Bioinspired Oxygen Atom Transfer: Light-Induced Activation of Molybdoenzyme Mimics

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Nature uses the ability of molybdenum to switch between oxidation states (VI), (V) and (IV) to catalyze oxygen atom transfer (OAT) reactions. By studying biomimetic *cis*-dioxo-molybdenum complexes with imine-containing ligands, we found that the catalytic activity of these complexes can be enhanced through irradiation with visible light (Figure 1). Inspired by the design of the molybdoenzymes, in which the two electrons that are released upon substrate oxygenation are moved sequentially, one at a time, by biological electron transfer units, we exploited ligand-to-metal charge transfer (LMCT) transitions that populate an antibonding Mo=O orbital to support photocatalytic OAT. As seen in thermal OAT chemistry, electron-withdrawing groups (EWGs) enhance activity, indicating the accumulation of negative charge in the rate-limiting transition state. A further important feature of the photoactive molybdenum complexes is that under the chosen experimental conditions the formation of inert [(O)Mo-O-Mo(O)] complexes is not restricting catalytic turnover. The catalysts, which can be synthesized from inexpensive, commercially-available precursors, are tolerant to both water and oxygen.

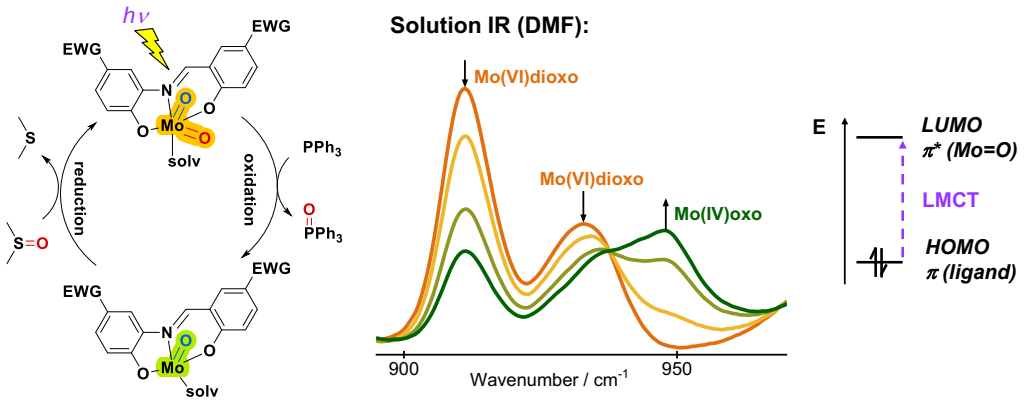

Figure 1. *Left:* General photocatalytic OAT cycle; *Centre:* FTIR spectra showing a Mo(VI)dioxo-catalyst before OAT (orange line) and the Mo(O)-intermediate formed after OAT (green line); *Right:* Schematic representation of catalyst activation *via* LMCT.

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The mitochondrial amidoxime reducing component (mARC) as basis for the clinical candidate N-succinyloxy dabigatran

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The mitochondrial amidoxime reducing component (mARC) is the most recently discovered molybdenum enzyme in mammals. In concert with the electron transport proteins, NADH cytochrome b5 reductase and cytochrome b5 it reduces N-oxygenated structures [1]. This plays a major role in *N*-reductive drug metabolism [2].

The discovery of the enzyme in my laboratory was connected to the activation of *N*-hydroxyamidines (= amidoximes) as prodrug for amidines [1-3]. The prodrug principle *amidoximes instead of amidines* [3] is based on the lower basicity of amidoximes, which are not protonated under physiological conditions and are thus absorbed from the gastrointestinal tract [1-3].

After absorption, the amidoximes are reduced to the active amidines by mARC.

In the lecture, the new prodrug N-succinyloxy-dabigatran will be introduced using amidoxime esters as prodrugs which are hydrolysed by esterases to amidoximes and then reduced to amidines by mARC1 and mARC2.

Dabigatran is an orally administered direct thrombin inhibitor and belongs to the class of anticoagulants. It has a zwitterionic structure and is not absorbed after oral administration. It is used as dabigatran etexilate, a carbamate prodrug, which is converted to dabigatran by esterase-catalysed hydrolysis in the liver and plasma [4]. Problems of dabigatran etexilate and the active dabigatran are mainly based on its poor solubility [5, 6]. We improved the oral bioavailability by means of different *N*-hydroxy prodrug strategies, including selected *O*-conjugates of the dabigatran amidoxime. These conjugates exhibited improved solubilities. All new prodrugs were orally available, with N- succinyloxy-dabigatran as the best candidate and a comparable *in vivo*- PK profile to dabigatran etexilate in rat and human. The prodrug N-succinyloxy dabigatran can be used both as an orally and for intravenous administration, the latter of which is not possible with the marketed dabigatran etexilate.

For oral application, no special formulation for N-succinyloxy dabigatran is required. The stability of N-succinyloxy dabigatran is excellent and no problems occurred during long-time storage. The suitability of the new prodrug has been shown in animal experiments and in a first study in humans. N-succinyloxy dabigatran is the first highly soluble representative of the new class of orally available anticoagulants.

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Enzymatic, spectroscopic and structural investigations of the respiratory arsenate reductase Arr and the arsenite oxidase Aio to progress in the understanding of arsenic conversion

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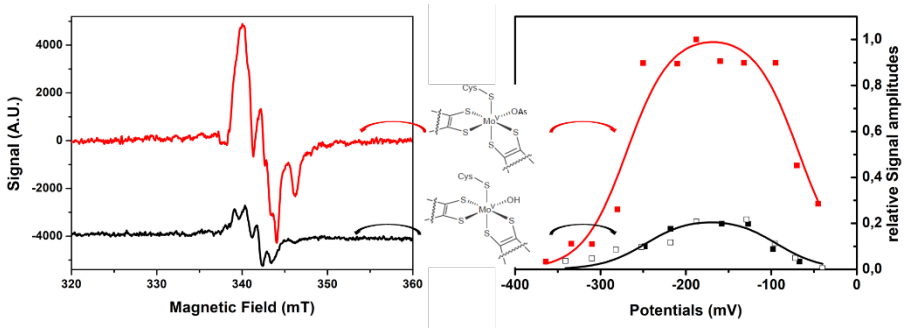
The respiratory arsenate reductase Arr is one of the three Mo-enzymes, with the arsenite oxidases Aio and Arx, involved in arsenic conversion [1-2]. Based on enzymatic, spectroscopic and structural data acquired so far, everything seems to oppose Aio and Arr [3-6], although both belonging to the so-called superfamily of Mo-*bis*PGD enzymes and both converting arsenic compounds. These totally distinct properties have been put forward to propose divergent catalytic mechanisms for arsenic conversion [5,7].
Here, by combining an in-depth enzymatic, spectroscopic, potentiometric and structural examination of the Arr from *Shewanella* sp. ANA-3, and by comparing with the Aio from *Pseudorhizobium banfieldiae* str. NT-26, we tried to reconsider the picture of the perfect opposition drawn up so far. Among others, our results establish for the first time the reversibility of the Aio activity. In addition, we characterized the spectral and redox properties of the Mo(V) species in Arr and discuss its significance in the catalytic cycle of the enzyme.



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From Mystery to Mechanism: Amidoxime Reducing Components as Players in Plant Nitric Oxide Synthesis?

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Key Words: Amidoxime reducing component (ARC), nitric oxide, nitrate reductase, plant physiology

The amidoxime reducing component (ARC) is the last identified molybdenum cofactor (Moco)-containing enzyme, found exclusively in eukaryotic organisms. In mammals and land plants, two *ARC* gene copies are consistently present, whereas certain non-mammalian animals (such as zebrafish), fungi, and green algae possess only a single *ARC* gene copy. Since its discovery in pig liver extracts in 2006, mammalian ARCs have been extensively studied in terms of structure and activity, but their precise physiological role remains unclear (1).

In contrast, studies on the ARC from *Chlamydomonas reinhardtii* (crARC) have shown that it is a soluble cytosolic protein capable of accepting electrons not only from the cytochrome b_5 reductase/cytochrome b_5 system, but also from another heme and FAD containing enzyme, nitrate reductase (NR). This interaction of crARC and NR turned out to be essential for the reduction of nitrite to nitric oxide (NO) in *Chlamydomonas* (2). We examined whether plant ARC functions as a dedicated NO-forming nitrite reductase in various higher plants. While our previous work demonstrated that this specific function could not be confirmed in the model plant *Arabidopsis thaliana* (3), functional analysis in the rhizobium-forming model plant *Lotus japonicus* suggested that *Lotus* ARCs may participate in NO production at a physiological level (4), however, the results are not as conclusive as for crARC. As mammalian mARCs are known to have a broad substrate spectrum, and recent research suggests a possible involvement in lipid metabolism, recent results on the search for potential substrates and metabolic functions will be presented.

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Molybdate high affinity transporters in plants and fungi

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Keywords: Molybdate, molybdate uptake, transport proteins

High affinity molybdate uptake in lower and higher eukaryotes is facilitated by a single transport protein MoT while in bacteria a multicomponent system is operating ($ModAB_2C_2$). In my presentation, I will summarize the work of our lab to clone and characterize the diverse molybdate high affinity transporters in higher plants and in fungi. There are two families of molybdate transporters known in eukaryotes, MoT1 and MoT2, showing different sequence motifs. While mammals harbor only MoT2, plants possess both transporter types, MoT1 and MoT2, and in the filamentous fungus *Neurospora crassa* we identified a transporter of the MoT1 type. In the model plant *Arabidopsis thaliana*, we identified altogether 5 MoT proteins that were characterized for their subcellular micro-compartmentation and their function in temporary vacuolar storage, in interorgan allocation of molybdate and developmental expression. I will also present the structures (AlphaFold2) of all plant and fungal MoT proteins.

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Where metabolism and virulence meet – sulfoxide reductases as determinants of bacterial survival in *Haemophilus influenzae*, *Escherichia coli* and beyond.

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When inferring metabolic properties of bacteria from new environments, protein sequence identity is often used to determine potential functions of new proteins. But – what happens if the inferred function makes 'no sense' in the environment a bacterium inhabits? This was the question that started our research into the cellular function of S-/N-oxide reductases such as the DmsABC DMSO reductase in *Haemophilus influenzae*, where we demonstrated that DmsABC is essential for survival in the host, but does not support anaerobic energy generation with DMSO, which is the accepted function of this enzyme in *E. coli*. A range of similarities between the *E. coli* and *H. influenzae* DmsABC enzymes such as high sequence similarities and similar regulatory patterns then led us to investigate, whether, in fact the *E. coli* DmsABC S-oxide reductase has a main functional role in anaerobic energy generation as has been postulated in the literature. Our results indicate that like its counterpart in *H. influenzae* the *E. coli* DmsABC S-/N-oxide reductase re-reduces oxidatively damage biomolecules such as precursors for NAD and pyrimidine formation and is specifically induced in the presence of host-produced antimicrobials, such as hypochlorite and hydrogen peroxide, suggesting a role in host-pathogen interactions rather than anaerobic energy generation. *E. coli* uses no less than 7 functionally related S-oxide reductases to protect itself from host-induced sulfoxide stress, and phylogenetic analyses reveal a large range of unstudied DmsABC-like S-oxide reductases including in Terrabacteria, indicating further and potentially different roles for these enzymes in bacterial physiology.

The Active Site of *Cupriavidus necator* Formate Dehydrogenase from X-ray Absorption Spectroscopy and Density Functional Theory Calculations.

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Key Words: Formate dehydrogenase, X-ray absorption spectroscopy, density functional theory

The formate dehydrogenase (FDH) enzymes catalyze the oxidation of formate to carbon dioxide (1). FDH enzymes with Mo and W are known, but in both cases the metal is coordinated to two molybdopterin dithiolene cofactors, with additional chalcogenide donors from cysteine or selenocysteine, depending on the enzyme, and an additional sulfur atom, which is thought to be a terminal sulfido (e.g. Mo=S) in the fully oxidized enzyme. Significantly, the active site appears to lack any oxygen coordination to the metal, which is consistent with current views on the mechanism (1). *Cupriavidus necator* formate dehydrogenase is a molybdenum enzyme with cysteine at its active site. We have used a combination of Mo K-edge X-ray absorption spectroscopy (XAS) (2) and density functional theory (DFT) to approach an improved understanding of the structures that active site of the enzyme can adopt. The possibility of a Mo=S group in the oxidized enzyme will be discussed, together with the structures for the reduced and superoxide inactivated forms of the enzyme.

