthetic group, five [4Fe4S] and two [2Fe2S] clusters that are located in α-, β- and γ-subunits.[6] In its resting state, the Mo^{VI} ion is hexacoordinated by four sulfurs from two MGD dithiolenes, a sulfido ligand and a cysteine ligand from the protein backbone. Upon two electron reduction, the Mo^{IV} ion is proposed to be pentacoordinated due to the displacement of the cysteine.[4,5]

One key aspect for the understanding of the catalytic mechanism is the elucidation of the first coordination sphere of the Mo ion, for which Resonance Raman spectroscopy was applied to monitor selective metal ligand vibrations. Herein, the presence of sulfido ligand in the oxidized was confirmed via the Mo=S bond, which is crucial regarding its potential involvement in the C–H bond cleavage of the substrate.[6] However, the question whether the substrate binds covalently during catalysis, and thus displaces the Mo ion attached cysteine ligand, remained unclear up to now and was the focus of our approach. To address the role of the second coordination sphere in substrate binding active site variants and charged IR sensitive inhibitors such as azide, that is isoelectronic with CO_2 and charged as formate, were studied. Herein, we could reveal that azide binds electrostatically in the vicinity of the Mo ion to two conserved Arg587 and a His387 but also interacts with the sulfido ligand.

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Expanding Artificial Metalloenzyme Toolbox with New Salophen-bound Myoglobin Biohybrid Catalysts

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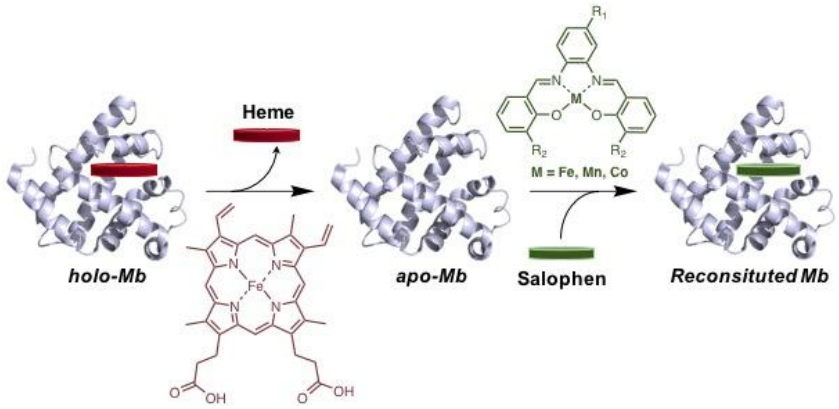
Planar salophen ligand-based catalysts demonstrate reactivity in a range of synthetic reactions, including water oxidation.[1] Precedent for such activity lies in the nature of bound transition metals which permits access to high-valent metal intermediate states, such as Fe(IV)=O. When bound within the stereogenic active site environment of the protein scaffold, such synthetic cofactors hold the potential of equipping the metal centre with enantio- and/or regio-selectivity. Up to this date, myoglobin-(Mb)-based artificial metalloenzymes (ArMs) bound to metal-salophen derivatives have only been employed in sulfoxidation reactions.[2] Hence, the reactivity of salophen-myoglobin biohybrid catalyst duo remains underexplored.



Herein we present that a sperm whale myoglobin mutant (T39I, R45D, F46L, I107F), previously reported to demonstrate a 25-fold increased peroxidase activity vs. its wild-type counterpart, acts as an efficient protein scaffold for supramolecular binding of a range of Fe-, Mn- and Co-salophen derivatives.[3] Reconstitution of recombinantly expressed quadruple myoglobin mutant with these metal-Schiff base complexes permits access to a new ArM biohybrid catalyst library. In addition, UV-vis spectroscopy and nanoflow electrospray ionisation mass spectrometry (native nESI-MS) are used to monitor changes in the ligand-protein assembly and confirm the binding of metal-salophen complexes within the protein scaffold. Further optimisation of salophen ligand backbone via installation of either hydrophobic (-CH_3) or H-bond acceptor/donor groups (-COOH) allows for improved metal-complex binding within the myoglobin scaffold.

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Strep-Tag II - Imprinted Polymer as Universal Platform for Bio(electro)catalysis

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Molecularly imprinted polymers (MIPs) are artificial recognition sites that bind target molecules with high affinity and selectivity[1]. MIPs are prepared by polymerizing functional monomer(s) in presence of the template. Its subsequent removal results in the formation of cavities in the polymer matrix that mirror the shape of the template. Due to limitations regarding size, stability, and conformational changes, the respective MIPs for proteins are nowadays preferentially formed from low-molecular-weight fragments of these biomolecular targets within the so-called epitope imprinting.[2] Herein, we successfully synthesized in such way a platform for the recognition of recombinant proteins carrying the commonly used affinity tag (Strep-Tag II®) following a three-step hierarchical approach. This involves the adsorption of the cysteine-extended Strep-Tag II peptide, followed by electro-polymerization of scopoletin and subsequent removal of the target peptide from the resulting polyscopoletin matrix. Thereby, imprinted receptor cavities are formed, which are highly selective for Strep-tagged proteins including membrane bound hydrogenase (MBH) and alkaline phosphatase (ALP), exhibiting relative low K_D values of about 1.06 nM and 33.08 nM, respectively. By means of electrochemical methods, atomic force microscopy, surface-enhanced infrared absorption (SEIRA) spectroscopy and molecular dynamic simulations the entire workflow was monitored and mechanic insights in the rebinding derived. Moreover, strep-tagged proteins retain their activity after binding to MIPs as demonstrated by the mediated catalytic current detected in the presence of their respective substrate, even after exchanging the entire electrolyte.

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In that way, we can provide a universal platform for specific binding and bio(electro)catalysis of Strep-Tag containing proteins.

Photocatalytic artificial metalloenzymes derived from LmrR

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Visible light photocatalysis is receiving increasing attention amongst chemists, intrigued by its powerful applications paired with mild reaction conditions and operational simplicity.[1,2] While already fascinating in a purely chemical context, this methodology offers yet another dimension of opportunities in combination with proteins. Inspired by this idea, we sought to build on former experiences in the Roelfes group[3,4,5] and set out to create photocatalytic artificial metalloenzymes by incorporating transition metal photocatalysts in the protein scaffold of LmrR.[6] Here we will present the construction of these artificial metalloenzymes and their application in photoredox catalysis.

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Semisynthesis of SmtA metallothionein using a highly efficient OaAEP1 ligase – comparison of metal binding properties of synthetic and E. coli-produced protein

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Protein semisynthesis constitutes a dynamically developing field of biochemistry. It relies on combining synthetic and recombinant peptide fragments in a controlled manner to acquire functional biomolecules, which have wide applications in many areas. Therefore, the development of new and efficient production strategies is of great interest and practical importance. Due to the high selectivity, efficiency, and excellent compatibility, the enzymatic method recently has gained tremendous popularity among other synthetic strategies, especially for difficult Cys-rich sequences[1]. The molecular target of this work is low molecular weight metallothionein (MT) from the cyanobacteria *Synechococcus elongatus* (SmtA). Due to the presence of numerous cysteine residues, it binds with Zn(II), Cd(II), and Cu(I) ions and is responsible for their storage, transport, and homeostasis in the cell[2]. In order to synthesize and carry out metal binding characteristics of the mentioned protein, we applied a new asparaginyl endopeptidase, OaAEP1, isolated from the plant *Oldenlandia affinis* and currently produced in a bacterial system[3]. The postulated activity of its OaAEP1 C247A mutant is around 160 times higher compared to the wild-type, and it forms an excellent alternative to other enzymes due to the use of a short C-terminal recognition motif -NGL, as well as faster hydrolysis of the intermediate thioester bond. The sequence of the target metallothionein contains a recognition motif for the mentioned enzyme, which enables applying this attractive and promising tool for the protein semisynthesis. Two peptide substrates were synthesized according to the SPPS strategy and then ligated by OaAEP1 ligase in order to obtain the target SmtA protein. Independently, mentioned metallothionein has also been overproduced in *E. coli*. MS and HPLC analyses confirmed the compliance of the final products obtained by different strategies. In addition, titration experiments presented their identical metal binding properties. As a consequence, this experimental work presents a fast, selective, and efficient synthetic strategy to gain SmtA using recombinant OaAEP1 ligase. It may provide an excellent path for producing other proteins, which via other pathways is not effective or still remains impossible.

The research was supported by the National Science Centre (2019/33/B/ST4/02428 to A.K.).

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Plant metallothioneins bind Zn(II) with different affinities

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The stress factors influence generating reactive oxygen species (ROS) and, in effect, cause protein misfolding, dysregulation of enzymatic activity, and nucleic acid damage. In response to stressors, plants possess defence and recovery solutions such as phytochelatins, metallothioneins, heat shock proteins, ubiquitin-proteasome systems, and autophagy[1,2]. The plant metallothioneins (MTs) are small cysteine-rich proteins responsible for binding essential like Zn(II), Cu(I), and toxic metal ions. They also participate in redox homeostasis, regulation of various metal-dependent pathways, and detoxification[1,3]. The Zn(II) is the second most abundant d-block metal in all living organisms. Due to its presence in all kinds of cells and cellular compartments is utilized in an enormous number of processes; therefore, keeping cytosolic free Zn(II) at the proper concentration is critical[3]. It is possible due to zinc transporters and MTs; however, their role in this process in plants is under debate. In mammals, MTs are involved in the zinc buffering process, which enables to keep free Zn(II) at the nano- to the picomolar range due to differentiated MTs affinities[3,4]. The fundamental question is whether or not the plant MTs bind their Zn(II) with various affinities and can buffer free Zn(II) at a similar range? Therefore in this study, we focus on the thermodynamics of Zn(II) binding to particular binding sites of MTs from *Musa acuminata*, *Oryza sativa*, and *Triticum aestivum*. Zn(II) binding constants of particular MTs were determined fluorometrically using the highly sensitive zinc probe ZnAF-2F. Due to its sensitivity, determination of the constants in nanomolar to the low picomolar range was possible. Obtained results indicate that, similar to mammalian, plant MTs bind Zn(II) in tight, moderate, and weak binding sites, which support their role in the zinc buffering mechanism.

Financial support by the National Science Centre of Poland (Opus grant 2018/31/B/NZ1/00567) is gratefully acknowledged.