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2016-00092). M.Z is a Fellow in the NSERC CREATE to INSPIRE (CREATE 555378-2021 to I.J.P., G.N.G. & others). Use of the Stanford Synchrotron Radiation Lightsource, SLAC National Accelerator Laboratory, is supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences under Contract no. DE-AC02-76SF00515. The SSRL Structural Molecular Biology Program is supported by the DOE Office of Biological and Environmental Research, and by the National Institutes of Health, National Institute of General Medical Sciences (including P41GM103393 and P30GM133894).

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Advancing our Understanding of mARC and Type I DMSO Reductase Catalysis

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Key Words: Molybdenum, DMSO reductase family, mitochondrial amidoxime reducing component

We will discuss our latest results regarding geometric and electronic structure contributions to catalysis in pyranopterin molybdenum and tungsten enzymes, with a specific emphasis on the mitochondrial amidoxime reducing component (mARC), periplasmic nitrate reductase (Nap), and new Type I DMSOR enzymes. We will present recent results from our laboratories that show how mARC differs from sulfite oxidase,(1) illustrate how the structure of *Campylobacter jejuni* nitrate reductase NapA contributes catalysis,(2) and enrich our understanding of an emerging pyranopterin molybdenum enzyme family from the human gut microbiota. A combined structural, biochemical, computational, and spectroscopic approach has been employed to define the salient geometric and electronic structure features necessary for catalysis in these enzymes. This has allowed for new insights into catalytically relevant intermediates and the relationship between active site geometric and electronic structure and how this promotes catalysis.

Acknowledgments
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Decoding and Taming Microbial Nitrogenases for CO_2 Conversion

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Key Words: Nitrogenase, N_2 fixation, CO_2 Reduction

Abstract: Nitrogenases are the only known enzymes that catalyze the reduction of molecular nitrogen (N_2) to ammonia (NH_3). The N_2 reduction drives the biological nitrogen fixation and the global nitrogen cycle. Besides the conversion of N_2, nitrogenases were recently shown to convert carbon dioxide (CO_2) to carbon monoxide, formate and hydrocarbons,(1-5) suggesting CO_2 to be a competitor of N_2 (Fig. 1). However, the impact of omnipresent CO_2 on N_2 fixation has not been investigated to date.

We comprehensively studied the competing reduction of CO_2 and N_2 by the two nitrogenases of *Rhodobacter capsulatus,* the molybdenum (Mo)- and the iron (Fe)-nitrogenase *in vitro* and *in vivo* (Fig. 1).[(5)] We identified significant differences among the Mo- and Fe-nitrogenase systems.[(5)] *In vitro,* the Fe-nitrogenase is almost threefold more efficient in CO_2 reduction and profoundly less selective for N_2 than the Mo-isoform under mixtures of N_2 and CO_2. Also *in vivo*, inside *R. capsulatus* the Fe-nitrogenase reduces CO_2 readily into formate and methane, under physiological CO_2 concentrations and in the presence of the main substrate N_2. The *in vivo* CO_2 activity of the Fe-nitrogenase facilitates the light-driven accumulation of formate and methane, one-carbon substrates for other microbes, and feedstock chemicals for a circular (bio)economy.

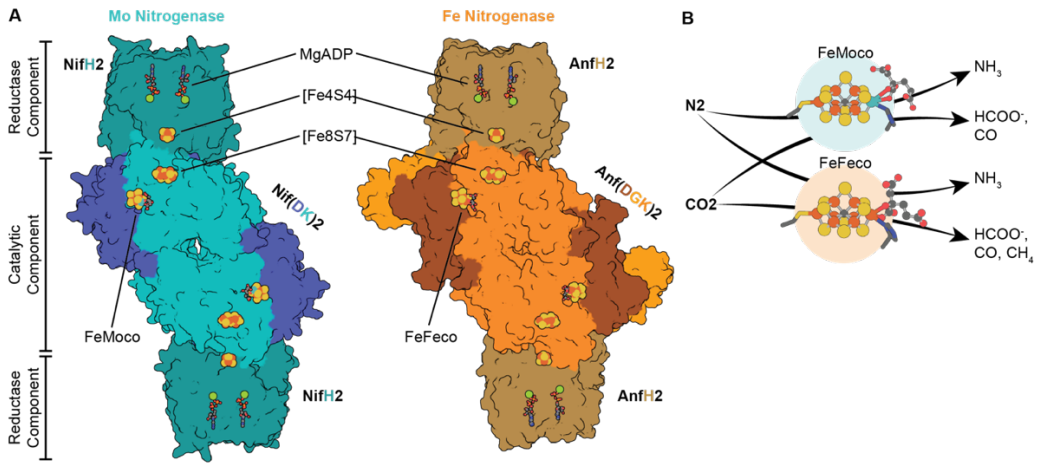

Figure 1. **The nitrogenase composition and activities of *R. capsulatus*.** (**A**) Architecture of the Mo- [left, (PDB): 7UTA] and Fe- (right, PDB: 8OIE) nitrogenase with the contained metalloclusters and cofactors highlighted in ball-and-sticks models. For both nitrogenases, electrons are delivered by the [Fe_4S_4] cluster of the reductase component and transferred via the [Fe_8S_7] cluster (P-cluster) to the FeMoco/FeFeco in the active site of the catalytic component. (B) Product spectrum of Mo- (top) and Fe- (bottom) nitrogenase for the reduction of N_2 and CO_2. Adapted from (5).

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Heterologous synthesis of a simplified nitrogenase analog in *Escherichia coli*

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The heterologous synthesis of a nitrogen-fixing system in a non-diazotrophic organism is a long-sought-after goal because of the crucial importance of nitrogenase for agronomy, energy, and the environment. Here, we report the heterologous synthesis of a two-component nitrogenase analog from *Azotobacter vinelandii*, which consists of the reductase component (NifH) and the cofactor maturase (NifEN), in *Escherichia coli*.[1] Metal, electron paramagnetic resonance, and activity analyses verify the cluster composition and functional competence of the heterologously expressed NifH and NifEN. Nuclear magnetic resonance, nanoscale secondary ion mass spectrometry, and growth experiments further illustrate the ability of the NifH/NifEN system to reduce N_2 and incorporate the reduced N into the cellular mass. These results establish NifEN/NifH as a simplified nitrogenase analog that could be optimized and engineered to facilitate transgenic expression and biotechnological adaptations of this important metalloenzyme.

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Modular synthesis of nitrogenase in *Escherichia coli* via a bioinorganic-synthetic biology approach

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Heterologous expression of nitrogenase has been actively pursued because of the far-reaching impact of this enzyme on agriculture, energy and environment. Yet, isolation of an active two-component, metallocentre-containing nitrogenase from a non-diazotrophic host has yet to be accomplished. Here, we report the heterologous synthesis of an active Mo-nitrogenase by combining genes from *Azotobacter vinelandii* and *Methanosarcina acetivorans* in *Escherichia coli*.[1] Metal, activity and EPR analyses demonstrate the integrity of the metallocentres in the purified nitrogenase enzyme; whereas growth, nanoSIMS and NMR experiments illustrate diazotrophic growth and ^{15}N enrichment by the *E. coli* expression strain, as well as accumulation of extracellular ammonia upon deletion of the ammonia transporter that permits incorporation of thus-generated N into the cellular mass of a non-diazotrophic *E. coli* strain. As such, this study provides a crucial prototype system that could be optimized/modified to enable future transgenic expression and biotechnological adaptations of nitrogenase.

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Insights into the catalytic mechanism of Arsenite Oxidase from crystallographic data on substrate-bound complexes, complemented by photo-reduction studies

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Key Words: Arsenite Oxidase; X-ray crystallography; radiation damage; metalloenzyme, molybdopterin

Arsenite Oxidase (Aio) is a Mo-dependent enzyme from the DMSOR family and exhibits unique features such as the lack of a protein ligand coordinating the active site metal as found in almost all members of the family [1]. The functional form of the enzyme consists of a large subunit, AioA, and a small subunit, AioB. The catalytic subunit AioA harbours the proximal and the distal molybdopterin guanine dinucleotide cofactor (P-MGD and D-MGD, respectively) that coordinate the Mo active site, where As^{III} is oxidized to As^{V} and one [3Fe-4S] centre. The smaller subunit AioB harbours one Rieske [2Fe-2S] centre that receives electrons from the [3Fe-4S] centre and shuttles them to specific electron acceptor proteins [2]. Aio has been identified in multiple prokaryotes, but experimental structural data is only available for Aio from *Alcaligenes faecalis* (*Af*) [1,4] and from *Pseudorhizobium banfieldiae* str. NT-26 [3,4]. Four high-resolution structures (up to 1.44 Å) of Aio were obtained in complex with As/Sb oxyanions bound to the active site. These allowed us to revisit the catalytic mechanism of As^{III} oxidation which combined with site-directed mutagenesis and activity assays, provided information on important catalytic-relevant residues [4]. Additionally, novel crystallographic data enabled to observe for the first time, the initiation step of the reaction, corresponding to Aio in its oxidized state (1.87 Å, unpublished data), related to the photo-reduction of the enzyme active site by monitoring the reaction as the radiation dose increases during X-ray data collection.

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Tuning Catalysis in Molybdopterin Enzymes

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Biology frequently uses molybdenum (Mo) and tungsten (W) as catalysts for oxygen atom or hydride transfers to a variety of carbon, sulfur and nitrogen centers. In the case of formate oxidation by formate dehydrogenase, the formate is oxidized by formal hydride transfer without the generation of non-productive byproducts such as H_2 through a carefully tuned redox center and proton transfer pathway.[1, 2] Periplasmic nitrate reductase, NapA, has a similar metal coordination environment, but has been proposed to catalyze formal oxygen atom transfer.[3] To understand these very different catalytic outcomes, despite superimposable peptide structure and high conservation between the enzymes, we need to look beyond the cofactors that share geometry, ligands, and oxidation state changes. Our recent work has identified two residues in the secondary coordination environment that facilitate this transition between two fundamentally different reactions. The implications of these residues on tuning the Mo center for catalysis will be discussed.

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Mo-Catalysed Nitrite Reduction? - an Electrochemical Perspective

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Key Words: nitrite, nitric oxide, voltammetry, molybdenum

Nitric oxide (NO) is an essential signaling molecule central to biological processes such as platelet aggregation and adhesion, innate immune response, vasodilation (blood vessel widening), cellular injury and post-translational protein modification (S-nitrosylation) [1]. The mammalian enzyme nitric oxide synthase (NOS) generates NO through an *oxidative* process using molecular oxygen and arginine as reactants. Without oxygen, NOS is inactive, yet NO is still produced in hypoxic tissues containing high concentrations of nitrite. Biochemical *reduction* of nitrite to NO (equation (**1**)) could account for these observations. While *bacterial* Cu- and heme-dependent nitrite reductase enzymes are well known [2], *no known nitrite reductase enzymes are found in plants or animals* leading to the hypothesis that they may possess enzymes that 'moonlight' as nitrite reductases under hypoxic conditions in addition to their normal function.

$$NO_2^- + 2H^+ + e^- \rightleftharpoons NO + H_2O \qquad (1)$$

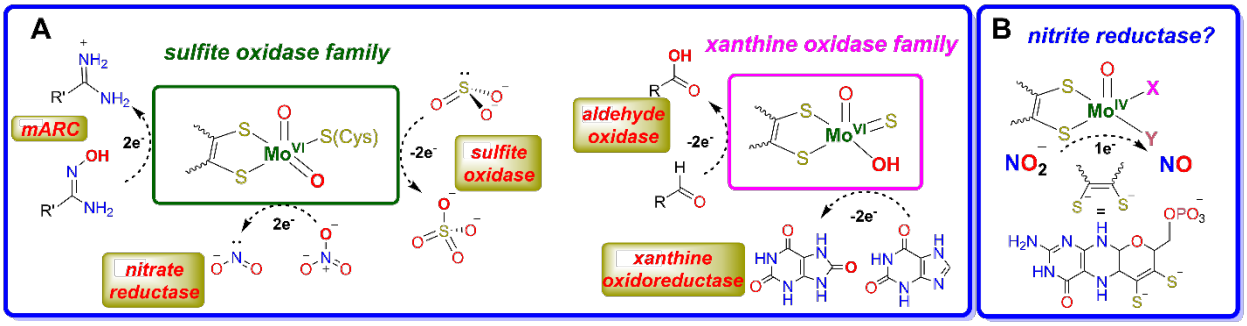

Figure 1 (**A**) the 5 eukaryotic Mo-enzymes and their reactions (**B**) nitrite reduction to NO.

The five eukaryotic Mo-dependent enzymes (**Fig. 1A**) have all been linked to NO synthesis through nitrite reductase activity (**Fig. 1B**) [3-8] with NO detection in biological tissues being the primary method of analysis. Given the complexity of this medium and the reactivity of nitrite, consensus is lacking on the relative abilities of the Mo-enzymes in **Fig. 1A** to act as nitrite reductases. We have used electrochemistry to examine the activity of some of the Mo enzymes in **Fig. 1A** to reduce NO_2^- to NO under physiologically relevant conditions and our latest results from this investigation will be presented.

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Structural and EPR spectroscopic investigations of pH-dependent Mo(V) species in *Thermus thermophilus* sulfite dehydrogenase

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Key Words: Keywords: sulfite dehydrogenase, Mo(V), EPR, HYSCORE, DFT

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 damage [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 (SDH) in prokaryotes. To decipher their catalytic mechanism, we have previously provided structural and spectroscopic evidence for direct coordination of HPO_4^{2-} to the Mo atom at the active site of the SDH from the hyperthermophilic bacterium *Thermus thermophilus* (*Tt*SDH), mimicking the proposed sulfate bound intermediate proposed to be formed during catalysis [3,4]. By solving the X-ray crystallographic structure of the unbound enzyme, we resolved the changes in the hydrogen bonding network in the molybdenum environment that enable the stabilization of the previously characterized phosphate-adduct [5]. In addition, the electron paramagnetic resonance (EPR) spectroscopic study of the enzyme over a wide pH range reveals the formation of pH-dependent Mo(V) species, a characteristic feature of eukaryotic SOEs. The combined use of continuous wave/pulsed EPR (HYSCORE) spectroscopies, H_2O/D_2O exchange and density functional theory calculations allowed the detailed characterization of a typical low pH Mo(V) species previously unreported in bacterial SOEs, underlining the conservation of the active site properties of SOEs irrespective of their source organism.

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Electrocatalytic Generation of Transition Metal Hydrides for CO_2 Reduction to Formate

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Key Words: CO_2 reduction, formate, hydride transfer

The thermochemistry for key bond-making and -cleavage steps for H+ reduction to H2 and CO2 reduction to HCO_2^-, and how to apply these properties to elicit product selectivity will be discussed.[1] Our recent work in this area has led to the discovery of a catalyst that reversibly catalyzes CO2 reduction and HCO_2^- oxidation.[2] Reversible reactivity is a property that had previously only been observed in the formate dehydrogenase enzymes, and indicates the catalyst is operating near the thermodynamic potential, or very low overpotential.

Synthetic approaches for inorganic molecular catalysts have exclusively relied on classic metal hydrides, where the proton and electrons originate from the metal (via heterolytic cleavage of an M–H bond). An analysis of the scaling relationships that exist in classic metal hydrides reveal that hydride donors sufficiently hydridic to perform CO_2 reduction are only accessible at very reducing electrochemical potentials, which is consistent with known synthetic electrocatalysts. By comparison, the formate dehydrogenase enzymes operate at relatively mild potentials. In contrast to reported synthetic catalysts, none of the major mechanistic proposals for hydride transfer in formate dehydrogenase proceed through a classic metal hydride. Instead, they invoke formal hydride transfer from an orthogonal or bidirectional mechanism, where the proton and electrons are not colocated. The thermodynamic advantages of this approach for favoring CO_2 reduction at mild potentials, along with guidelines for replicating this strategy in synthetic systems will also be discussed.[3]

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Synthesis and Reactions of Mo(VI)-Cu(I) Complexes Supported by Heterodinucleating Ligands as Models of Mo-Cu CODH

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Key Words: Mo-Cu CODH, carbon monoxide, isocyanide, heterobimetallic complexes

Our research focuses on the development of biomimetic Mo-Cu heterobimetallic complexes that serve as functional models of Mo-Cu CO dehydrogenase (CODH). Mo-Cu CODH from aerobic bacteria such as *Oligotropha Carboxidovorans* is an enzyme that oxidizes CO to CO_2 according to the following equation: $CO + H_2O \rightarrow CO_2 + 2H^+ + 2e^-$. Deciphering the molecular mechanism of Mo-Cu CODH can facilitate the development of efficient aerobic catalysts to remediate dangerously high local concentrations of toxic CO. The enzyme features a unique heterobimetallic active site containing nucleophilic Mo(VI)-oxo linked through a single-atom bridge (sulfido) to a low-coordinate Cu(I). Both metals are required for the oxidation of CO and related substrates (i.e. isocyanide), suggesting cooperative bimetallic cooperativity. While several feasible mechanisms have been postulated based on structural, computational, and spectroscopic studies, these mechanistic hypotheses have not been probed empirically in a functional model. We hypothesize that a functional CODH model can be assembled using an appropriate heterodinucleating ligand containing hard and soft chelates for the selective coordination of the respective metals. Our previous models (employing xanthene-based heterodinucleating ligands) enabled (1) cooperative Mo(VI)-Cu(I) bimetallic reactivity and (2) isolation of a stable heterobimetallic complex. However, we were not able to create a model which enabled both. Our current research focuses on isolable heterodinuclear complexes, supported by new xanthene-based and dibenzobarallene-based ligands, that are capable of reactions with the desired substrates. Structural, spectroscopic, and computational studies will be presented.

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Bioinspired Mo-Containing Metal-Sulfur Clusters for Small Molecule Activation

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Key Words: Metal-Sulfur Cluster, Nitrogenase, Small Molecule Activation, Catalysis

Metal-sulfur clusters, which are composed of multiple metal and sulfur atoms in their inorganic cores, represent a key class of natural transition metal complexes. Owing to their flexible redox properties, certain metal-sulfur clusters serve as active sites in enzymes that catalyze the reduction of small molecules such as N_2 (nitrogenase) and CO_2 (CO dehydrogenase).

We have been exploring the activation of inert small molecules using synthetic metal-sulfur clusters. Our recent work includes the synthesis of a series of cubic $[Mo_3S_4M]$ clusters with a defined reaction site M,[1,2] and their application in N_2 activation[3] as well as catalytic N_2 reduction.[4,5] This presentation will primarily focus on the N_2 activation by molybdenum-containing metal-sulfur clusters. In addition, this presentation will address preliminary results from our ongoing studies on the direct catalytic conversion of CO_2 into CH_4, an area of significant interest due to the potential use of CO_2 as a carbon feedstock, building upon our previous finding that a $[MoFe_5S_9]$ cluster catalyzes the conversion of CO_2 to short-chain hydrocarbons with limited turnovers.[6]

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Probing oxygen sensitivity of nitrogenase to assist engineering of nitrogen-fixing crops

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Key Words: synthetic biology, biotechnology

A long-standing goal of plant biotechnology has been to engineer bacterial nitrogenase into plants so that they may be self-sufficient for their nitrogen requirements. The extreme oxygen sensitivity of nitrogenase is a major barrier to achieving this goal. One strategy that has been employed is to express nitrogenase proteins in the mitochondria as the rapid rate of oxygen consumption through respiration could theoretically provide the anoxic conditions required. There is some encouraging precedent, from tests in yeast, suggesting mitochondria can provide the anaerobic conditions required for nitrogenase.

To date however, nitrogenase proteins expressed in plant mitochondria are purified without any metal clusters and therefore show very low levels of activity. This raises the question: is the mitochondria in plant cells sufficiently anaerobic for nitrogenase function? Furthermore, what happens when nitrogenase components are exposed to oxygen, can they be repaired or are they irreversibly damaged?

We probe the effects of oxygen on some key nitrogenase components; namely NifH, AnfH and the iron-cluster biosynthesis protein NifU. The effect of oxygen is tested using activity assays and other spectroscopic techniques such as Mössbauer spectroscopy, EPR and circular dichroism in an effort to answer some of these questions.

Bioinspired Molybdenum Complexes for Sulfur Atom Transfer Catalysis

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The growing interest in homogeneous sulfur atom transfer (SAT) catalysts arises from their applications in crude oil desulfurization and the conversion of abundant elemental sulfur into valuable organosulfur feedstocks. While catalysts based on Group 6 elements have shown significant potential, they often suffer from instability in the presence of oxygen. In recent years, we have developed efficient molybdenum SAT catalysts with enhanced tolerance to oxygen and water compared to existing systems. We present a series of μ-oxo-bridged dinuclear Mo(V) complexes that remain dormant until activated by the presence of substrates. To gain deeper mechanistic insights, we have decoupled the catalytic cycle and isolated key catalytic intermediates, characterizing them through spectroscopic and crystallographic methods. The presentation will also explore the roles of ligand denticity, ligand electronics, and exogenous ligands, as well as the catalyst's dynamic behavior.

Oxygen atom transfer reactions of bio-inspired molybdenum compounds: C-H hydroxylation, sulfide oxidation, and more

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Key Words: xanthine oxidase, DMSO reductase, C-H activation, oxygen atom transfer

This oral presentation will cover two related topics. First, we have targeted functional mimics of the xanthine oxidase active sites toward the long-term goal of Mo-catalyzed C-H hydroxylation. In doing so, we have conducted reactivity studies with $O=W^{VI}=S$ complexes previously reported by Holm[1] and a new $O=W^{VI}=Se$ complex prepared in our lab that serves as a structural model for the nicotinate dehydrogenase active site.[2] Based on the propensity of these complexes to exchange dithiolene ligands and form dimeric species, we shifted focus to $O=Mo^{VI}=O$ complexes supported by tetradentate dithiolate ligands.[3] This led to a breakthrough result in which one such complex was observed to hydroxylate C(sp^3)-H bonds of hydridic character, using substrates inspired by the tetrahedral intermediates proposed to form from xanthine in the native system.[4] Second, we have revisited previously reported bis(dithiolene) complexes of Mo and W,[5] here re-purposing them as catalysts for oxygen atom transfer. Successful catalytic transformations include selective sulfoxide formation from sulfides, including a sulfur mustard analogue,[6] and oxidative C=C cleavage of aryl alkenes.[7]

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Tungsten Complexes as Mimics for Acetylene Hydratase

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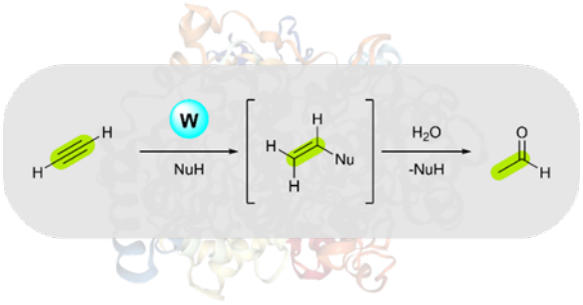
Key Words: Biomimetic, Tungsten, Acetylene, Hydration

Tungsten-dependent acetylene hydratase (AH) catalyzes the net-hydration reaction of acetylene to acetaldehyde. As the mechanism of this transformation is unclear, we develop biomimetic model chemistry as a useful tool for a better understanding.

In recent years we have developed strategies to selectively prepare tungsten acetylene compounds. A series of bidentate nitrogen sulfur donor ligands have been investigated allowing the elucidation of factors leading to selective acetylene coordination. The reactivity towards extra- and intramolecular nucleophilic attack is presented. Our studies reveal that tungsten(IV)-acetylene adducts readily react with nucleophiles to yield η¹-vinyl complexes, consistent with the expected behavior of two-electron donor alkyne ligands. By contrast, tungsten(II)-acetylene adducts exhibit unexpected reactivity, leading to the formation of stable carbyne complexes. This surprising outcome, which arises from a challenging 1,2-H shift, required detailed spectroscopic and computational studies.[1] Depending on the nucleophile, the intermediate vinyl species undergoes hydrolysis under formation of acetaldehyde.[2] The latter represents the first early transition metal compound known to facilitate acetylene hydration, making it a functional model of acetylene hydratase (AH).

With our studies a new perspective on the AH mechanism arises including vinylation of a neighboring amino acid in the active site by acetylene insertion into a W−S or W−O bond before hydrolysis to acetaldehyde.



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W-BioCat – heavy metal enzymes for sustainable industrial biocatalysis

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Key Words: tungsten, aldehyde oxidoreductase, carboxylic acid reduction

Mo and W share remarkable chemical similarity, which extends to biology as these elements share highly similar cofactors (1). Mo enzymes are ubiquitous in nature and can be found among representatives of all kingdoms of life, while W enzymes enzymes are predominantly found in anaerobic bacteria and archaea and have no known representatives in eukaryotes. A critical difference between Mo and W in enzymes are the midpoint potentials of the cofactors, which are significantly lower for W than for Mo (2). *Pyrococcus furiosus* is a hyperthermophilic archaeon that is dependent on W for its growth. This organism has a strong preference for W over Mo that can be attributed to selective uptake, regulation and cofactor biosynthesis (3). *P. furiosus* produces five different tungsten containing aldehyde oxidoreductases (AORs) which are capable of oxidizing a wide range of aldehydes to their corresponding carboxylic acids (1). The enzyme can also catalyze the reverse reaction to produce aldehydes from carboxylic acids under specific conditions (4). This reaction is thermodynamically challenging and requires low redox potential electron donors and capture of the produced aldehyde. Given the strong industrial interests in biotechnologically produced aldehydes, e.g. as natural flavor or fragrance compounds, tungsten containing AORs offer an interesting alternative to conventional chemical routes as well as established carboxylic acid reductases that depend on ATP and NADPH. W-BioCat is a EU funded research project to develop novel expression systems for W-AORs, which will enable exploration of the potential of AORs for chemical synthesis, the development of a hydrogen driven carboxylic acid reduction process, as well as deep mechanistic investigations.