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Metal-binding properties of the eukaryotic Rad50 hook domain variants – insights into the sequence-stability relationship

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Rad50 protein is a part of the MRN complex, the key player in the machinery that safeguards genome stability through DNA damage sensing and repair[1]. Rad50 consists of a bipartite ATPase domain and a 50 nm-long antiparallel coiled-coil chain that ends with a sharply bent β-hairpin called the hook[2].This simple protein fold facilitates the dimerization of Rad50 molecules *via* tetrathiolate coordination of Zn(II) by a CXXC motif. Two such motifs establish an extremely stable interprotein zinc site with a femtomolar Zn(II) affinity[3]. Genetic analyses have revealed that mutations in the hook area impair a broad range of MRN functions[4]. Besides the invariant Cys residues from the CXXC motif, significant conservation of specific amino acid residues within the hook sequence is observed among different groups of eukaryotes. To our knowledge, no systematic physicochemical analyses of eukaryotic hook homologs have been reported so far. This has encouraged us to design and characterize the Zn(II) coordination properties of several hook models representing distinct motifs found in plant and animal kingdoms. We have applied a combination of spectroscopic and mass spectrometry techniques to investigate the complex stoichiometry, metal-dependent structurization, and Zn(II)-to-hook affinity. The study has shown that the natural amino acid alterations within the hook significantly impact the stability, structure, and overall Zn(II)-binding properties of the entire domain. The eukaryotic hook models manifested distinct folding pathways induced by Zn(II), which resulted in differences in the overall hook fold. The estimated affinity of Zn(II) for the Rad50 homologs also differed, which might be related to the intrinsic Zn(II) availability within the source organisms. This systematic study illustrates how the evolution shapes the structure and metal-binding properties of the hook domain to adapt the Rad50 functioning to various intracellular conditions.

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New insights into the electronic structure basis of biological nitrogen reduction

The nitrogenase enzymes catalyze the difficult redox reaction of dinitrogen to ammonia. The mode of action of these enzymes remains poorly understood, however, despite decades of detailed structural characterization studies. The MoFe protein of molybdenum nitrogenase contains a metal-sulfur cluster, FeMoco, where N_2 binding and reduction takes place. The resting state of FeMoco (E_0) has been characterized by crystallography, multiple spectroscopic techniques (XAS, Mössbauer, EPR) and theory (broken-symmetry DFT). The highly complex electronic structure, involving spin coupling of 8 high-spin metal ions, delocalized electrons and weak metal-metal bonding, is not completely understood [1-4] and even less is known about the other redox states of the cofactor (E_1-E_8). Spectroscopic studies have proposed hydrides to be present in E_2-E_4, importantly prior to binding of dinitrogen, and reductive elimination of H_2 via these hydrides explains the obligatory H_2 formation upon N_2 binding. Our theoretical studies for the past few years are aimed at characterizing in electronic-structure detail the redox states of FeMoco. Our QM/MM BS-DFT protocol gives a calculated E_0 structure of FeMoco in excellent agreement with the high-resolution crystal structure, and reveals a strong sensitivity to both redox state and spin-coupling treatment [4]. Recently, we have shown that the strong sensitivity of FeMoco metal-metal distances on DFT approximations is also found in simpler [Fe_2S_2] dimers and can be traced back to the calculated covalency of the Fe-S bond [5]. The potential energy surfaces of the E_2 and E_4 redox states have been explored by QM/MM calculations [6,7]. Bridging hydrides are found to be preferentially stabilized between belt Fe ions due to the hemilability of protonated belt sulfides. Interestingly, our model for the E_4 state reveals favorable N_2 binding and offers a chemically intuitive explanation for why N_2 binding occurs in this state and not in others. Furthermore, the geometry of the N_2-bound state reveals a plausible reductive elimination step (H_2 elimination via hydrides) leading to a reduced FeMoco state that partially activates N_2 for protonation [7]. Finally a systematic study of the mechanism of CO inhibition has recently been described [8].

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Structural Basis of Human Melanogenic Enzymes

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The production of the pigment melanin in human requires the activity of at least three melanogenic enzymes, tyrosinase (TYR) and tyrosinase-related proteins 1 (TYRP1) and 2 (TYRP2), which regulate the type and amount of melanin produced. Despite their essential role, the catalytic mechanism and specificity are still under strong debate. The lack of structural data hampers the understanding of their molecular bases and the design of specific inhibitors to treat pigmentation disorders or melanoma[1]. Recently, the solved crystal structure of human TYRP1 challenged previous assumptions claiming it is a redox enzyme[2,3]. In view of the existence of pathological mutations for all three melanogenic enzymes, it is thus very likely that each enzyme plays a unique function in human melanogenesis[4]. Therefore, it is crucial to obtain 3D structures of all three melanogenic enzymes at atomic level as well as to identify their respective metabolites in order to improve our current knowledge on the human melanin biosynthesis pathway. With all these information, compounds of great efficiency and specificity can then be designed to treat melanogenesis disorders.

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Tungsten containing Aldehyde Oxidoreductase from *Aromatoleum aromaticum* and Maturation of the Tungsten Cofactor

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Aldehyde Oxidoreductase from *Aromatoleum aromaticum* EbN1 is a tungsten containing enzyme of the family of bacterial AORs that reversibly catalyzes the oxidation of various aldehydes to the corresponding carbonic acids. It consists of three different subunits and contains a tungsten bis-molybdopterin cofactor in the active site, from where electrons are transferred via five Fe_4S_4-clusters to a FAD cofactor. *In vitro*, AOR uses either NAD^+ or benzyl viologen as electron acceptors. The enzyme also possesses hydrogenase activity that allows the use of hydrogen as electron donor for the reduction of NAD^+, benzyl viologen or carbonic acids. Using the closely related *Aromatoleum evansii* as host organism, we established recombinant production of affinity-tagged AOR that allows to easily obtain large quantities of purified enzyme for further biochemical and structural characterization.

Besides the enzyme itself and its potential biotechnological applications, we are interested in the maturation process of the tungsten cofactor. As biosynthesis of the W-bis-MPT in *A. aromaticum* follows the well investigated pathway of molybdenum cofactor synthesis, the organism has to discriminate between molybdenum and tungsten. We show that this discrimination process includes two paralogues of molybdopterin molybdotransferase, MoeA1 and MoeA2. Our recombinant system allows expression of tagged AOR in genetically altered strains of A. evansii in order to investigate the role of these gene products.

TsrM, a unique B12-dependent Radical SAM Methyltransferase Catalyzing Non-radical Reactions

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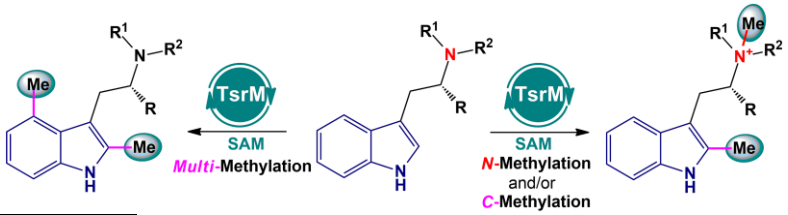
Radical SAM enzymes are arguably the largest and most functionally diverse superfamily of enzymes playing key biological functions in all kingdoms of life from viruses to bacteria and mammals. These metalloenzymes are particularly abundant in the biosynthetic pathways of the so-called ribosomally synthesized and post-translationally modified peptides (RiPPs) where they catalyze chemically challenging post-translational modifications including epimerization, complex rearrangements, carbon-carbon and thioether bond formation, as well as C-methylations. Among these enzymes, the vitamin B12 (cobalamin)-dependent radical SAM enzymes constitute the largest group (200,000 proteins) and perform notably methyl transfer to *sp2*- and *sp3*-hybridized carbon atoms. However, to date we have little information regarding their complex mechanisms and their biosynthetic potential. By using X-ray absorption spectroscopy (XAS), mutagenesis and synthetic probes, we obtained novel insights into the mechanism of the B12-dependent radical SAM enzyme, TsrM involved in the biosynthesis of the antimicrobial RiPP, Thiostrepton A. Notably, we established that TsrM catalyzes not only C- but also N-methyl transfer reactions, further expanding its synthetic versatility. In addition, we showed that TsrM has the unique ability to directly transfer a methyl group to the benzyl core of tryptophan, including the least reactive position C4. Finally, our study supports a major role of the [4Fe-4S] cluster for the configuration of the cobalamin cofactor and demonstrates that despite being a radical SAM enzyme, TsrM catalyzes non-radical reactions. Collectively, our work establishes the usefulness of radical SAM enzymes for novel biosynthetic schemes including serial alkylation reactions at particularly inert C-H bonds.[1]



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The structural influence of Sulfonated Moco on the Moco-related *moaA* riboswitch

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A riboswitch is a highly conserved sequence of noncoding mRNA that upon binding of a specific cellular metabolite changes its tertiary structure. This change in structure controls the expression of downstream genes, displaying an exemplary representation of structure meets function.
The *moaA* riboswitch from *E. coli* is involved in the regulation of molybdenum cofactor (Moco)-related genes and was proposed to respond to Moco.[1] Moco, which has a pyranopterin moiety linked to a Molybdenum (VI) center, is highly oxygen sensitive and cannot be obtained in its free form. Hence, its direct interaction to the *moaA* riboswitch could not yet be proven.
We isolated sulfonated Moco from the molybdenum dependent enzyme Xanthine Oxidase via heat treatment and showed with in-line probing that under anaerobic conditions sulfonated Moco induces a change on the *moaA* riboswitch structure. This influence cannot be observed under aerobic conditions, suggesting that the effect is indeed caused by the oxygen sensitive sulfonated Moco. In earlier experiments, we showed that all stable biosynthetic precursors of Moco as well as synthetic structural analogs containing a pterin moiety showed no influence on the *moaA* riboswitch structure.[2] Our results strongly indicate that at least one of the additional functional moieties of Moco – the complexed molydenum and/or the phosphate group – are compulsory for the recognition by the riboswitch.