Acknowledgments
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Exploring the mechanistic pathways of tungsten and molybdenum enzymes by means of chemical imagination and multiscale modelling

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The incorporation of tungsten or molybdenum pterin complexes into the active site of enzymes enormously expands the available repertoire of possible chemical transformations. The central metal facilitates two electron redox processes due to stable IV and VI oxidation states and acts as a Lewis acid (with its open ligation position), which can directly activate a bound reagent. Meanwhile, the other ligands of the Mo/W ions participate in the reactions by (i) providing additional means to activate recalcitrant bonds (e.g. in homo or heterolytic C-H cleavage), (ii) enabling hydroxylations without molecular oxygen (by O transfer to activated intermediates), (iii) introducing strong acid/base catalysis (accepting protons or enabling proton transfer electron transfer processes), (iv) tuning the Mo/W redox potentials the by varying their positions, or (v) even participate directly in the redox processes by providing a reservoir for additional electrons. Moreover, many Mo/W-enzymes contain additional redox-active cofactors assembling into “nanowire” structures, which connect the Mo/W-cofactors with other active sites within the enzyme. As a result, many chemically highly challenging reactions are catalysed by Mo/W-enzymes, such as direct reduction of aromatic rings, reduction of CO_2 to formate or of non-activated carboxylic acids to aldehydes, hydroxylation of alkylaromatic or heterocyclic compounds, hydration of acetylene to acetaldehyde, or oxidation of molecular hydrogen. The extremely versatile nature of Mo/W-metallopterin cofactors makes them a fascinating, but also challenging study object which requires application of a wide array of research techniques and flexible chemical imagination, which is always the first step in formulating mechanistic hypotheses. With such hypotheses in place, we can apply both experimental and theoretical tools to test them, improve them and provisionally accept them or discard them entirely as improbable. In this presentation we want predominantly to concentrate on results of our mechanistic investigations conducted for tungsten aldehyde oxidoreductase from *Aromatoleum aromaticum,* which unexpectedly turned out to be able to oxidize molecular hydrogen. This so far unique feature of AOR_{Aa} enables to revert its natural activity towards the reduction of acids, or to provide a cheap tool for NADH recycling. We will present QM and QMMM modelling results showing possible pathways for hydrogen activation and reduction of carboxylic acids by AOR[1]. Furthermore, we will show QMMM modelling results on periplasmic nitrate reductase (NAP) from *Campylobacter jejuni*[2], as well as MD simulations of its interaction with nitrate and substrate analogues. Finally, we will present some speculative, yet untested mechanistic proposals describing the reactions catalysed by aliphatic sulfonate:ferredoxin oxidoreductase (formerly WOR5) from *Pyroccocus furiosus* and acetylene hydratase from *Pelobacter acetylenicus*[3].

Acknowledgement

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Enzymatic conversion of alkanes to chiral alcohols at MoCo: structure, function and chaperon-dependent MoCo insertion of alkane hydroxylase

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Key Words: DMSOR family, alkane hydroxylation, chiral alcohols, chaperone, TAT secretion system

Selective oxyfunctionalisation of alkanes is of paramount importance in organic chemical synthesis, yet remains one of the greatest challenges[1]. Enzymatic hydroxylation of alkanes is typically associated with monooxygenases or peroxygenases. In this study, we identified a previously unknown member of the DMSOR enzyme family in a sulfate-reducing, alkane-degrading Deltaproteobacterium, which uses water as the hydroxylating agent to selectively hydroxylate C10–C18 alkanes to chiral alcohols and dialcohols in presence of ferricyanide as an artificial electron acceptor. Active alkane hydroxylase was produced heterologously together with a specific chaperone (AhyD). It is composed of the AhyABC subunits, with AhyA harbouring the bis-MGD active site cofactor. Cryo-electron microscopy structures of AhyABC with hexadecane bound were consistent with stereospecific hydroxylation of alkanes at C2 and C14. Additional structures were obtained of the AhyABC-AhyD chaperone complex in both the bis-MGD cofactor-bound and -free states. The specific interaction of the AhyD chaperone with the TAT sequence was revealed, providing unprecedented insights into chaperone-dependent MoCo insertion into apo-AhyABC and interaction of the matured enzyme with the TAT secretion system.

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Wolfram jaws – The best way to crunch CO_2?

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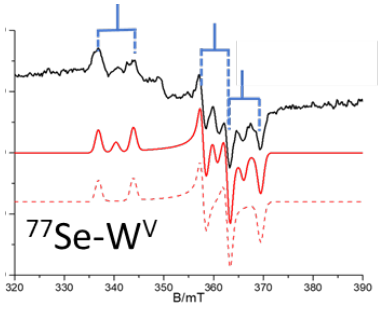
Key Words: Formate dehydrogenase, tungsten cofactor, EPR, HYSCORE, DFT, Catalysis

Molybdenum (Mo) and tungsten (W) enzymes are found in virtually all living organisms where they catalyze a wide diversity of redox reactions. In prokaryotes, most of these enzymes harbour a large Mo/W-*bis* pyranopterin guanosine dinucleotide cofactor in which the metal ion cycles between the +IV and +VI redox states during catalysis. However, in spite of numerous crystallographic and spectroscopic studies, the structure of active site intermediates and catalytic mechanisms are still largely debated. In the case of the +V redox state which is observable by EPR, several species are often detected and their relationships with structural data, enzyme activity and catalytic intermediates remain unclear.

The formate dehydrogenase (FdhAB) isolated from the bacterium *Desulfovibrio vulgaris* Hildenborough is a tungsten and SeCys dependent enzyme showing remarkable properties of CO_2 reduction and O_2 tolerance [1]. Depending on substrate or reductant used, several W(V) species could be observed but their structure could not be directly determined by X-ray crystallography. By using a combination of site directed mutagenesis, EPR analysis of enzyme preparations both in solution and in crystal state, relationships between these species and enzyme active forms could be established [2]. Moreover, by combining *g*-tensor and hyperfine coupling analysis (^{183}W), isotope labeling (^{77}Se) and DFT calculations, structural models of these species could be obtained. The results indicate clearly that SeCys remain bound to the W ion throughout the catalytic cycle [3]. In addition, the protonation state of the W-center was also investigated by HYSCORE spectroscopy and interesting differences in the catalytic mechanism by comparison with Mo-Fdhs will be discussed.



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Nitrogenase: Inside the Black Box

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Key Words:
Nitrogen fixation, iron sulfur enzymes

The bacterial enzyme nitrogenase has the remarkable ability to catalyze the reduction of dinitrogen to ammonia to under physiological conditions. The mechanistic questions related to how nitrogenase overcomes the kinetic stability of the NN triple bond to fix dinitrogen under ambient conditions have intrigued chemists for the past century. We have applied a structure-based approach to examine how nitrogenase uses iron-sulfur metalloclusters and ATP-dependent electron transfer to reduce dinitrogen and other substrates. A puzzling feature of the nitrogenase mechanism has been how to reconcile the relative stability of the FeMo-cofactor with the reactivity towards dinitrogen. Our studies have established that binding of ligands to nitrogenase under turnover conditions can be accompanied by the distortions and rearrangements of the catalytic FeMo-cofactor; these rearrangements may provide clues how the active site is activated during the catalytic cycle. The complementary strengths of X-ray crystallography and electron microscopy are being used to illuminate the mechanistic foundations of this process.

Acknowledgments
The many contributions of, and discussions with, Dr. Rebeccah Warmack, Dr. Jens Kaiser and Prof. Limei Zhang (University of Nebraska, Lincoln) are gratefully acknowledged.

Handle with Care: Trafficking and Maturation of the Nitrogenase FeMo Cofactor

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Key Words: Nitrogen Fixation; Nitrogenase Cofactor; Cofactor Biogenesis; Maturation Machinery; Molybdenum Insertion

There is a remarkable divergence between a postulated core set of nine gene products that are indispensable for producing a functional nitrogenase and the – somewhat hypertrophic – more than one hundred reading frames in the different nitrogenase clusters of the paradigmatic diazotroph *A. vinelandii*. For molybdenum nitrogenase, the assembly of the [Mo:7Fe:9S:C]:homocitrate cofactor requires at least the NifSU, NifB, NifEN and NifV proteins, but many additional factors are involved in the process, for many of which a precise role remains unclear. With several protein complexes involved, the assembly intermediates are mostly shuttled by designated cluster chaperones. Many of these are structurally related and similar to the NifX protein, a designated carrier of the common precursor cluster of all nitrogenases (L-cluster, or NifB-co) from its place of formation on NifB to the maturation scaffold NifEN. Here we discuss the features of the family of NifX-like proteins in *A. vinelandii*, their different roles in cofactor shuttling and the initial steps of cluster transfer to the NifEN scaffold. Furthermore, we find that the requirement for many additional factors in *A. vinelandii* is quite strongly dependent on the metabolic state of the cell and will only reveal a phenotype upon deletion under a specific condition of growth.

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Structure and Mechanism of Nitrate Reduction in NapA: Evidence of oxygen atom transfer and Reversibility.

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Key Words: Nitrate reductase, Oxygen atom transfer, Mechanism, Reversibility

The catalytic subunit of the periplasmic nitrate reductase (NapA) belongs to the DMSO reductase (DMSOR) family of molybdenum cofactor (Moco) containing enzymes. Members of this family catalyze a wide array of reactions, and in some cases, the enzymes function reversibly. Periplasmic nitrate reductases are important for human health, environment, and biotechnological applications. The structure and mechanism of NapA has been described in the literature. At least three mechanisms involving the oxygen atom transfer (OAT) process have been proposed for NapA-catalyzed nitrate reduction. Here, we discuss the Cryo-EM structure of *Campylobacter jejuni* NapA. The structure of the Mo-center is defined with the help of EXAFS and EPR spectroscopic studies. We also provide the biochemical demonstration of OAT in NapA. We disclose the reversible action of NapA, i.e., oxidation of nitrite to nitrate. This is the first demonstration of reversible interconversion of nitrate and nitrite by a nitrate reductase.

Acknowledgments
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Role, insertion and protection of the sulfido ligand in molybdoenzymes from the DMSO reductase family

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Molybdoenzymes are widespread in all domains of life and catalyze key steps in carbon, sulfur and nitrogen metabolism. In the DMSO reductase family of molybdenum enzymes present only in prokaryotes, the molybdenum coordination sphere generally is composed of two dithiolene groups from two molybdopterin (MPT) guanine dinucleotide (MGD) molecules (referred to as bis-MGD cofactor), one amino acid ligand from the protein backbone and a Mo=O group as sixth ligand. Moco biosynthesis in *E. coli* seems to be more complex than previously suggested, since a novel form of the cofactor has been identified recently in addition to modifications in the ligand sphere of the molybdenum atom. As novel Moco intermediate, bis- Mo-MPT has been revealed, a cofactor that is used by the YdhV protein. Additionally, in recent studies it became obvious that several molybdoenzymes harbor a sulfido-ligand instead of the oxo group at the bis-MGD cofactor. This modification was recently identified to be present in the *Escherichia coli* TMAO reductase TorA when purified under strictly anaerobic conditions. The role of the sulfur ligand for the reaction mechanism of these enzymes is not clear so far. Inhibitors like azide or nitrate are routinely employed to protect the active site from oxidative damage in FDH enzymes. For TorA no inhibitor has been identified so far. Also in nitrate reductase, a sulfido ligand has been identified. In FDH enzymes, we were recently able to establish *in vitro* sulfido ligand incorporation to the active site of the oxo-containing bis-MGD cofactor in FDH from *Rhodobacter capsulatus,* that completely reactivated the enzyme. This in vitro sulfuration has been initially established for XDH enzymes. Reductive treatment with either sulfide or bisulfite, or with sodium dithionite under weakly acidic conditions in the strict absence of O_2 resulted in comparable enzymatic activity to FDH purified after heterologous expression in *Escherichia coli*. Confirmation of the inserted sulfido ligand was proven by EPR spectroscopy of a Mo^V intermediate species associated with MoS_6 coordination. Specific insertion of a ^{33}S sulfido ligand to the bis-MGD Mo evidenced the chemical insertion of the sulfido ligand and confirmed its role to serve in defining the electronic character of the sulfurated bis-MGD Mo^V-SH state. These results provide an important benchmark by which targeted *in vitro* sulfuration can be applied in other enzymatic systems without an apparent association of maturation enzymes identified to biosynthetically install a sulfido ligand.

Cryptic Covalent Carbon Carrying Mechanism of Pterin Formation in Molybdenum Cofactor Biosynthesis

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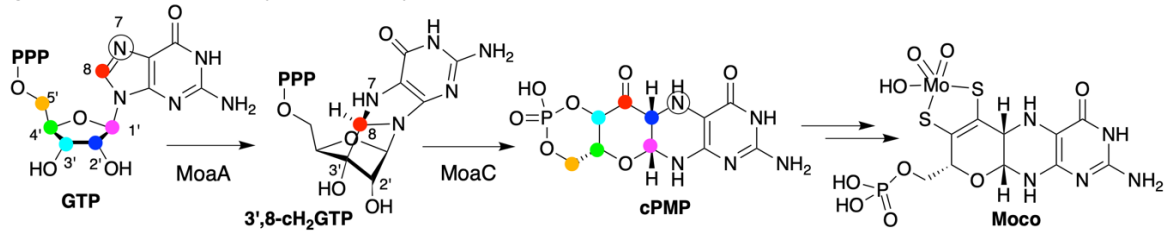
Key Words: Biosynthesis, molybdenum cofactor, MoaC, covalent catalysis, carbon insertion

The characteristic pyranopterin structure of molybdenum cofactor (Moco) is formed by the MoaC enzyme through a complex rearrangement of guanosine 5′-triphosphate (GTP), involving the insertion of the C8 atom between C2′ and C3′ of ribose (1-3), one of the most complex rearrangement reactions in Nature. However, its catalytic mechanism remains ambiguous. Here, we report that, unlike other pterin biosynthetic pathways, the pyranopterin construction by MoaC proceeds through unexpected and cryptic covalent catalysis. Using a chemical reductant in the MoaC reaction with its native substrate or its analog, we successfully trapped and characterized four chemically distinct covalent intermediates that previously eluded detection or even prediction. We also solved crystal structures that demonstrate the covalent linkage between K131 of MoaC and the C8 of the substrate and suggest that this covalent linkage carries the C8 to catalyze the C-C insertion reaction. Together with a comprehensive kinetic characterization, our data reveal the unprecedented use of covalent catalysis as a carbon carrier and reaction navigation role. The results together reveal a novel covalent mechanism of pterin cofactor biosynthesis and extend our knowledge of covalent catalysis in enzymes.



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Neuroprotective Potential of Xanthine Oxidoreductase Inhibitors

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Key Words:
Xanthine oxidoreductase; XOR inhibitor; neuroprotection; ATP maintenance; energy metabolism; purine metabolism

Abstract
Xanthine oxidoreductase (XOR) is a molybdenum-containing enzyme involved in the final steps of purine metabolism, catalyzing the oxidation of hypoxanthine to xanthine and xanthine to uric acid. XOR inhibitors are widely used in clinical practice to manage gout by suppressing uric acid production. However, recent studies have suggested a broader role for XOR inhibitors, implicating their involvement in the pathogenesis and treatment of neurodegenerative and cardiovascular diseases. While previous studies have reported results using mice, this study employed human-derived cells and tissues to bring the findings to closer to clinical application.

In this presentation, we will introduce the latest findings that reflect human-specific metabolic environments regarding the effects of XOR inhibitors on metabolism, particularly the mechanism of neuroprotection through ATP maintenance. Furthermore, we will discuss approaches to enhance the protective effects of XOR inhibitors and their potential applications, along with future prospects.

Acknowledgments

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Reversible CO_2 Reduction by Formate Dehydrogenase. Direct and Mediated Electrochemical Catalysis

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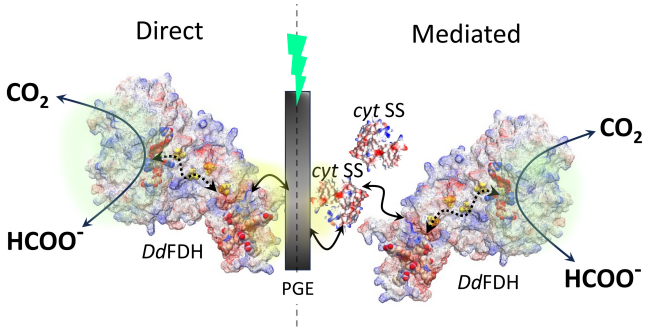
The electrocatalytic reduction of carbon dioxide (CO_2) to formate by the enzyme formate dehydrogenase (FDH) makes use of the enzyme's observed reversibility, offering a promising strategy for the mitigation of CO_2 and the production of value-added compounds. To enhance the catalytic potential of *Desulfovibrio desulfuricans* FDH (*Dd*FDH), a range of artificial and natural redox cofactors were investigated using electrochemical methods. These studies included direct (non-mediated) conditions and mediated conditions employing viologens (methyl and benzyl viologens), and small heme proteins (cytochromes). Methyl viologen, acts as efficient mediator for CO_2 reduction achieving very high current density. The studies of the different small proteins, namely cytochrome split-soret (cyt SS), cytochrome c3 (cyt c3) and cytochrome c552 (cyt c552), allow the identification of the potential natural physiological partners.



Scheme showing non-mediated (direct) and mediated electron transfer with *Dd*FDH (a small cytochrome, *cyt* SS [Split Soret] is used as an example for MET).

Acknowledgments

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On the Mechanism of Action of Formate Dehydrogenases

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Key Words: formate dehydrogenase, hydride transfer, oxygen exchange

The molybdenum- and tungsten-containing formate dehydrogenases from a variety of microorganisms catalyze the reversible interconversion of formate and CO_2[1], several in fact function as CO_2 reductases in the reverse direction under physiological conditions[2, 3]. CO_2 reduction catalyzed by these enzymes occurs under mild temperature and pressure rather than the elevated conditions required for current industrial processes. Given the contemporary importance of remediation of atmospheric CO_2 to address global warming, there has been considerable interest in the application of these enzymes in bioreactors. Equally important, understanding the detailed means by which these biological catalysts convert CO_2 to formate, a useful and easily transported feedstock chemical, might also inspire the development of a new generation of highly efficient, biomimetic synthetic catalysts[4]. Here we have examined the ability of the FdsDABG formate dehydrogenase from *Cupriavidus necator* to catalyze the exchange of solvent oxygen into product CO_2 in the course of formate oxidation under single-turnover conditions. Negligible incorporation of ^{18}O is observed when the experiment is performed in $H_2{}^{18}O$, indicating that bicarbonate cannot be the immediate product of the enzyme-catalyzed reaction, as has been previously concluded[5]. These results, in conjunction with the observation that the reductive half-reaction exhibits mildly acid-catalyzed rather than base-catalyzed chemistry, are consistent with a reaction mechanism involving direct hydride transfer from formate to the enzyme's molybdenum center, directly yielding CO_2. Our results are inconsistent with any mechanism in which the initial product formed on oxidation of formate is bicarbonate.

Acknowledgments

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Bacterial formate hydrogenlyase enzymes

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Key Words: formate dehydrogenase; [NiFe] hydrogenase; hydrogen-dependent carbon dioxide reduction; complex I-like; membrane-bound hydrogenase

Formate hydrogenlyase (FHL) is a widespread family of membrane-bound enzymes that brings together a molybdenum- or tungsten-dependent formate dehydrogenase and a second metalloenzyme that belongs to the 'Group 4' of nickel-dependent hydrogenases. FHL complexes share an evolutionary origin with the Complex I NADH dehydrogenase and are predominantly found in prokaryotes associated with the ability to carry out anaerobic fermentation (Peters & Sargent, 2023).

A well characterized member of the FHL family is the formate hydrogenlyase-1 (FHL-1) complex from *Escherichia coli* (Steinhilper *et al*. 2022). The structure of the enzyme reveals a catalytic arm, containing all the metal centers and both active sites, and a membrane arm, which is a minimalist version of the Complex I membrane arm containing just two integral membrane proteins. FHL-1 can function both as a hydrogen-producing enzyme (Metcalfe *et al*., 2022) and as a hydrogen-dependent carbon dioxide reductase (HDCR) capable of capturing gaseous CO_2 as aqueous formic acid (Roger *et al*., 2021). Current projects are focusing on engineering synthetic metabolic pathways into *E. coli* in order to enable growth on H_2 and CO_2 only, and thus ultimately facilitate sustainable production of commodity biochemicals from waste gases.

A second member of the FHL family is formate hydrogenlyase-2 (FHL-2). A functional version of FHL-2 is found in *Pectobacterium atrosepticum* (Finney *et al*., 2019) and comprises a larger membrane arm of five proteins, which is much closer to the structure of bacterial Complex I and other membrane-bound hydrogenases. In this work, a 15 kbp gene cluster from *P. atrosepticum* has been cloned and shown to be functional in an *E. coli* host. Unlike FHL-1, FHL-2 is not reversible *in vivo* and does not generate formic acid from CO_2.

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Catalytic mechanism and oxygen tolerance in a W/Sec-dependent formate dehydrogenase from *Nitratidesulfovibrio vulgaris* Hildenborough

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Key Words: CO_2 reduction, Selenocysteine, Tungsten coordination, active site variants

Metal-dependent formate dehydrogenases (Fdh) catalyze under mild conditions the reversible reduction of CO_2 to formate with high activity and specificity. One of the most active CO_2 reductases is the W/Sec-FdhAB isolated from *Nitratidesulfovibrio vulgaris* Hildenborough. This enzyme has a simple composition and high CO_2 reduction activity, and has been widely used in biocatalytic applications for CO_2 reduction. The expression of the W/Sec-FdhAB is upregulated by W, and the presence of both W and selenocysteine at the active site contribute to its high CO_2 reduction activity. Contrary to most W-Fdhs, this enzyme is relatively oxygen-tolerant and can be purified and handled aerobically. Recently, we showed that the activity of FdhAB is controlled by a redox switch based on an allosteric disulfide bond [1]. Here, we will present the molecular basis for this mechanism and the first structure of FdhAB in the active state. The redox switch mechanism operates in vivo and prevents enzyme reduction by physiological formate levels, conferring a fitness advantage during transient cell exposure to O_2.
In order to get further insights into the CO_2 reduction mechanism we constructed several variants of the W/Sec-FdhAB, by mutating the Sec and other conserved residues close to the active site. The variants were characterized in terms of kinetic performance, EPR spectroscopy and structurally by X-ray crystallography. The results provide important insights into the role of first and second coordination residues in catalysis and the overall mechanism, with Sec playing a critical role in the high activity and oxygen tolerance of the enzyme.

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Expanding the landscape of formate dehydrogenases

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Molybdenum- and tungsten-dependent redox enzymes participate in a wide range of microbial cellular processes and are best known for their central role in energy metabolism. Within this broad class, formate dehydrogenases (FDHs) stand out for their remarkable diversity. Yet, our current understanding of FDH functional and structural variety remains limited, owing to the small and insufficiently diverse set of systems studied to date.

In this context, we identified and structurally characterized a novel FDH, ForCE, which reveals alternative bacterial strategies for electron transfer [1]. Structural analyses, combining X-ray crystallography and cryo-EM, uncover key deviations from canonical FDH features—including substitution of the conserved His ligand at the Mo active site by a Glu residue—challenging conventional sequence-based classifications.

This discovery stems from a systematic bioinformatic investigation of FDH modularity, which also revealed new multidomain architectures and previously unrecognized protein partners, thereby expanding the repertoire of potential energy-conserving assemblies. Finally, our phylogenetic reconstruction highlights three major branches of FDH diversification, of which only a handful of clades have been functionally or structurally characterized so far.

Altogether, our findings call for a broader reassessment of FDH diversity, encompassing not only catalytic mechanisms but also their structural and physiological integration in microbial systems.

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The Role of Selenocysteine in the MopB Family

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Key Words: selenocysteine, MopB superfamily, formate dehydrogenase

Selenocysteine (Sec or U) is the 21st amino acid encoded by the opal codon UGA, normally a stop codon.(1) As a result, many selenoproteins are often missed as current search engines and bioinformatics programs fail to recognize the codon or misread the "U" as the nucleotide uracil.(2) Selenocysteine plays an important role in the MopB superfamily of metalloproteins as it serves as the coordinated ligand in several enzymes.(3) These include FdhG, cytoplasmic Fdh, and FwdB/FmdB. The fact that many of the formate dehydrogenases are selenoproteins and catalyze what could be the oldest reactions within the MopB superfamily (e.g., formate oxidation, CO_2 reduction) stimulated us to construct phylogenies of the three families. Consistent with our comparative metagenomic study of the MopB superfamily, we found that the FwdB/FmdB and FdhG families formed coherent monophyletic clades, however, the cytoplasmic Fdhs remained a polyphyletic assemblage.(3) The largest radiation contained cytoplasmic Fdhs that function in acetogenesis and hydrogenotrophic methanogenesis, the Wood-Ljungdahl pathway for carbon fixation, the formate hydrogen lyase complex, and an aerobic FdsA formate dehydrogenase. Sec was likely the ancestral state for the FdhG and cytoplasmic Fdh families within Bacteria, further supporting an early origin for Sec utilization in this domain and involved in the carbon biogeochemical cycle through deep time.(4)

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Tree of life scale protein analyses: using omics tools to prioritize targets for wet lab experiments

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Key Words: Bioinformatics, protein family evolution, mopB superfamily

Metagenomics enables recovery of genome sequences of uncultured organisms at large scale. Annotation of these genomes largely determines our interpretation of physiology and ecology of uncultured organisms. Annotation can be error prone due to long phylogenetic distances from experimentally characterized organisms, or a high degree of functional diversity within protein families. Improving our interpretation of microbial genomes requires improvements to the current annotation tools, as well as prioritizing experiments based on available genome data.