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Adduct of Aquacobalamin with Hydrogen Peroxide

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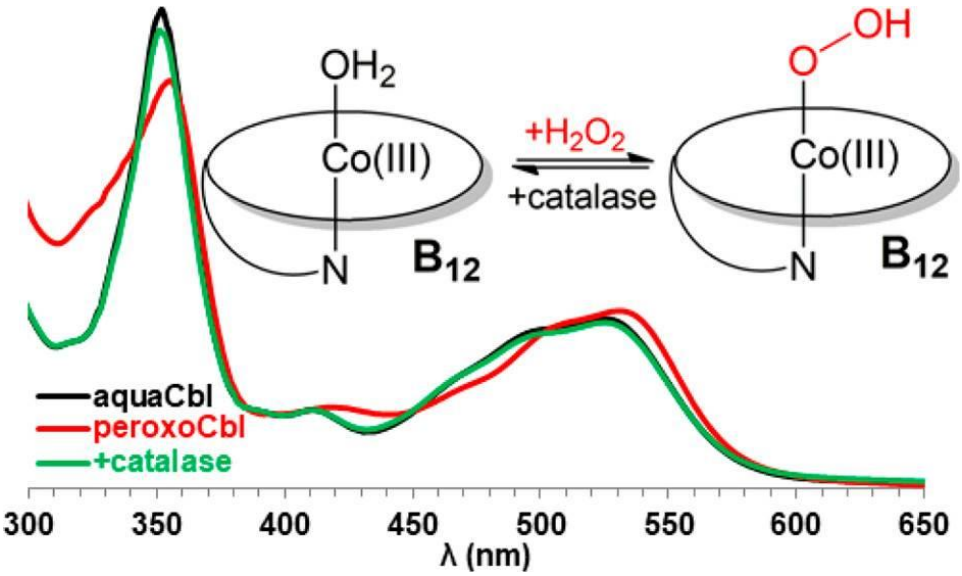
Aquacobalamin binds hydrogen peroxide reversibly to form a cobalt(III) hydroperoxo adduct with a 0.25 mM dissociation constant, as evidenced by UV−vis absorption spectroscopy and corroborated by NMR, Raman spectroscopy, stopped-flow UV−vis measurements, and density functional theory calculations.[1]



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Multimetal Methyltransferase Making Methanogenesis

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B12-dependent radical SAM enzymes are one of the largest groups of radical SAM enzymes with more than 200,000 members. This family of metalloenzymes are unique in their ability to form carbon-carbon bonds between unactivated sp3-hybridized carbons. Using biochemical and biophysical techniques including X-ray crystallography and electron paramagnetic resonance we have investigated methanogenesis marker protein 10 (Mmp10). Mmp10 has been shown to perform a post-translational methylation of an arginine within the active site of methyl-coenzyme M reductase. This is one of many post-translational modifications that are profoundly important for the methane production by microbes using methyl-coenzyme M reductase. Our crystallographic snapshots of Mmp10 reveal unique features including four distinct metal centres and a tyrosine that can coordinate the radical SAM clusters fourth iron allowing it to switch between radical and nucleophilic chemistry[1].

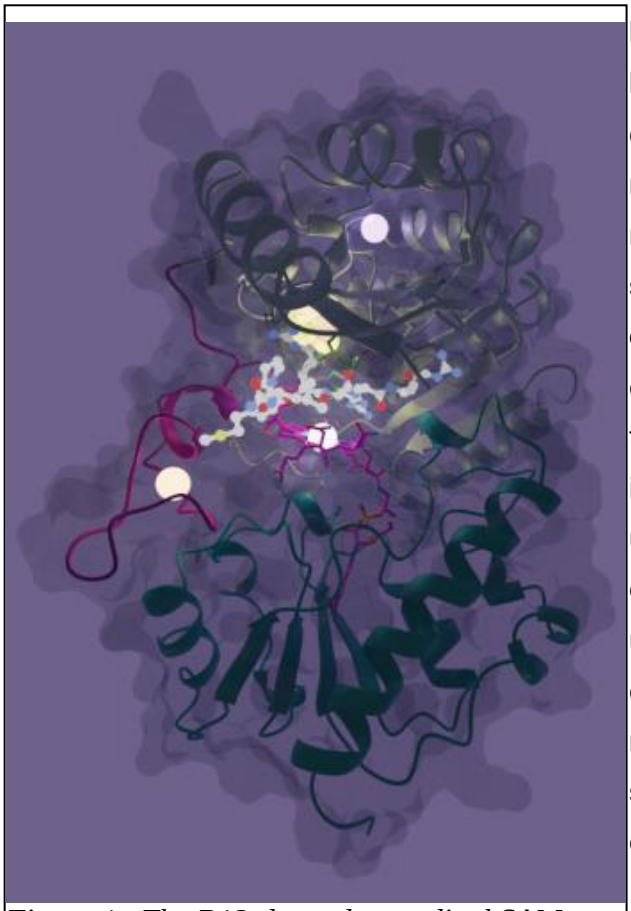

Figure 1. The B12-dependent radical SAM methyltransferase, Mmp10, with its four metal centres and bound peptide substrate highlighted.

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Dithiolene Containing Peptides: Synthesis and Characterisation of their Molybdenum Complexes.

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The industrial revolution has increased the atmospheric concentration of CO_2, which makes it a major concern for the environment. The abundance of this polluting gas can be tackled in a useful manner by CO_2 valorization, that is its conversion to high value-added chemicals and fuels. This valorization can be the basis of a greener and more sustainable chemical industry. However, the thermodynamic and kinetic stability of CO_2 makes its activation a challenging task. Formate dehydrogenases are enzymes present in Nature which have a Mo or W metal center in their active site bound to two pyranopterin guanosine dinucleotide units, known as the molybdenum cofactor (Moco). These enzymes can reversibly convert formate to CO_2 and thus, they have attracted a lot of interest in recent years.[1] Many research groups have tried to develop biomimetic and bioinspired models of these enzymes, but only a few are hardly functional.[2] Our aim is to develop a new generation of bioinspired Mo complexes by incorporating dithiolene units in peptides and studying their structures, properties and reactivities using both experimental and theoretical approaches.

The commercially available tripeptide glutathione was chosen as starting peptide and its Cys residue was modified to introduce dithiolene units. The Mo complexes were prepared either in DMF/water and DMSO/water solvent mixtures and were characterized by UV-Vis and, EPR spectroscopies, and by theoretical calculations based on Density Functional Theory (DFT). Finally, the electrochemical properties were studied via Cyclic Voltammetry (CV) and confronted with DFT computations to get insight into the nature of the species formed during the redox processes. Interestingly, our data indicate the formation of different Mo complexes, namely a Mo(V)-oxo and a Mo(VI)-dioxo complex in DMF/water and DMSO/water mixtures, respectively. The electrochemical studies show that these Mo complexes are capable to cycle between the (+VI), (+V) and (+IV) redox states, all being relevant for CO_2 valorization reactions.

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Optimising Artificial Enzymes by Altering the Nature of the Active Metal Complex

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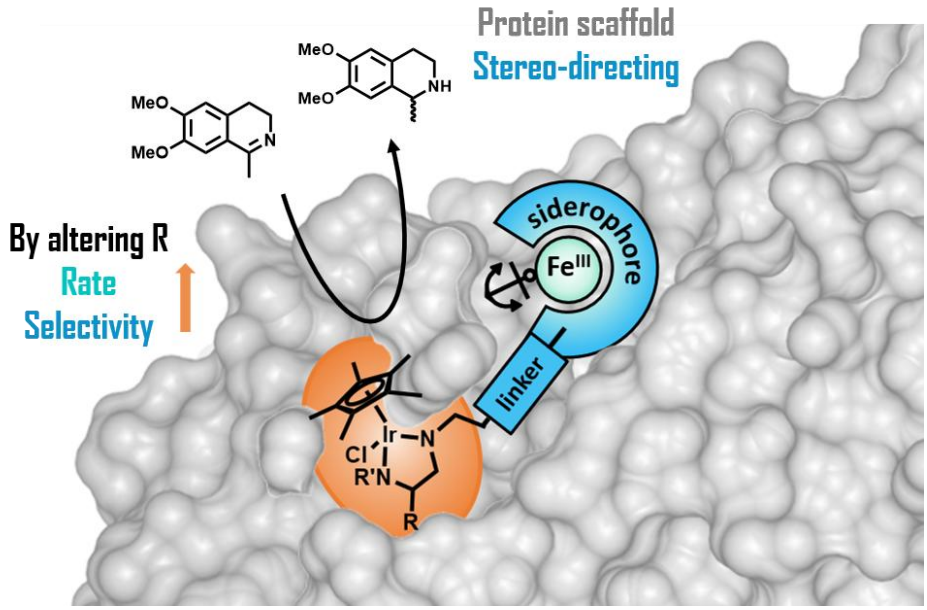

Artificial metalloenzymes have potential to expand the scope of green biocatalysis,[1] opening up inventive alternative applications[2]. Several approaches to designing artificial metalloproteins are being investigated, with catalytically active metal sites being incorporated into proteins by a variety of methods[3]. Our design utilizes a small protein involved in the iron-uptake pathway in some microorganisms[4]. By attaching a catalytically-active metal complex to an iron chelator, we can position the catalyst inside our protein scaffold. An iridium transfer hydrogenation catalyst was selected to target imine reduction, and in combination with our artificial metalloenzyme design initially achieved moderate catalytic rate and selectivity[4]. Through tuning of the steric and electronic properties of the iridium-coordinating ligands, both the selectivity and catalytic rate of the reaction were improved. In addition, the use of similar thermophilic proteins have further improved artificial metalloenzyme performance.

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Understanding the key factors governing the reactivity of methyl-coenzyme M reductase for generation of methane

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Methyl-coenzyme M reductase (MCR) is capable of catalyzing biological methane production and anaerobic oxidation of methane[1 – 4]. The catalytic site of MCR contains a nickel hydrocorphinate F430 cofactor with Ni in its low-valent oxidation state +1. The current consensus on the mechanism for methane generation involves formation of a methyl – Ni(II) intermediate which abstracts a hydrogen atom from coenzyme B to generate methane and the mixed disulfide CoMSSCoB[5]. Here, we performed density functional theory (DFT) calculations to understand the mechanism for methane generation by native F430 cofactor. We also interrogated the reactivity of intermediate states along the biosynthetic pathway of F430 coenzyme to understand how nature optimizes metanogenesis. DFT calculations on a cluster model derived from XRD, revealed that native F430 cofactor features the lowest activation energy barrier for formation of the methyl radical – Ni(II) intermediate as compared to four other intermediates in the biosynthetic pathway of this cofactor. We investigated the effect of reduction potential of $Ni^{I/II}$ and bond formation between Ni(II) and sulphur of the co-substrate SCoM to the overall activation free energy. We found that Ni-S bond formation is the driving step that controls the reaction barrier and it the most favorable for the native F430 over its biosynthetic precursors.

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Spectroscopic characterizations of OrpR, a σ^{54}-dependent activator that uses an iron-sulfur cluster for redox sensing

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To survive, living organisms must adapt to new environments or changes in their current environment. At the molecular level, the regulation of transcription is a fundamental mechanism that underlies adaptive processes for all domains of life. In bacteria, Enhancer Binding Proteins (EBPs) are key players of σ^{54}-regulation that control transcription of genes expression in response to environmental signals[1]. We recently discovered a novel type of EBP, called OrpR, which is involved in signal transduction and regulation of redox responses in anaerobic bacteria, such as *Desulfovibrio vulgaris* Hildenborough. We found that OrpR uses a redox-sensitive Fe-S cluster in its sensory domain to detect redox potential variations and regulate expression of target genes specific of anaerobic life[2] (**Fig 1**).

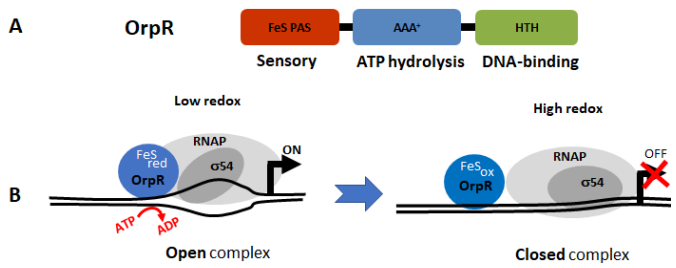

Fig 1 A. Three-domain structure of OrpR, a new member of EBPs family.
B. Proposed mechanism for regulation of redox responses by OrpR in the anaerobe *D. vulgaris* Hildenborough. Change in the medium redox potential is detected by OrpR via its Fe-S cluster. An increase in redox potential (redox stress) leads to a switch from OrpR ON state (active) to OFF state (inactive).

UV/vis, EPR and Mössbauer experiments on both purified protein[2] and whole-cells were consistent with the presence of a $[4Fe\text{-}4S]^{2+}$ cluster when OrpR is in "active" form (ON state, **Fig 1B**). The $[4Fe\text{-}4S]^{2+}$ cluster is bound in the sensory PAS domain via three conserved cysteines[2], the fourth ligand could be an unusual glutamate residue or a non-proteic ligand. Anaerobic oxidation of OrpR *in vitro* using mild oxidants (ferricyanide, DCPIP) revealed that one iron ion of the $[4Fe\text{-}4S]^{2+}$ cluster is labile, leading to a $[3Fe\text{-}4S]^{1+}$ species that may be related to OrpR in its "inactive" form (OFF state, **Fig 1B**). We will present our last studies using a panel of spectroscopic, biochemical and structural methods aiming 1) to solve the nature of the fourth ligand, 2) to identify which iron of the Fe-S cubane is labile, 3) to elucidate the inactive Fe-S form and the mechanism of redox sensing by OrpR.

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Study of cobalamin's adducts with cysteine and its derivatives

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Linkage isomerism is an expanding topic in bio-inorganic chemistry. We previously showed/confirmed this occurrence in an enzyme for the first time, i.e nitro/nitrito in nitrite reductases and in globins. Other examples include the complexes with nitric oxide, peroxynitrite, or with chlorine or sulfur oxides/oxyanions. In the present work, we investigate the reactivity of cobalamin towards with cysteine and S-oxidized cysteine derivatives (sulfenic, sulfinic and a sulfonic derivative with disulfide bond), comparing these with reactivity towards related nitrogen, oxygen and sulfur-based ligands and focusing on the concept of linkage isomerism. In order to perform such comparisons, a detailed investigation and correlation of experimental spectrometric data with those obtained through DFT and TD-DFT calculations is undertaken. UV-Vis, resonance Raman and NMR spectroscopy, complemented by DFT and TD-DFT calculations, show that cysteine and its derivatives do yield adducts at pH values ranging from 3 to 12, with a preference for binding to the cobalt through the sulfur.[1,2]

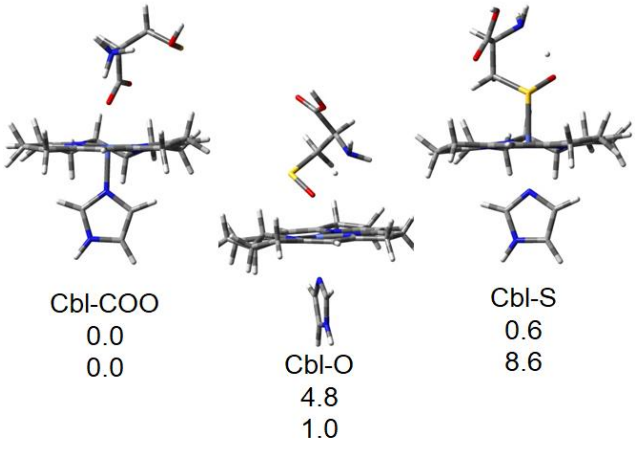

Figure 1. DFT data for linkage isomers of cobalamin complexes with cysteine's sulfonic acid. Relative energies are given in kcal/mol from calculations employing a truncate model of cobalamin, (top), or the full structure (bottom).

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Functionalization of carbon nanotubes by thiol-yne chemistry for enzyme electrocatalysis

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Click chemistry reactions are known for their attractive characteristics: simplicity, regioselectivity, high reaction rate, mild reaction conditions, functionality tolerance.[1] Among them, the thiol-yne reaction, involving the addition of a thiol to an alkyne, offers some compelling advantages as a tool for the post-functionalization of materials and biomolecules: it is metal-catalyst free, the reaction triggered by UV light can be controlled in a spatial and temporal way and the double hydrothiolation of alkynes is possible by its nature allowing an increased degree of functionalization. Furthermore, it is possible to take advantage of the thiol groups already available on proteins. Carbon nanotubes (CNTs), due to their large surface area and conductivity, are ideal substrates for bioelecrochemical applications. Furthermore, we have already proven the efficiency of clik reaction such as oxime ligation and copper(I)-catalyzed azide–alkyne cycloaddition for the immobilization and wiring of cyclopeptidic scaffolds and multicopper oxidases.[2,3] To the best of our knowledge, the photoinduced thiol–yne reaction has not been investigated yet on alkyne modified electrodes for enzyme electrocatalysis. We performed the thiol-yne reaction on two types of alkyne-modified CNTs, covalently grafted by 4-ethynylbenzenediazonium salt and non-covalently modified by 1-Ethynylpyrene. The immobilization of thiol-modified ferrocene and phenanthrolinequinone redox mediators onto the modified electrodes led to a considerable improvement in the mediated electrocatalysis of immobilized enzymes such as FAD-GDH for the oxidation of glucose or copper-containing laccase for the reduction of oxygen for biofuel cell applications.

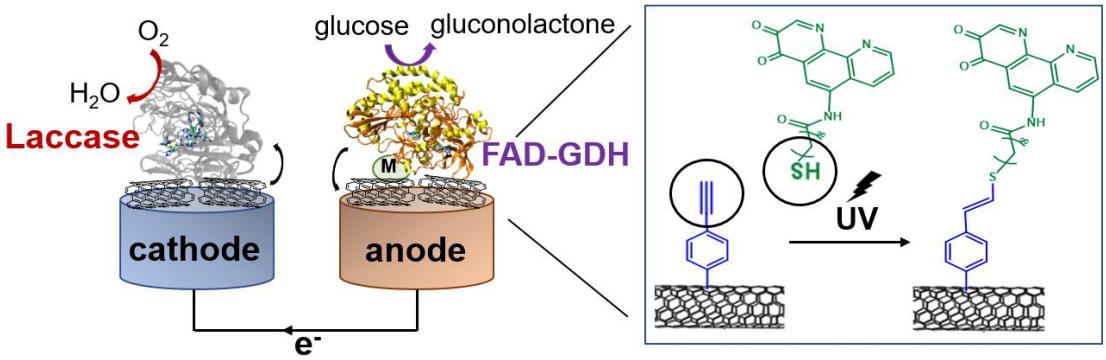

Figure 1: Scheme of an enzymatic biofuel cell constituted by laccase enzyme at the cathode and FAD-GDH enzyme at the anode after immobilization of thiol redox mediators onto alkyne modified carbon nanotubes by thiol-yne chemistry.

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Investigating protein structures of catalytic intermediate of [NiFe] hydrogenases via electrochemical poising

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[NiFe] hydrogenase enzymes reversibly catalyse the oxidation of dihydrogen to protons and electrons utilising an earth-abundant metal active site which operates with low overpotential. This has attracted interest from researchers seeking to develop bio-inspired synthetic catalysts and biotechnology systems. A catalytic cycle for [NiFe] hydrogenases has been proposed based on insight from comprehensive biophysical studies. Although the mechanism of catalysis is generally accepted, obtaining structures of [NiFe] hydrogenases and other metalloenzymes in precise catalytic intermediate states is still challenging. Currently, methods for controlling the redox state of protein crystals include adding substrate/inhibitors or reductant and oxidant;[12] or crystallising pre-equilibrated protein.[3] However, these methods often lack precision in generating single redox states of enzymes. We have previously reported the speciation of catalytically-relevant intermediate states of *Escherichia coli* (*E. coli*) [NiFe] hydrogenase 1 (Hyd1) at different electrochemical potentials and pH environments via a spectroelectrochemical technique. This method utilizes precise electrochemical control on single protein crystals and *in-situ* IR microspectroscopy to identify redox state(s) present at each applied potential and pH. This maps out potentials and pH conditions at which high purity of redox states are present. In this work, single protein crystals of *E. coli* Hyd1 and Hyd2 at different redox states were generated via electrochemical poising with circulated redox mediator cocktails at desired potentials. The poised crystals were then isolated and frozen to allow structural determination by X-ray crystallography, yielding high diffraction resolution. *E. coli* Hyd1 and Hyd2 show a common set of catalytic states and architecture at the active site but different redox states speciation at a given potential. We hence obtain a hydrogenase structural library which includes most of catalytic intermediate states of [NiFe] hydrogenases. Furthermore, this unprecedented control of redox levels allows us to establish several conformational changes at the proximal FeS cluster at a single redox level of the active site (Ni-B). Overall, this work provides mechanistic and structural insight into [NiFe] hydrogenases, as well as demonstrating a novel methodology to obtain crystals of redox enzymes in intermediate states relevant to catalysis that is readily transferrable to other redox active systems.