I present an outlook on this problem using the widespread and functionally diverse mopB protein superfamily (also known as DMSO reductase, or Mo/W-bis-PGD superfamily) as a use case. On average there are roughly three members of the mopB superfamily in a bacterial or archaeal genome. Members of the superfamily enable respiration of many electron acceptors other than molecular oxygen, play a crucial role in the microbially mediated biogeochemical cycling of carbon, nitrogen, sulfur, arsenic, selenium, chlorine, and iodine, and have evolved into subunits of several respiratory chain complexes.

We use the GlobDB (https://globdb.org/), a species-dereplicated dataset of >300,000 genomes to build a comprehensive database of the mopB superfamily (>1,000,000 sequences) using an alignment score ratio approach and classify these proteins into (sub)families using matrix clustering. The distribution of these (sub)families across the entire genome dataset is indicative of the eco-evolutionary success of each protein (sub)family. Furthermore, we determine the consensus genomic context for all members of the proposed families, revealing putative novel protein complexes. Novel complexes can be prioritized for further experimental study by prevalence, taxonomic distribution, novelty, or quality of predicted function.

Acknowledgments

The GlobDB is hosted on the life science compute cluster (LiSC) of the University of Vienna.

Biological N_2-fixation at 92 °C: unveiling the molecular secrets of an archaeal hyperthermostable nitrogenase

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Key Words: Nitrogenase, N_2-fixation, Hyperthermophile, FeMo-cofactor.

Methanogenic archaea contribute to the carbon cycle by generating half the atmospheric methane yearly. Recent studies indicate that methanogens are also important to the nitrogen cycle by fixing N_2 in particular ecological niches (1). The work of our group is to understand how energy-limited hydrogenotrophic methanogens (relying on H_2 and CO_2 to derive their cellular energy) cope with the process of N_2 fixation. Marine *Methanococcales* are of particular interest due to their physiology and because their nitrogenase has been postulated to be of an ancient type (2). The combination of microbial physiology and protein biochemistry provided insights into the metabolic reshuffling occurring during the N_2 fixation transition (3). Moreover, by focusing on a hyperthermophile isolated from deep-sea volcanoes (4), we obtained snapshots of the N_2 fixation machinery operating close to the water boiling point. Interestingly, the hyperthermostable nitrogenase is almost inactive at room temperature, allowing us to describe new states of the metallocofactors at near-atomic resolution. This work highlights the importance of exploring the natural reservoir of catalysts from the microbial world to decipher elementary metabolic processes that can be applied in biotechnology.

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Life outside of enzymes: Understanding the roles of pterins as signaling molecules in bacteria

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Pterins are well-known as cofactors that facilitate diverse and complex enzymatic reactions. However, various pterin species serve a range of roles in nature including insect pigments, redox regulators, and signaling molecules. Here, we describe a novel pterin-dependent regulatory pathway that controls biofilm formation in a model α-proteobacterium, *Agrobacterium tumefaciens*. The pathway involves a protein termed PruR that shares low sequence similarity to MoCo-binding proteins as well as a pteridine reductase termed PruA. We demonstrate that PruR is a periplasmic protein that binds transiently to several pterin species and prefers the fully reduced tetrahydropterin forms. This regulatory protein modulates the activity of a dual function diguanylate cyclase-phosphodiesterase, leading to decreased c-di-GMP concentration and thus reduced propensity for biofilm formation. Bioinformatic analyses and identification of excreted pterins suggest that pterin-dependent signaling pathways are widespread in bacteria.

Poster Abstracts

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1. Recombinant expression of W-containing aldehyde:ferredoxin oxidoreductase (AOR) in *Escherichia coli*

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Key Words: Aldehyde:ferredoxin oxidoreductase, AOR, W-cofactor, recombinant protein expression

Transition metals, such as tungsten, are key components in several enzymes as a result of their ability to accept and donate electrons. As a result of this redox activity, these metals supply catalytic power and can facilitate a wide range of chemical reactions. To supply this catalytic power, a tungsten ion is ligated to at least one metal binding pterin (MPT) molecule to form a tungsten cofactor (W-cofactor) (1). The use of many of these metalloenzymes is held back by limitations in availability, which is mostly due to the difficulty of growing the original host organisms in which the enzymes originate, and the low enzyme yield obtained. Most characterized tungsten-containing enzymes (W-enzymes) originate from the anaerobic hyperthermophilic archaeon *Pyrococcus furiosus* (*P. furiosus*) (2). This organism has a growth optimum around 100 °C and is therefore requires specialized stainless steel fermenters to grow the archeae. Furthermore, the amount of genetic manipulation tools for *P. furiosus* are scarce, so introducing mutations in the proteins to study or alter their reactivity will be challenging. To overcome this issue, we focus on the production of W-enzymes using alternative protein production methods. One of these alternatives is a recombinant expression in *Escherichia coli* (*E. coli*). An enzyme that will be specifically focused on is the aldehyde:ferredoxin oxidoreductase (AOR). AOR is capable of oxidizing various aldehydes to their corresponding carboxylic acids with ferredoxin as natural electron acceptor. Next to that, it has also been shown to be capable to catalyze the thermodynamically challenging reverse reaction, the reduction of non-activated carboxylic acids to aldehydes aldehydes ($E' \approx -560$ mV), if low-potential electron donors are available (3–5). To ensure a functional AOR enzyme will be produced in *E. coli*, several bottlenecks in the biosynthesis of the W-cofactor need to be overcome, since *E. coli* does not naturally produce W-containing enzymes. To start, several *E. coli* strains have been screened on W-cofactor incorporation and activity of the produced AOR. This revealed sub-stoichiometric incorporation of Mo and W in recombinant AOR and enzyme activity up to 1% of the native enzyme activity using specific *E. coli* strains.

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2. Synthesis of new heterodinucleating xanthene-bridged ligand with aminopyridine chelate and its Mo(VI)/Cu(I) reactivity

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Key Words: Mo-Cu CODH, dinucleating ligand, heterobimetallic complexes

The development of a functional model for the active site of the Mo-Cu CODH (CODH = Carbon Monoxide Dehydrogenase) enzyme is of significant interest. The Mo-Cu CODH enzyme plays an important role in regulating atmospheric concentrations of toxic CO, transforming it into more benign CO_2. CO oxidation is carried out at the Mo(VI)-Cu(I) heterobimetallic site using cooperative reactivity of both metals. Spectroscopic and computational studies suggest that coordinatively unsaturated Cu(I) is necessary for the coordination and activation of the substrate. To develop a functional model of CODH, our lab is investigating heterobimetallic complexes supported by xanthene-based heterodinucleating ligands. We have previously demonstrated that a tridentate dipyridylamine chelate leads to an oversaturated Cu(I), which prevented cooperative reactivity with Mo(VI)-oxo. In the current work, we prepared a new heterodinucleating aminopyridine ligand (LH_2) which contains catecholate moiety as a donor for Mo(VI), and bidentate aminopyridine as a donor for Cu(I). The reactions of LH_2 with Cu(I) precursors form unstable Cu(I) complexes, that decompose upon recrystallization. However, treatment of the Mo(VI) complex $[MoO_3(L)]^{2-}$ with Cu(I) forms heterobimetallic $[CuMoO_3(L_2)]^-$, obtained as a dimer in the solid state. Our attempts to treat this complex with CNR or CO result in its transformation into a neutral trimetallic $[Cu_2MoO_2(L^2)_2]$, whose structure was also confirmed by X-ray crystallography. In this presentation, I will describe the syntheses and structures of the heterobimetallic complexes, and their reactions with small molecules within the context of Mo-Cu CODH reactivity.

Acknowledgments
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3. Redox Potentials Elucidate the Electron Transfer Pathway of NAD^+-dependent Formate Dehydrogenases

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Keywords: Carbon dioxide; Electron transfer; Formate; Iron-sulfur; Molybdenum; Redox potentials

Metal-dependent, nicotine adenine dinucleotide (NAD^+)-dependent formate dehydrogenases (FDHs) are complex metalloenzymes coupling biochemical transformations through intricate electron transfer pathways. *Rhodobacter capsulatus* FDH is a model enzyme for understanding coupled catalysis, in that reversible CO_2 reduction and formate oxidation are linked from the bis-metal (Mo) binding pterin guanine dinucleotide (bis-MGD) cofactor active site to a flavin mononuclotide (FMN)-bound diaphorase module via seven iron-sulfur (Fe-S) clusters as a dimer of heterotetramers. Insights regarding the proposed electron transfer mechanism between the bis-MGD and the FMN have been complicated by the discovery that an alternative pathway might occur via intersubunit electron transfer between two [4Fe-4S] clusters within electron transfer distance (1). To clarify this difference, the reduction potentials of the bis-MGD and the Fe-S clusters were determined via redox titration by EPR spectroscopy (2). Reduction potentials for the bis-MGD cofactor and five of the seven Fe-S clusters could be assigned. Substitution of the active site residue Lys295 with Ala resulted in altered enzyme kinetics, primarily due to a more negative reduction potential of the A1 [4Fe-4S] cluster that could clearly be assigned. Finally, characterization of the monomeric FdsGBAD heterotetramer exhibited slightly decreased formate oxidation activity and similar iron-sulfur clusters reduced relative to the dimeric heterotetramer. Comparison of the measured reduction potentials relative to structurally defined Fe-S clusters support a mechanism by which electron transfer occurs within a heterotetrameric unit, with the interfacial [4Fe-4S] cluster serving as a structural component toward the integrity of the heterodimeric structure to drive efficient catalysis.

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4. Assembly or disassembly of human MPT synthase complex: dynamic relocalisation of MOCS2A between MOCS2B and MOCS3

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Keywords: cofactor synthesis, MPT synthesis, MOCS2B, disease, mechanism, relocalisation

The molybdenum cofactor is an essential compartment of different enzymes in all kingdoms of life and part of four proteins in humans. While its redox function secures the detoxification of sulfite by enabling the sulfite oxidase to catalyze its reaction to soluble sulfate, my PhD project focusses on the synthesis of the cofactor itself[1]. A mutational study was performed to show the influence of different amino acid exchanges in MOCS2B on the catalytic activity. By this it is possible to identify important residual elements and mutations possibly triggering MoCD type B. It can be used in the future for prenatal prediction of the disease, which is especially important, because of the early fatality of the illness. However, the main project should shed light on the reason for the existence of two active centers, which are created by the dimerization of MOCS2B and insertion of MOCS2A[2]. Is it possible that one of these centers catalyzes the first thiolation of cPMP to thiopyranopterin, while the second center catalyzes the finalization to MPT? This should be tested by the creation of a hybrid, where both large subunits are connected via a linker and one of the two active centers is deactivated. The last part of my project is about the localization of the MPT synthase and MOCS2B. It is known, that the product MPT is relatively unstable while its unbound, so its reasonable to assume that it somehow needed to be transported to the subsequent enzyme (gephyrin) for its finalization to Moco[3]. We assume a direct recruitment of the MPT synthase complex to gephyrin itself for the prevention of MPT degradation. This should be shown by imaging analyzis, using upregulated gephyrin. My most recent interest in a possible moonlighting function of MOCS2B was sparked by the work of Tamaki Suganuma[4]. Here I would like to delve deeper into the role of MOCS2B in transcription and translation, especially the possible relocalization of MOCS2B to the nucleus. There it could then function as part of the ATAC complex. Together, these studies aim to clarify the mechanistic details and broader biological roles of human MPT synthase, deepening our understanding of both enzyme function and disease relevance.