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Differences in Zn^{2+} binding properties of human metallothioneins natural variants

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Human metallothioneins (MTs) are small proteins with a high amount of cysteine residues involved in zinc and copper homeostasis, detoxification of heavy metals, reactive oxygen species (ROS), and alkylating agents. MTs family consists of four main isoforms and their numerous subisoforms named MT1-MT4, which have 20-21 cysteine residues, allowing them to bind up to seven Zn^{2+} ions playing a buffering function[1]. The reason for such divergence is still poorly understood. Moreover, various single nucleotide polymorphisms (SNPs) of MTs have been proved to occur in the human genome. In small proteins such as MTs, SNPs might lead to changes that impact metal binding. Interestingly, recent research demonstrates the relation between MTs polymorphisms and pathological processes. Thus, two polymorphisms in the coding sequence of MT1A gene were mentioned in literature with the effect of amino acid change T27N and K51R. They were associated with IL-6 plasma concentrations, hair mercury levels, cardiovascular disease, type 2 diabetes, and hepatocellular carcinoma. The most interesting natural variants containing aromatic amino acids occur for MT4, present in the skin. Moreover, polymorphism of MT4 was linked to susceptibility to the toxic effects of lead. Our research is focused on polymorphism as a consequence of amino acid change to investigate its impact on Zn^{2+} binding properties. We produced and purified natural variants of MT1A: T27N, K51R; MT1G: A10del; MT1M: T20K, and MT4: C30Y, R31W, G48D. We analyzed their basic properties, such as Zn^{2+} - binding stoichiometry, and investigated the acidity of cysteine residues. We also determined differences in Zn^{2+} - binding affinities comparing natural variants to wild-type proteins using competition assays with fluorescent zinc probe ZnAF-2F. Differences were also observed in susceptibility to oxidation with thiol-reactive compounds (DTNB) and mild Zn^{2+} chelator (PAR). The results show that a change of one amino acid in a sequence can significantly impact the Zn^{2+} binding properties of MTs. The obtained results shed a new light on interpreting MTs diversity which can affect biological outcome and might help in further investigation of MTs polymorphism.

Financial support by the National Science Center of Poland (Opus grant no 2018/31/B/NZ1/00567) is gratefully acknowledged.

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Ruminococcus gnavus' Sactipeptide RumC1; a Possible Alternative to Conventional Antibiotics?

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Antibiotic resistance is considered as one of the main health challenges around the world. The WHO is alerting that the death toll caused by antimicrobial resistance might reach 10 million cases in 2050 (1). One of the possible alternatives to conventional antibiotics is a class of antimicrobial peptides called RiPPs (Ribosomally synthesized and post-translationally modified peptides) produced by bacteria. *Ruminococcus gnavus*, a strictly anaerobic commensal bacterium residing in the human colonic microbial community (2), possesses a regulon encoding for five peptide isoforms called RuminococcinC (RumC1-C5) (3). These peptides belong to the "Sulfur-to-Alpha Carbon Thioether Peptides" class, also known as "Sactipeptides". In other words, they carry intramolecular thioether bonds between the sulfur atom of a cysteine residue and the Cα of a partner amino acid. These thioether bonds are inserted by Radical-SAM enzymes, called sactisynthases. Two genes encoding for such enzymes are present in the regulon (3).

We recently demonstrated that RumC1 presents a double hairpin structure held by four thioether bonds inserted onto the precursor peptide during the maturation step by a radical-SAM enzyme (4). We also reported that RumC1 possesses a strong antimicrobial efficacy with minimal inhibitory concentrations (MIC) that are similar to or less than those of the reference antibiotics used for priority pathogens including *Clostridium difficile*, *Enterococcus faecalis* and *Streptococcus pneumoniae* (5,6) . Here we will present the biochemical characterizations of the maturation enzyme and the RumC1 peptide, as well as the biological activity of the mature peptide.

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Structural basis for catalytic mechanism and O_2 sensitivity of [FeFe]-hydrogenases

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[FeFe]-hydrogenases are most active H_2-converting catalysts. Understanding its catalytic mechanism and addressing its extreme O_2 sensitivity are among most urgent and challenging tasks. By using X-ray crystallography, the photobiotehnology group in Bochum has made several important progresses in the recent years in the field. The structures of catalytic cofactor variants[1-4] and site-directed mutagenesis variants targeting on the proton transfer pathway[5-7] provided us important information on its catalytic mechanism and properties. The detailed structural analysis on the O_2-exposed [FeFe]-hydrogenases shed light on its degradation process upon O_2 contact[8]. Furthermore, the structure of recently isolated [FeFe]-hydrogenase from *Clostridium beijerinckii* reveals its unique molecular morphing mechanism by which the enzyme reversibly transits between the active states and inactive but O_2-protected state[9]. To further tackle these questions, X-ray crystallography in the aim of obtaining sub-angstrom structures and neutron crystallography is under progress.

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Thiol-ene/Oxidation tandem reaction by heterogeneous artificial metalloenzymes

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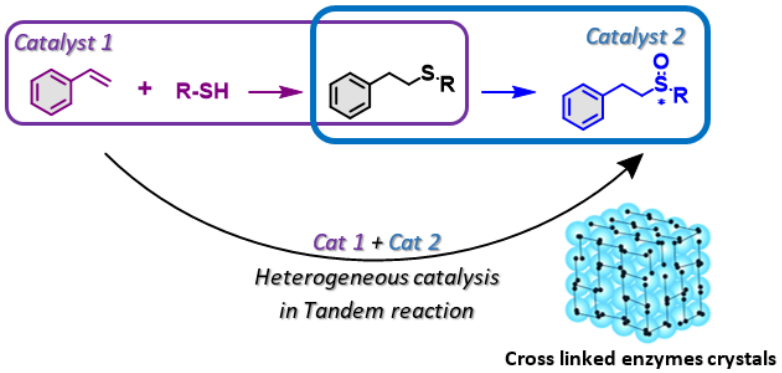
Nowadays, industrial processes relies on many toxic reagents for both health and environment, especially concerning oxidation reactions. Our interdisciplinary project aims to propose greener and more sustainable alternatives to these synthesis conditions through a new heterogeneous catalytic methodology based on the design of bio-inspired artificial metallo-enzymes (ArMs).[1] To go further, the project aims at a cascade of reactions, including a hydrothiolation followed by a sulfoxidation in order to add a sulfoxide function on an alkene (see scheme). Our strategy relies on the use of an Fe(III) complex capable of an efficient thiol-ene reaction with an excellent yield and the use of a V(IV) complex, known to transform a sulfide into a sulfoxide.[2] However, when the tandem reaction is performed with these two catalysts in solution (homogeneous catalysis), enantiomeric excesses and yields are quite moderate. The first aim of this project is then to improve the sulfoxidation step by finding a new catalyst to increase the *ees* and yields. For this purpose, catalysis will be performed under heterogeneous conditions by using cross-linked enzyme crystals (CLEC) *via* the insertion of complexes into crystals of NikA, a Ni importer from *E. coli*, allowing thus to work with harsher conditions (low pH, high temperatures, organic solvents…).[3] To do that, we are modifying Bölm's VO chiral aminophenolate complexes and analogs by introducing a carboxylate moiety essential for their anchoring into NikA. Currently, four new vanadium complexes have been synthesized and their efficiency in NikA-based CLEC is under study.



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L-tyrosine-bound ThiH structure reveals C-C bond break differences within radical SAM aromatic amino acid lyases

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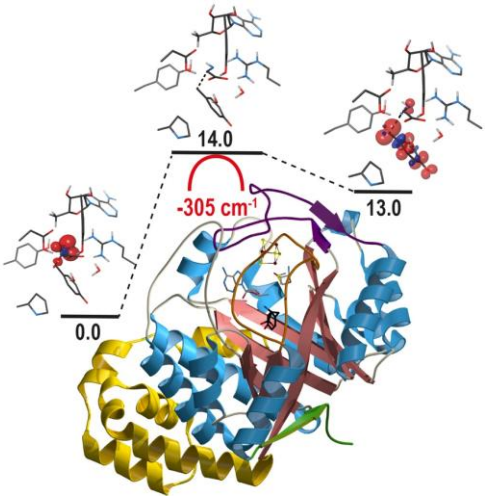
The radical S-adenosyl-L-methionine (SAM) L-tyrosine lyase ThiH catalyzes the L-tyrosine Cα-Cβ bond break to produce dehydroglycine and *p*-cresol[1-3] while the radical SAM L-tryptophan lyase NosL cleaves the L-tryptophan Cα-C bond to produce 3-methylindole-2-carboxylic acid.[4] Understanding which features condition one C-C bond break over the other one has long remained an open question because the two enzymes display significant primary structure similarities[5] and presumably similar substrate-binding modes. The crystal structure of L-tyrosine bound ThiH from *Thermosinus carboxydivorans* reveals an unusual protonation state of its substrate upon binding. Structural comparison of ThiH with NosL[3] and computational studies of the respective reactions they catalyze show that substrate activation is eased by tunneling effect and that subtle structural changes between the two enzymes affect, in particular, the hydrogen-atom abstraction by the 5´-deoxyadenosyl radical species, driving the difference in reaction specificity.[6]



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A Theoretical Exploration of the Electronic Structure, Bonding, and Reactivity of Dinickel(II)-Dichalcogenide {Ni_2E_2} complexes

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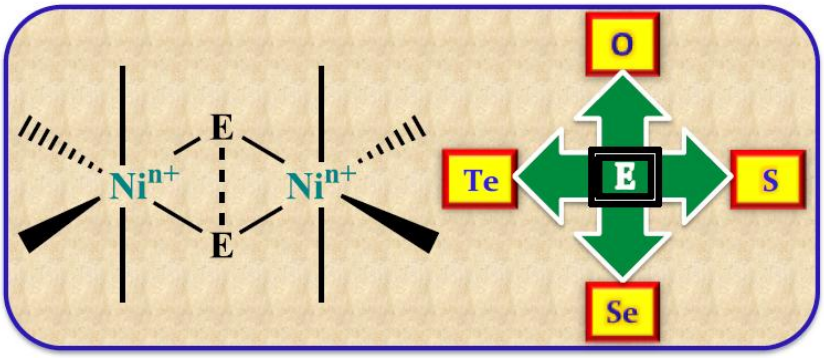
Here, we have performed theoretical studies on the dinuclear nickel complexes with dichalcogenides {Ni_2E_2} for exploring their bonding, structure, and reactivity characteristics. For this study we have chosen four different dinickel dichalcogenide {Ni_2E_2} complexes (where, E=O, S, Se, Te) *viz.* {Ni_2O_2}(**1**), {Ni_2S_2}(**2**), {Ni_2Se_2}(**3**) and {Ni_2Te_2}(**4**) which are supported by 1,4,7,10-Tetramethyl-1,4,7,10 tetraazacyclododecane ligand. Here, we have attempted to predict the correct spin state of all the **1**, **2**, **3,** and **4** complexes with {Ni_2E_2} core, electronic structure, and bonding using the Density Functional Theory tool. Several reports of C—H activation reaction by Ni_2O_2 core with many different ligands are there but C—H activation with dinickeldiselenide complex {Ni2Se2} is rarely studied. Keeping in mind this fact further reactivity tests for all the four Ni_2O_2, Ni_2S_2, and Ni_2Se_2 and Ni_2Te_2 complexes have been tested towards DHA.



Structural and dynamical characterization of Gram-positive copper-sensitive CopY transcription factor using EPR spectroscopy.

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Transition metal ions are essential co-factors for many enzymes, but they can also be highly toxic. Therefore, bacterial metal ion homeostasis is extremely important to ensure sufficient intracellular levels of metal ions for use as co-factors, but also limit excess intracellular level to prevent toxicity. Metal transcription factors are proteins that regulate the in-cell metal concentrations in bacteria. These metal transcription factors have evolved metal coordination sites that “sense” specific metals ions by forming specific coordination complexes; this, in turn, functions to activate or inhibit DNA binding or transcription activation, thereby controlling the expression of genes that mediate what must be an exquisitely selective adaptive response. Copper is also an important transition metal for most organisms, albeit toxic at high levels. CopY is a copper responsive repressor found in Gram-positive bacteria. At low ambient copper concentrations, CopY is present as a Zn(II)-containing homodimer and is bound to the operator-promotor region of the operon. When the level of medium copper is raised, Cu(I) displaces Zn(II), and CopY is released from the DNA, allowing the transcription of CupA and CopA. the 3D structure of the full S. pneumoniae CopY has not been resolved yet, leaving many open questions regarding the structure-function relation of this transcription factor. In this study we utilized Electron paramagnetic resonance (EPR) spectroscopy to follow changes in the structure and dynamics of the protein in the absence and presence of DNA as a function of the copper/zinc binding. This work provides mechanistic information on the CopY transcription factor.

In cristallo Cascade Oxidation reaction using cross-linked metalloenzyme bearing two active sites

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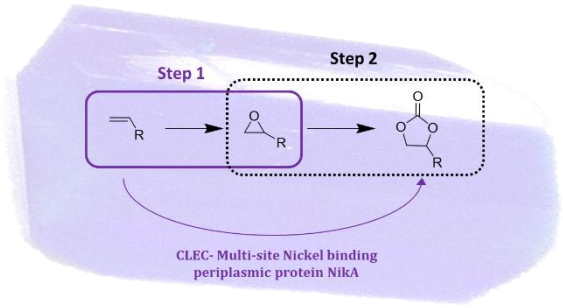
Catalysis represents a corner stone of sustainable chemistry and among all the different strategies, biocatalysis is one of the most promising. Nevertheless, it is still challenging due to the low stability of enzymes in organic media and their narrow reaction scope. Artificial metalloenzymes (ArMs) are a merging concept as an alternative to enzymes. Although it was first investigated and published in the early 70s, the applications in the field of oxidative catalysis remains scarce.[1–3] In an attempt to fill the gap in this domain, we aim to apply the concepts of ArMs, carbon dioxide revalorization and bioinspired heterogeneous catalysis to the synthesis of cyclic carbonate through cascade oxidation reactions.



Scheme 1

The synthesis of cyclic carbonates from alkene derivatives involves an epoxidation and a carbonatation step and thus requires two catalysts (scheme 1). Therefore, starting from the periplasmic Nickel binding protein NikA, we aim to produce a heterogeneized ArM following the method previously optimized by the team that will include two active sites.[4] Accordingly, the first catalyst will occupy the protein's native cavity via supramolecular interactions as proven by previous results and the second site will be created by covalently anchoring our desired catalyst following a Michael's addition on a cysteine residue.[4]

Since wild type NikA does not contain any cysteine residue, we produced several NikA cysteine mutants through directed mutagenesis. On the other hand, we have designed and synthesized cobalt and manganese Schiff-base complexes bearing a Michael's acceptor. We then proceeded to perform anchoring assays on cysteine mutants in protein crystals. We have obtained promising results on the epoxidation step using cross-linked crystals of the NikA-cysteine mutant on which the catalysts of our interest has been grafted. We are also carrying through catalysis investigations on CO2 insertion and will present our attempts on the tandem reaction.

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A multicopper oxidase as ultimate artificial photosynthetic mimic

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Electron transfer (ET) reactions are of fundamental importance in biological processes like photosynthesis. Understanding how electrons are transported in these complex systems may help chemists to discover new tools to perform multielectronic sustainable chemical transformations. Currently, it is a major interest to design photocatalytic systems that use light energy to drive natural enzymes to perform both chemical oxidation and reduction reactions.[1] The multicopper oxidase laccase couples the mono-electronic oxidation of a wide range of substrates (at a T1 surface located Cu^{2+} center) to the 4-electron reduction of O_2 into H_2O (at an embedded tri-nuclear Cu^{2+} center TNC) via successive intra-molecular ETs. Here, a strategy is presented for achieving a controlled orientation of a ruthenium-polypyridyl photosensitizer graft at the surface of a fungal laccase. Laccase variants are engineered by site-directed mutagenesis with unique surface-accessible lysine residues (designated UNIKs) located at a desired position on the protein' surface. Ruthenium-polypyridyl-modified UNIKs are obtained by reductive alkylation of lysine residues,[2] allowing to trigger and probe photoinduced ET processes from discrete surface grafting points relative to the redox-centers (both T1 and TNC). In none of these Ru-UNIK hybrids the presence of the graft compromises significantly the catalytic efficiency of the enzyme on the substrate 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and on the O_2 reduction. In the presence of a sacrificial electron donor and under constant white light irradiation, the Ru-UNIK hybrids undergo photoreduction, in which both rate and extend of reduction seems to be intrinsically dependent on the grafting location. In addition, under these experimental conditions, electrons can be directly injected in the TNC to be, subsequently, transferred to the T1 center in a reverse intra-molecular ET process. The same effect is observed for O_2 reduction, the catalytic efficiency of which hybrid is improved under continuous irradiation depending on the grafting position. These results suggest that this approach is useful to study intra-molecular ET and the repartition of electrons on the 4 Cu^{2+} ions.

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Thermally Induced Spin Transition in Nitrito-Myoglobin as Revealed by Resonance Raman Spectroscopy

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During the last years, nitrite (NO_2^-) was highlighted as a physiological storage pool supporting NO signalling during metabolic stress and its bio-activation involves enzymatic and non-enzymatic reactions in blood and tissues under hypoxic conditions.[1] Specifically, NO_2^- is reduced to bioactive NO via reaction with metalloproteins possessing nitrite reductase (NIR) activity or through acidification. Myoglobin is among the heme proteins that demonstrate NIR activity and contributes to NO_2^- reduction. The reaction pathway involves NO_2^- binding to the heme Fe and thus, elucidating the structural properties and electronic configuration of heme-NO_2^- adducts is of profound importance. In this work, we employed resonance Raman spectroscopy to characterize the heme-NO_2^- adduct formed upon the reaction of metmyoglobin with NO_2^-, as previous crystallographic and spectroscopic studies have provided conflicting evidence regarding the binding mode (O-nitrito versus N-nitro) and spin configuration.[2-6] The resonance Raman experiments show that nitrite binding to the heme Fe forms a high-spin heme Fe-O-N=O nitrito species at room temperature, which undergoes a thermally-induced spin transition at low temperature with retention of the O-nitrito binding.

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Analysis of the Binding Mode of Dipeptidic Molecules and Non-native Substrates to Cytochrome P450BM3

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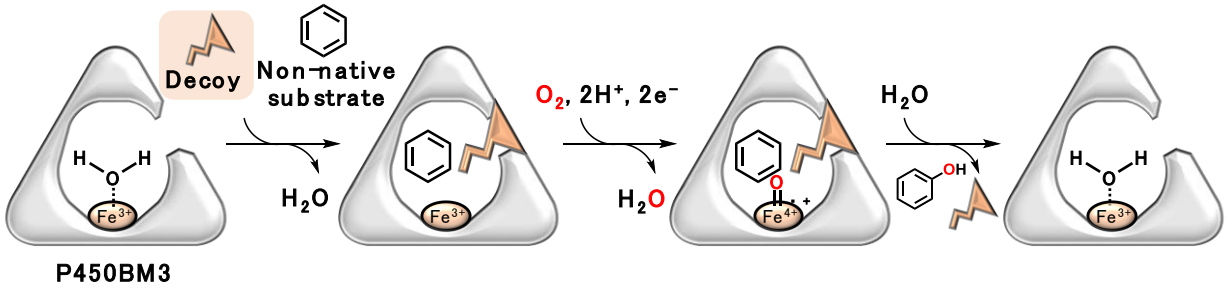
Cytochrome P450BM3 (CYP102A1, P450BM3) is a heme enzyme which catalyzes hydroxylation of long chain fatty acids at an extremely high rate. The turnover rate of P450BM3 is the highest among reported P450s. While the enzyme does not hydroxylate non-native substrates such as benzene, we achieved benzene hydroxylation by wild-type P450BM3 by adding amino acid derivatives.[1] We named such functional molecules “decoy molecules.” Decoy molecules activate P450BM3 by binding to the substrate access channel of P450BM3 in a similar manner to native substrates. However, decoy molecules themselves are not hydroxylated because of shortage of chain length. The small reaction space for non-native substrate hydroxylation is therefore formed at the catalytic site (Figure). The structure of decoy molecules has been improved to enhance catalytic activity of P450BM3. Recently, we demonstrated that the systematic screening of dipeptidic derivatives is effective way to discover more effective decoy molecules in benzene hydroxylation.[2] However, activation mechanism in the reaction system is still unclear.