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5 The inter-subunit transfer during nitrate reduction by *E. coli* NapAB

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Key Words: NapA, NapB and ping-pong

Abstract content: The catalytic subunit of periplasmic nitrate reductase NapA from *E. coli* contains a Mo-bis-pyranopterin guanine dinucleotide cofactor (molybdenum cofactor, Moco) and a [4Fe4S] cluster located closer to the surface of the protein.[1] NapA is associated with a diheme cytochrome (c-type) protein NapB. It has been suggested that NapA gets electrons from NapB during nitrate reduction in NapB-dependent NapA (e.g., *E. coli* NapA).[2] Consistent with this idea is a conserved Tyr residue between the heme of NapB and the [4Fe4S] cluster of NapA. Here, we demonstrate the electron transfer from NapB to NapA during nitrate reduction using UV-vis spectroscopy. Further, we have performed cyanide inhibition kineties using different cyanide concentrations. The double reciprocal plot at different cyanide concentrations resulted in a set of parallel lines, suggesting a ping-pong mechanism. A few DMSOR family members (e.g., biotin sulfoxide reductase and DMS dehydrogenase) have been suggested to function via a ping-pong mechanism involving two reactive sites.[3,4] We propose that these two sites are oxidized and reduced forms of NapA containing Mo(VI)=O and Mo(IV), respectively. Finally, it has been suggested that the ping-pong mechanism may explain the low substrate specificity of BSO reductase.[4] NapA from other organisms (e.g., *C. jejuni*) is known to accept non-native substrates. Thus, the ping-pong mechanism could be a general mechanism for NapA as well as DMSOR family members.

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6. Modeling *xanthine oxidase* family, structurally and functionally

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Key Words: Structural and functional modelling of Molybdenum containing enzymes, Bio-Inspired reactivities.

Abstract Content : The xanthine oxidase family of molybdenum-containing enzymes performs C-H hydroxylation of various substrates. Even though some progress has been made in the past on structural modelling of these, no reactivity studies, showing successful C-H hydroxylation has been reported with previous model compounds. Here, we report a dioxomolybdenum (VI) compound that shows the precise functionality of this class of enzymes. The poster will discuss the reactivity studies performed with bio-inspired substrates. Our research also spans making a structural model of the nicotinate dehydrogenase, which has a terminal selenide ligand at its active site and falls under the xanthine oxidase family. The reactivity of that compound towards hydride donors will also be discussed.

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7. Oxidative damage of Mo/W formate dehydrogenases

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Key Words: Mo- and W-Formate dehydrogenases; CO_2 reduction; Oxygen damage; Inactivation mechanism; X-ray crystallography

Mo- and W-Formate dehydrogenases (Mo/W-Fdhs) catalyze the reversible reduction of CO_2 to formate. The sensitivity of these enzymes to O_2 has hampered their industrial use as biocatalysts. However, the chemical/structural implications of O_2-induced damage remain largely unknown, yet are crucial for devising protective mechanisms. Our focus is on studying the W-FdhAB from *Nitratidesulfovibrio vulgaris*, which serves as an excellent model for biocatalytic applications in CO_2 reduction due to its robustness and high catalytic activity (1, 2).
Our recent study (3), combining biochemical, spectroscopic, and structural analyses of W-FdhAB, reveals that O_2 inactivation is promoted by the simultaneous presence of either substrate (CO_2 or formate) and led to the formation of a new active site species, consistently captured in all crystal structures. This process involves the displacement of the catalytic Sec residue from tungsten coordination and replacement by an O_2 or H_2O_2 molecule (Fig. 1). Additionally, we proved that oxidative inactivation does not require Mo/W reduction, as widely assumed, occurring also in the oxidized state in the presence of CO_2.

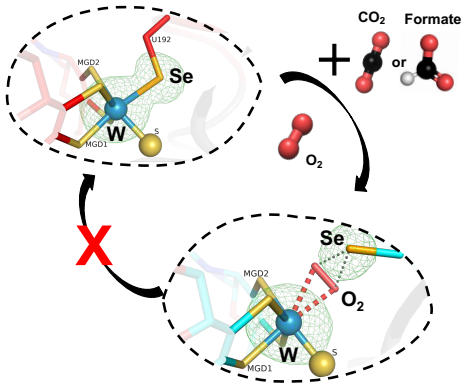

Fig.1 Irreversible inactivation of *N. vulgaris* W-FdhAB in the simultaneous presence of substrate and oxygen. The Sec residue is displaced from the W coordination and replaced by an O_2 or H_2O_2 molecule.

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8. Resonance Raman Studies of Trimethylamine-N-Oxide Reductase

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During anaerobic respiration, *Escherichia coli* can use Trimethylamine-N-Oxide (TMAO) as an alternative electron acceptor to oxygen. The reduction of TMAO is catalysed by TorA, a molybdoenzyme that binds a *bis*-Molybdopterin-Guanine-Dinucleotide (*bis*-MGD) cofactor. Previous work has shown that TorA activity is largely affected by the cultivation conditions of *E. coli* and the resulting Molybdenum ligation, suggesting an inactive Mo=Oxo ligation and an active, Sulfido-ligated form obtained by anaerobic cultivation.
The poster will present the use of Resonance Raman spectroscopy (RR) to elucidate the ligation of the Molybdenum in highly active TorA, as well as other enzymes binding Molybdenum cofactor (Moco). Furthermore, the previously found stability of Moco under strictly anaerobic conditions has been exploited and developed on, to conduct measurements on isolated cofactor and reconstituted TorA. Towards the assignment of Raman bands to the various ligands, spectra from enzymes with a ligating Cystein (in place of the Serine found in TorA) are compared. Additionally, in some of them, an exchange of Molybdenum with Tungsten was performed. Shifts resulting from these changes are interpreted using Density Function Theory (DFT) calculations.
The range of different enzymes and high resolution of the spectra further the establishment of Resonance Raman spectroscopy for the analysis of metal ligation in Molybdenum cofactors.

9. Oxidation of the FdsDABG formate dehydrogenase from *Cupriavidus necator* by NAD^+

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Key words: formate dehydrogenase, oxidative half-reaction, rapid-reaction kinetics

Formate dehydrogenase DABG from *Cupriavidus necator* performs the reversible reaction of oxidizing formate to form carbon dioxide (CO_2) via a molybdenum center that that consists of the metal coordinated by two equivalents of MGD, a cysteine ligand provided by the polypeptide and a catalytically essential terminal sulfido ligand. The reduction of FdsDABG has been well studied and documented, with oxidation of formate to CO_2 catalyzed by a well-supported hydride transfer directly from formate to the catalytically active sulfido ligand coordinated to the molybdenum, thereby reducing the Mo from Mo^{VI} to Mo^{IV} (1). Here we have examined the remaining, oxidative half of the catalytic cycle, the oxidation of reduced enzyme by NAD^+, a reaction that takes place at the enzyme's FMN. Stopped-flow kinetic work indicates that enzyme reoxidation occurs with a hyperbolic dependence on [NADH], yielding an apparent rate constant of 380 s^{-1} and an apparent K_d^{NADH} of 800 μM at 10° C. During an enzyme-monitored turnover experiment, the enzyme is in partially reduced in the steady-state, likely at the level of $FdsDABG_{4e}$-. Parallel electron paramagnetic resonance experiments demonstrate that several the seven Fe/S clusters that intervene between the molybdenum center and the FMN become transiently reduced during turnover and electron transfer among them does not appear to be at all rate-limiting to turnover. The reductive half-reaction is principally rate-limiting, as by the substantial isotope effect seen with 1-^{2}H-formate as substrate not only the reductive-half reaction kinetics but on also steady-state turnover as well.

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10. Characterisation of the Formate Hydrogenlysae 2 From *Pectobacterium atrosepticum* In an *Escherichia coli* Host.

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Global climate change, resulting from greenhouse gas emissions, is having unprecedented effects around the world. Global efforts are being made to reduce carbon dioxide emissions via carbon capture storage and utilisation technologies to reach NetZero by 2050. *Escherichia coli* expressing the formate hydrogenlyase-1 (FHL-1) complex has already shown promise as a potential biological carbon capture technology. Under experimental conditions, FHL-1 can function as a hydrogen-dependent carbon dioxide reductase (HDCR) capable of producing formic acid at levels comparable to that of native organisms. One biotechnological draw-back of utilising FHL-1 is its dependence on a catalytic selenocysteine residue which prevents efficient over expression.

In addition to FHL-1, *E. coli* possesses the genetic information required to produce a second FHL complex, FHL-2. However, literary reports regarding the functionality of *E. coli FHL*-2 are contradictory and confusing. Therefore, the *E. coli* FHL-2 complex is still not greatly understood and cannot be definitively classified as active. Although the production and activity of the *E. coli* FHL-2 complex is still debated, important research has identified unambiguously functional FHL-2 complexes in other organisms including *Pectobacterium atrosepticim*. The FHL-2 complex from P. atrosepticum may prove to be a more appropriate complex for biotechnological applications as it is not reliant on a catalytic selenocysteine. Although the *P. atrosepticum* FHL-2 is functional, *P. atrosepticum* is not a suitable host for biotechnological applications as it grows slowly over 40 hours at 24 °C. Therefore, the genes encoding *P. atrosepticum* FHL-2 have been cloned for expression within an *E. coli* host.

11. An engineered soluble periplasmic formate dehydrogenase from *Cupriavidus necator*

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Key Words: formate dehydrogenase, FDH, catalysis, MoCo, O_2 tolerance, oxo ligand, reactivation

While several studies have focused on characterizing the cytoplasmic NAD^+-reducing formate dehydrogenase (Fds) [1], relatively little is known about the membrane-bound, periplasmically oriented FDHs of *Cupriavidus necator*. Two membrane-bound enzymes, Fdo and Fdh, have been identified, and it is known that one of them contains molybdenum and the other tungsten as the metal in its active center. However, it is still unclear which enzyme carries which cofactor [2].

For cathode-driven formate synthesis, we succeeded in overproducing the formate dehydrogenase (Fdo) from *Cupriavidus necator* in a soluble, membrane-detached form in the cellular periplasm. This enzyme has been characterized both *in vivo* and *in vitro* with regard to its catalytic activity and the composition of its active site.

The overproduced Fdo comprised only the FdoG and FdoH subunits and it lacked both the FdoI subunit and the membrane-anchoring α-helix of FdoH. The enzyme was purified from *C. necator* under aerobic conditions without the use of protective agents such as nitrate or azide.

Inductively coupled plasma mass spectrometry (ICP-MS) confirmed molybdenum as the functional metal at the active site, while Raman spectroscopy and activity measurements revealed an inhibited state in part of the enzyme fraction in which the active site molybdenum carried an oxo ligand (Mo=O). In contrast to most other characterized FDHs, however, the *C. necator* Fdo could be reactivated by simple anaerobic incubation with formate. Upon reactivation, the Mo=O ligand was removed, and the catalytic activity increased fourfold.

The possibility to purify the enzyme aerobically without protective agents and its successful reactivation by adding substrate suggest an unusual O_2 tolerance mechanism that is worth investigating.

It was demonstrated both photometrically and electrochemically, using benzyl viologen as a redox mediator, that *C. necator* Fdo is active *in vivo*. This provides compelling evidence that soluble, periplasmic Fdo interacts with electrodes via redox mediators, which is a crucial step toward the development of electrosynthesis systems based on *C. necator.*

Acknowledgements

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12. Life at 90°C: Tungsten Enzymes as Engines of Bio-Based Production

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Microbial enzymes are key to advancing greener, energy-efficient chemical processes. In particular, the enzymatic reduction of carboxylic acids to alcohols could offer a sustainable alternative to traditional chemical methods. Tungsten-containing aldehyde oxidoreductases (W-AORs) from the hyperthermophilic anaerobic archaeon *Pyrococcus furiosus* offer unique potential to catalyze such low-potential reactions. These enzymes catalyze the reversible conversion of carboxylic acids to aldehydes; however, the molecular basis of their substrate specificity remains poorly understood. Here, we cultivated *P. furiosus* anaerobically at 90 °C in 15 L bioreactors, yielding 1.5 ± 0.3 g cells · L^{-1} after 20 ± 3 h of growth on carbohydrates. We successfully purified two W-AORs from native cells with distinct substrate preferences and catalytic profiles. Using spectrophotometric assays, we explored their activity in both oxidative and reductive directions. Structural predictions guided by AI identified key residues involved in substrate recognition and specificity. To confirm the accuracy of AI-based structural predictions, protein crystallization is in progress for subsequent X-ray crystallography analysis. Our findings represent a crucial step in the European W-BioCat project, which aims to develop recombinant W-AORs for scalable, bio-based chemical production. Our work highlights the untapped potential of tungsten enzymes in enabling a sustainable future for the European chemical industry.