Herein, we performed isothermal titration calorimetry (ITC) analysis of the binding of ligands such as fatty acids and decoy molecules to P450BM3 to discuss the difference of the binding mode between fatty acids and the dipeptidic decoy molecules developed by the screening. We utilized liposome-ITC method for the titration of the hydrophobic ligands.[3] The thermodynamic parameters indicated that the binding of the ligands is entropy driven. In addition, the binding conformation of non-native substrate was also clarified by X-ray crystal structure analysis of co-crystal of P450BM3, decoy molecule and non-native substrate. From the results of the



experiments, we discuss the activation mechanisms of P450BM3 by decoy molecules

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Directed Evolution of Cytochrome P450BM3 to Be Optimized for Natural Decoy Molecules

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Cytochrome P450BM3 (P450BM3) isolated from *Priestia megaterium* is a heme enzyme that catalyzes hydroxylation of long-chain fatty acids at their sub-terminal positions.[1] P450BM3 shows the highest catalytic hydroxylation activities toward natural substrates among P450 enzymes because of its self-sufficient structure and its active site optimized for the natural substrates. Despite its high activity, P450BM3 does not hydroxylate non-native substrates like small organic molecules due to its high substrate specificity. Our research group has reported that synthetic dummy substrates "decoy molecules" enable efficient hydroxylation of non-native substrates such as propane and benzene by P450BM3 without mutation.[2] P450BM3 misrecognizes and binds decoy molecules which are shorter than natural substrates and it results in formation of space for non-native substrates above heme in its active site. Subsequently, non-native substrates are accommodated above heme and hydroxylated by the active species of P450BM3. Although some decoy molecules induce high benzene hydroxylation by P450BM3 (at most 405 min^{-1}),[3] synthesis and addition of decoy molecules remains required, which causes environmental load owing to organic solvent or chemical wastes.

In this research, we applied directed evolution in order to engineer P450BM3 mutants recognizing natural products as decoy molecules.[4] Herein we will discuss the catalytic activities, X-ray crystal structures of evolved mutants and the effects of natural decoy molecules.

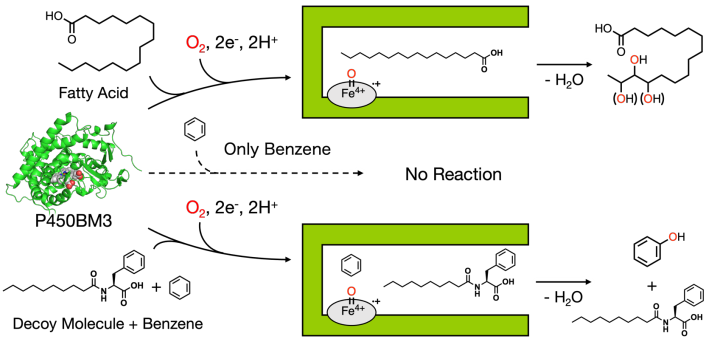

Figure 1 Hydroxylation of long-chain fatty acid (upper) and benzene (lower) by P450BM3

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Artificial hemoproteins incorporating porphyrin derivatives bearing metal coordination sites

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Heme substitution is one of the most powerful methods to change the function of hemoproteins. Generally, the structures of the incorporated synthetic complexes are limited to those like heme because heme-binding sites of hemoproteins are designed mainly for heme. In contrast, heme acquisition system protein A (HasA) having its unique heme-binding site highly exposed to the solvent (Fig 1-a) can capture various synthetic complexes other than heme such as iron(III)-salophen and iron(III)-phthalocyanine.[1] Recently, we have found that HasA can capture iron(III)-tetraphenylporphyrin (Fe-TPP), which is the first example of a stable complex between a hemoprotein and a TPP derivative (Fig. 1-b, c).[2] Since TPP derivatives have been applied to catalysts and building blocks of supramolecular architectures in the field of coordination chemistry, it is expected to develop new functions of HasA based on the various TPP derivatives reported. Focusing on the crystal structure of Fe-TPP HasA complex (Fig 1-c), Fe-TPP is coordinated by His32 and Tyr75 of HasA in the same manner as heme, and two phenyl groups of Fe-TPP are exposed to the solvent. In this study, we have attempted to introduce metal coordination sites to HasA using TPP derivatives.[3]

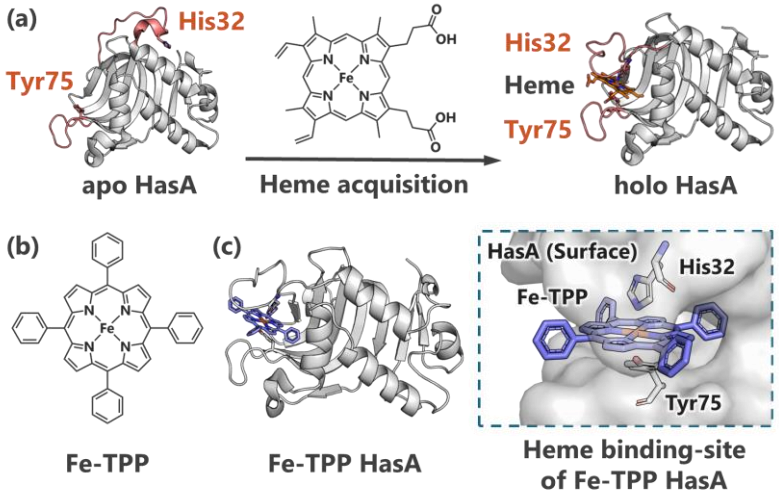

Fig. 1 (a) Heme acquisition system protein A (HasA), (b) Fe-TPP, (c) Crystal structure of Fe-TPP HasA

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Understanding the assembly machinery of the nitrogenase active site

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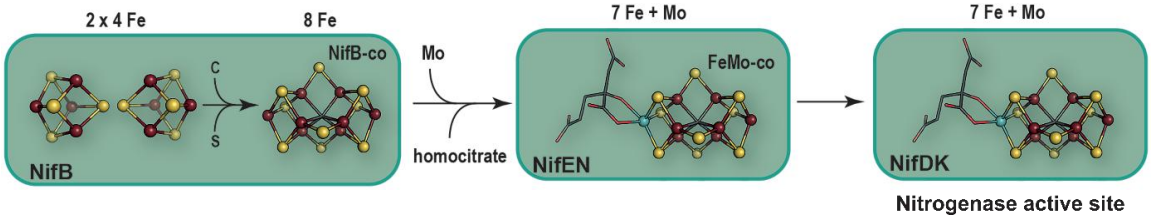
Reduced nitrogen in the form of ammonia (NH_3) is fundamental to all life and many industrial processes. The Haber-Bosch process, which converts hydrogen and nitrogen to ammonia, made ammonia fertilizer widely available and significantly increased crop yield in a short time. However, it is an exceedingly energy-demanding process that requires high temperature and pressure, largely driven by fossil-fuel and leaves a massive carbon footprint throughout the production[1].

Interestingly, nature has always been a huge player in nitrogen fixation, yet in a more sustainable way. Indeed, about half of the nitrogen intake of the human body comes from an enzyme called nitrogenase – the only enzyme known to be capable of reducing N_2 to NH_3 at ambient temperature and pressure. The FeMo-co active site of nitrogenase is a [$MoFe_7S_9C$-*R*-homocitrate] center – perhaps one of the most sophisticated metalloclusters that exist in nature. It is synthesized by different accessory proteins that constitute the NIF (NItrogen Fixation) assembly machinery[2]. NifB is considered as the key enzyme in this mechanism because it is responsible for the fusion of two [Fe_4S_4] centers, combined with a carbide ion insertion and the addition of a sulfide ion to produce a [Fe_8S_9C] precursor termed NifB-co[3]. By combining X-ray crystallography, spectroscopy and *in vitro* analyses, we have identified the presence of a unique 8-Fe intermediate prior to the formation of the NifB-co[4]. In addition, the scaffolding protein NifEN also plays an important role in the machinery as it receives NifB-co from NifB protein and tailors the cluster into the final nitrogenase cofactor FeMo-co[3]. Using computational modelling approach, we were able to predict some interactions between NifB and NifEN. This result can be combined with practical structural study and functional analysis of the NifEN-NifB complex to elucidate the intermolecular interactions between these components, as well as define the order of mechanisms involved in components recruitment and cluster transfer.

This study provides a deeper understanding of the biosynthesis of the nitrogenase active site and its unique chemistry. This in turn may help inspire the development of more efficient catalysts for the production of ammonia.



Schematic illustration of the biosynthesis of the nitrogenase FeMo cofactor

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EPR spectroscopy on the selenium labeled nitrogenase FeMo-cofactor. A strategy to analyze intermediate states?

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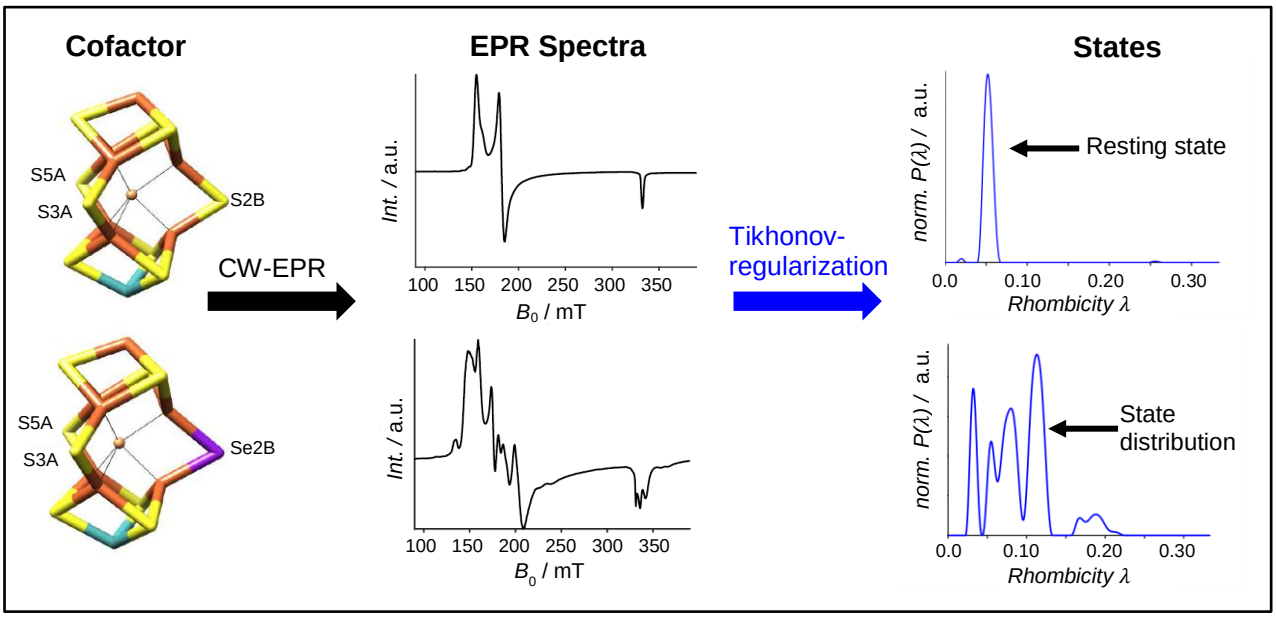

Nitrogenase is an enzyme that catalyses the fixation of N_2 at ambient temperature and pressure. Understanding its mechanism is therefore of high interest. The metal center that is responsible for nitrogen fixation is called FeMo-cofactor and is a [7Fe:9S:Mo:C]:homocitrate moiety. A kinetic scheme for the reaction of the enzyme was devised by Lowe and Thorneley in the 1980s. In this model, the FeMo-cofator requires 8 electrons and at least 8 protons for a single turnover cycle of a N_2 molecule[1]. Some years ago, the sulfides in the equatorial belt of the cofactor, especially at the 2B-position, were shown to be exchangeable by selenium during turnover of the enzyme[2-4]. Based on these observations, we used EPR spectroscopy to analyze the selenium-labeled FeMo-cofactor. Compared to the non-labeled cofactor in its resting-state, we observed several different, coinciding states rather than a single signal. To analyze and interpret these EPR spectra, we applied a Tikhonov-regularization. With this method we were able to obtain a probability distribution of a series of different states of the FeMo-cofator, and we show that in accordance with published data, these states can be attributed to early states in the Lowe-Thorneley-scheme [5-7]. Therefore, we consider the strategy of selenium-labeling together with EPR spectroscopy and Tikhonov-regularization of the resulting spectra as a promising method to analyze intermediate states of the nitrogenase FeMo-cofactor.

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Low pH hinders N_2OR biogenesis and causes N_2O emissions

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Several microbially mediated processes within the nitrogen cycle can generate nitrous oxide (N_2O). Of these, denitrification is the dominant source in most ecosystems. The only known physiological sink for this greenhouse gas is the enzyme N_2O reductase (N_2OR), which reduces it to harmless N_2. The N_2O/N_2 product ratio of denitrification shows strong, negative correlation with soil pH. This empirical knowledge arose from both field and laboratory observations. We have demonstrated that the problem is due to a post-transcriptional hampering of the synthesis of this protein at pH ≤6.1[1], but the exact mechanism remains unresolved. We studied the model denitrifiers *Paracoccus denitrificans* (Pd) and *Pseudomonas stutzeri* (Ps). The bacteria exhibit the same subordination of N_2O reduction to ambient pH as seen in nature, i.e. they reduce N_2O to N_2 at pH above 6.1, but not below. We have traced the N_2OR in Pd cells grown under denitrifying conditions at pH 6 and pH 7 and analyzed the proteomes of these cells. Our results demonstrate the presence of N_2OR in the periplasm in comparable amounts in cells grown under both pH regimes. However, no N_2O reduction took place in cultures that synthesized the enzyme at pH 6.0. Moreover, we expressed N_2OR from Ps[2] in *E. coli* cells grown under strictly controlled pH regimes ranging from pH 5.7 to 7.3 and characterized it by UV-Vis spectroscopy and ICP mass spectrometry. The detailed spectroscopic analysis of Ps N_2OR revealed the absence of copper in N_2OR developed at pH below 6. A fully matured functional enzyme contains twelve copper atoms per homodimer. Our findings corroborate the hypothesis of impaired N_2OR assembly in the periplasm under suboptimal low pH

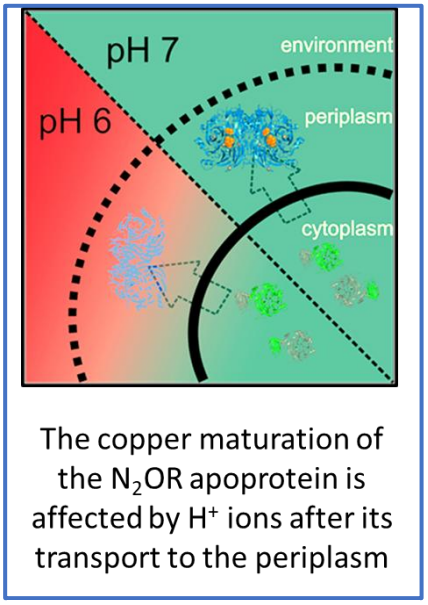

Figure 1. Hypothesis

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conditions, stressing the importance of soil pH management in order to mitigate the N_2O emissions.

Structural insight into the RNF complex, a widespread membrane-bound NADH:ferredoxin oxidoreductase

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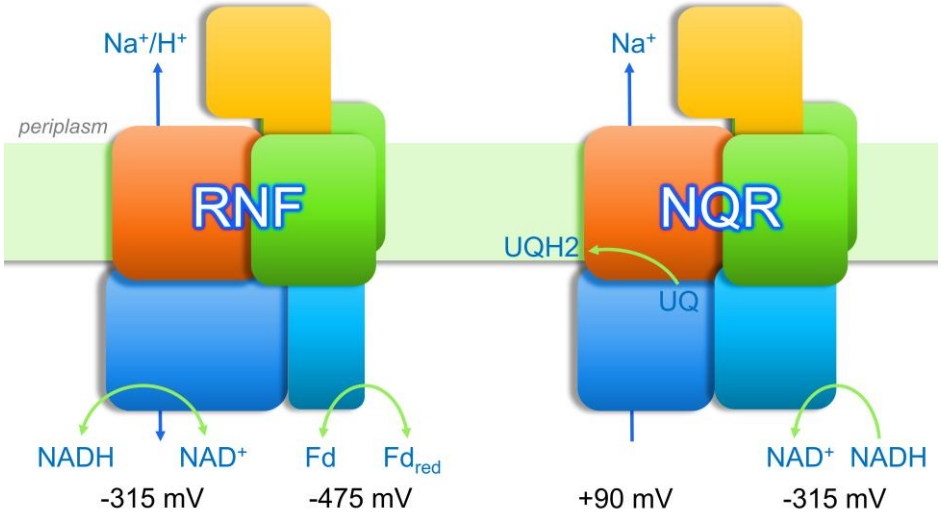
The *rnf* gene cluster was first discovered in ***R****hodobacter capsulatus* for their essential role in **n**itrogen **f**ixation[1], and soon found present in many prokaryotes[2]. The RNF complex is a redox-driven ion (Na^+ or H^+) pumping transmembrane oxidoreductase which mediates the following reaction[3]: NADH + 2 Fd_{ox} + $\Delta\mu H^+/Na^+$ ⇋ NAD^+ + 2 Fd^-_{red} + H^+. In most cases, the Rnf complex has been considered to oxidize reduced ferredoxin, transfer the electron across the cell membrane and back to reduce NAD^+, and contribute to the formation of an ion gradient coupled with ATP synthesis[4, 5]. In diazotrophs, the reverse electron flow from NADH to ferredoxin driven by exploiting the proton motive force facilitates the generation of low-potential electron for nitrogen fixation. However, the architecture of the RNF complex has not been elucidated. Here we report the cryo-EM structure of the nitrogenase-associated RNF complex of *Azotobacter vinelandii*, a seven-subunit membrane protein assembly that contains multiple flavin and iron-sulfur cofactors. Although the RNF complex shares certain similarity to the NQR complex[6], their functionalities work in different redox spans.



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Utilizing EPR spectroscopy to resolve metal-sensitive transcription mechanisms

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CueR is a metalloregulator protein that “senses” Cu(I) ions with very high affinity, thereby stimulating DNA binding and the transcription activation of two other metalloregulator proteins. The crystal structures of CueR when unbound or bound to DNA and a metal ion are very similar to each other, and the role of CueR and Cu(I) in initiating the transcription has not been fully understood yet. Using nitroxide spin labeling (MTSSL) and double-histidine (dHis)-based CuI(I) spin labeling, double electron-electron resonance (DEER) measurements and structure modeling, we investigate conformational changes that CueR undergoes upon binding Cu(I) and DNA in solution. We observe three distinct conformations, corresponding to apo-CueR, DNA-bound CueR in the absence of Cu(I) (the ‘repression’ state), and CueR-Cu(I)-DNA (the ‘activation’ state). We suggest that more than one Cu(I) ion binds per CueR monomer, leading to changes in site-specific dynamics at the Cu(I) binding domain and at the distant DNA binding site. We propose a detailed structural mechanism underlying CueR’s regulation of the transcription process. The mechanism explicitly shows the dependence of CueR activity on copper, thereby revealing the important negative feedback mechanism essential for regulating the intracellular copper concentration.

Structural insights into darobactin biosynthesis

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Darobactin A is a recently discovered antibiotic that proved to be effective against Gram negative pathogens[1]. It belongs to the family of ribosomally synthesized and posttranslationally modified peptides (RiPP) and is unique because it contains an ether and a C-C crosslink that stabilize its structure. Darobactin is synthesized by the radical SAM (rSAM) protein DarE, through a complex mechanism involving the formation of an ether crosslink, between two tryptophans of the propeptide DarA, that originates from molecular oxygen[2]. Since its discovery, several variants of darobactin have been identified from DarA and DarE homologs. Analysis of both, DarE and DarA, sequences suggests that the coevolution of the substrate and the tailoring enzyme confers a specificity of darobactin against different bacteria. By combining sequence analysis and in silico models we identified structural determinants of DarE and DarA involved in substrate recognition that are essential for darobactin A biosynthesis. We experimentally demonstrate the essential role of the N-terminal of DarE for activity and that substrate recognition is mediated by highly conserved amino acid residues in DarA and DarE.

References

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2. Nguyen, H. *et al*. Submitted. 2021.