13. Heterologous expression of active Mo-nitrogenase in a non-diazotrophic model organism

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Key Words: nitrogenase, FeMo-co

Endowing cereals with nitrogen fixation activity is considered a holy grail of synthetic biology. However, previous attempts in the literature fail to demonstrate the expression of functional nitrogenase and related proteins in a heterologous host. Recently, we have reported the isolation of active *Azotobacter vinelandii* Mo-nitrogenase from a non-diazotrophic model organism, *Escherichia coli*. Using a bottom-up approach, we were able to successfully reconstitute the metallocofactor assembly pathways of Mo-nitrogenase's two complex metallocofactors in *E. coli*. This work allowed us to, conclusively, endow *E. coli* with N_2-fixing ability. Biochemical and spectroscopic analyses further demonstrate the functionality of the isolated proteins and confirm the assembly of these metallocofactors. Intermediates in the metallocofactor assembly pathway of nitrogenase were also isolated, providing insight into Mo-nitrogenase's metallocofactor assembly pathways.

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14. Integrative Structural Modeling of the YcbX-CysJ-CysI Complex

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Key Words: Protein-Protein Interactions, Electron Transfer, Iron-Sulfur Cluster, MOSC domain

MOSC domain proteins are a large superfamily of molybdoenzymes that occur in all domains of life (1). Understanding of their biological function remains poor, although involvement in detoxification reactions and lipid metabolism have been suggested (2). The most prominent members studied so far are molybdenum cofactor sulfurase (MOCOS), the human mARC enzyme and its putative *E. coli* orthologue YcbX (2). mARC and YcbX have similar enzymatic activities (3, 4) and spectroscopic studies reveal virtually identical geometric structures of the molybdenum complexes located in their active sites (5, 6). Another shared property of mARC and YcbX is their requirement for additional electron carrier proteins in order to become active enzymes. Here, we have used an array of biophysical and structural techniques in order to understand how YcbX its electron transfer partner, the diflavin reductase CysJ, and sulfite reductase CysI form an approx. 1.1 MDa hetero-20-mer with various redox-active prosthetic groups that transfer electrons among each other (8 x FMN, 8 x FAD, 8 x Mo-PDT, 8 x [2Fe-2S], 4 x sirohaem, 4 x [4Fe-4S]). We applied a vast array of biophysical techniques including small-angle neutron scattering with contrast variation (CV-SANS), cryogenic electron microscopy (Cryo-EM). Our findings allow a comprehensive understanding of the mechanism of electron transfer within the YcbX-CysJ-CysI complex, which is also a good starting point for mechanistic investigations into the disease-relevant human mARC enzymes and its interaction partners.

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15. Tungsten-dependent aldehyde oxidoreductase: biotechnological applications

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Molybdenum and tungsten are transition metals that are both present in biological systems. These metals occur in the active site of Mo- and W-dependent enzymes where they are coordinated to a metallopterin cofactor and are usually involved in the catalysis of various redox reactions. Although molybdenum and tungsten share similar chemical properties, they differ in the redox potentials of their biologically relevant oxyanions[1]. Therefore, some reactions of this enzyme family are exclusively catalysed by tungsten-containing enzymes[2]. This includes the direct reduction of carbonic acids to the corresponding aldehydes without any need for prior activation. One example of these W-dependent enzymes is the aldehyde oxidoreductase from *A. aromaticum* EbN1[3]. This enzyme is a member of the bacterial subfamily of AOR and has been shown to catalyze the oxidation of various aldehydes as well as the reverse reaction[4]. The reverse reaction provides a versatile tool in the reduction of various acids of interest to the corresponding aldehydes, especially in coupled reactions with other enzymes, like alcohol dehydrogenases or aminotransferases. Many organisms producing W-enzymes employ selective pathways for synthesizing Mo- and W-containing enzymes simultaneously. Here we introduce potential applications of AOR as well as the potential mechanisms involved in the highly selective incorporation of tungsten during cofactor maturation of AOR.

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16. Formate handling and CO_2 wrangling in a W-formate dehydrogenase: impacts on catalysis

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Key Words: CO_2 reduction, Metal-dependent formate dehydrogenase, Substrate diffusion tunnel, X-ray crystallography, High-Pressure Crystal Soaking.

The reversible CO_2/formate interconversion by Mo/W-Formate dehydrogenases (Fdhs) is a promising route not only for green-house gas sequestration but to sustainably produce fuel. Formate is a safe option for hydrogen storage/delivery (53g H_2/L) in cell power applications (1).
W-FdhAB (periplasmic heterodimer; W active site: bisMGD, Se(Cys), S ligand; 4x[4Fe-4S]) is the main responsible for CO_2 reduction in *Nitratidesulfovibrio vulgaris* (2) and an excellent model for CO_2 reduction biocatalytic applications due to its robustness and high catalytic activity (3, 4).
Previous studies have suggested the existence of two independent substrate access tunnels (3). However, the existence of a CO_2-specific tunnel was not confirmed experimentally. We report a new crystallographic study on W-FdhAB crystals pressurized (5) with Kr, O_2 and CO_2 that enables the mapping of gas diffusion tunnels in the enzyme. These experiments allowed the identification of a substrate binding site on the main tunnel, which is commonly occupied by small molecules, verified by additional soaking experiments. Mutagenesis and activity assays support the proposal of a substrate retention site, capable of transiently holding substrates, playing a key role in CO_2 binding and catalysis. Moreover, a novel branch of the main substrate tunnel could be identified, and its specific role for CO_2 diffusion was confirmed through structure-based mutagenesis. Overall, the results indicate that each substrate has a dedicated pathway/tunnel to reach the proposed retention site and both substrates share the final segment of the main tunnel to access the buried W active site.

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17. Structure and mechanism of 1-testosterone dehydrogenase, a novel member of the xanthine oxidase family

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Key Words: xanthine oxidase family, sex hormone degradation, mechanism of XO family enzymes

Steroids are widely abundant environmental contaminants, and the steroidal sex hormones in particular cause severe problems due to their endocrine-disrupting activity. At anoxic environments, bacterial degradation of androgens and estrogens proceeds via the central intermediate 1-testosterone (and its oxidized form androsta-1-en-3,17-dione), which is then oxidized to a 1,3-diketone of ring A (androsta-1,3,17-trione) [1,2]. This reaction of anaerobic steroid degradation is catalyzed by 1-testosterone dehydrogenase, a novel member of the xanthine oxidase family. The heterologously produced enzyme, which was purified from a denitrifying steroid degrading bacterium, was characterized as a hetero trimer that binds MCD (α-subunit), 2 [2Fe-3S] clusters (β-subunit), and an FAD (γ-subunit). The cryo-electron microscopy analysis of the enzyme in the presence of the substrate revealed a possible reaction intermediate with an oxygen atom at C1 bridging the substrate to the Mo-atom. Together with the results of EXAFS analyses and DFT calculations, we present a robust foundation for understanding the mechanism of 1-testosterone dehydrogenase, thereby validating the proposed mechanism for members of the xanthine oxidase family that act on heterocyclic substrates.

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18. What pathways load 4Fe4S clusters onto NifH and AnfH in bacteria and the mitochondria of yeast and plants?

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Key words: synthetic biology, plant biotechnology

There is significant biotechnological interest in the engineering of nitrogenase into plants. To succeed, metalloclusters need to be formed and loaded into the active sites of various components of nitrogenase pathways. A traditional model of nitrogenase biogenesis places NifU as the central "server" of 4Fe4S clusters that are transferred to downstream "clients", including apo-NifH and apo-AnfH. Given the complex genetics of nitrogenase assemblies perhaps the most conclusive evidence of this model is *in vitro* biochemical assays using purified enzymes where the 4Fe4S clusters on NifU are rapidly transferred to apo-NifH forming active NifH.

The obligate requirement for NifU for loading apoH has been challenged in both yeast and bacterial models. In the mitochondria of yeast both *Azotobacter* NifH and AnfH were found to be active 'as isolated' without co-expression of NifSU. These results in yeast are mirrored also in *E. coli* where transgenic expression of both *Klebsiella* NifH and *Azotobacter* NifH can be isolated as active and loaded with 4Fe4S clusters also in the absence of NifSU. The results from both *E. coli* and yeast indicate that NifSU is not the exclusive pathway for loading apoH and that non-NifSU endogenous pathways are capable of loading these transgenic enzymes with 4Fe4S clusters.

Here we present our results regarding the functionality of NifH and AnfH expressed in the mitochondria of plant leaves. We demonstrate that a soluble version of *Klebsiella* NifH was only partially active 'as isolated' and this activity was reduced even further when co-expressed with NifSU. Similarly, a soluble version of *Azotobacter* AnfH was not active as isolated with co-expression with NifSU. Both versions of leaf-isolated NifH and AnfH were capable of accepting 4Fe4S clusters when donated in vitro from NifU. These results indicate that the transgenic proteins were correctly folded in the plant mitochondria, but were essentially lacking clusters.

We will briefly outline the approaches we have taken to express soluble nitrogenase proteins in plants combined with biochemical characterisation of the isolated proteins, and discuss our results in plants relative to bacteria- and yeast-based assays in other labs.

19. Active site structure and mechanism of a molybdenum catechol dehydroxylase

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KEYWORDS. Molybdenum enzyme, enzyme mechanism, catechol dehydroxylase.

ABSTRACT: The dehydroxylation of catechols represents an important chemical transformation facilitated by gut bacteria in mammals.[1,2] This reaction is catalyzed by pyranopterin molybdenum enzymes that belong to the DMSO reductase family.[3-5] Despite their chemical and biological significance, the structure and mechanisms of catechol dehydroxylases remain uncharacterized. In this study, we interrogated the active site structure of hydrocaffeic acid dehydroxylase from the gut bacterium *Gordonibacter urolithinfaciens* (*Gu* Hcdh) using a combination of Mo K-edge X-ray absorption near-edge structure (XANES) spectroscopy and extended X-ray absorption fine structure (EXAFS) analyses.[6] In the oxidized state, the Mo(VI) ion is coordinated by a terminal oxo atom, a cysteine thiolate, and four sulfur atoms from the two bidentate pyranopterin dithiolene (PDT) ligands. A combination of protein sequence analyses and site-directed mutagenesis experiments reveal that Cys157 is ligated to the Mo ion and is catalytically essential. In the reduced Mo(IV) state, the Mo coordination environment remains hexacoordinate with the terminal oxo ligand being protonated to yield a hydroxyl ligand. Reaction coordinate computations on the EXAFS-derived structure suggest the likely role of an active site base in facilitating substrate dearomatization and product formation. An Alphafold-2 predicted structure shows that this active site base is an aspartate, which is oriented in the substrate access funnel for interaction with the substrate. Site-directed mutagenesis experiments reveal that Asp210 is a catalytically essential amino acid. Taken together, our study on Hcdh enriches our understanding of an emerging pyranopterin molybdenum enzyme family from the human gut microbiota.

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20. Characterization of S-/N-oxide reductase substrate specificity by electrochemical methods

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Key Words: DMSO reductase; S-/N-oxide reductase; enzyme kinetics; electrochemistry

TorZ from *E. coli* is a periplasmic molybdenum enzyme and was previously shown to efficiently reduce trimethylamine N-oxide (TMAO) (1). However, the full spectrum of its substrate specificity remained incompletely characterized, as kinetic parameters for its activity toward other S- and N-oxide substrates had not been determined.

In this study, we used both traditional, solution-based enzyme assays and electrochemical methods to systematically evaluate the kinetic properties of EcTorZ towards a range of S- and N-oxide substrates. We optimized the electrode preparation protocol for electrochemical analysis by comparing immobilization of the enzyme on the electrode surface and direct addition of the enzyme to the reaction buffer rather. With the improved experimental setup—featuring optimized mediators and enzyme concentrations—the electrochemical system displayed a clear substrate concentration-dependent increase in catalytic current. In contrast to previous protocols, where K_M values determined by electrochemistry were typically an order of magnitude higher than those obtained from solution-based spectrophotometric assays, the revised method produced K_M values that were in close agreement with spectrophotometric data. This consistency supports the reliability and validity of the updated electrochemical approach for evaluating enzyme kinetics. The results revealed that EcTorZ exhibited lower K_M values for N-oxide substrates compared to S-oxides. Notably, the K_M for PNO (pyrimidine N-oxide) was the lowest among all tested substrates, indicating a particularly high substrate affinity in this case.

In addition to EcTorZ, MsrP (previously named YedY) from *E. coli* is another well-studied molybdenum enzyme. Although classified within the sulfite oxidase enzyme family, MsrP exhibits S- and N-oxide reductase activity (2). MsrP has been shown to act on protein-bound methionine sulfoxide (MetSO); however, the experimentally determined K_M values for free MetSO was the highest among all tested substrates, indicating relatively low substrate affinity. Using our electrochemical approach to evaluate the substrate specificity of MsrP, we found that the K_M values for PNO was the lowest among the compounds examined, suggesting that PNO may be the preferred substrate under the conditions tested. A similar observation was made for S-/N-oxide reductases from different enzyme families, which may broaden our understanding of the true physiological roles of S-/N-oxides.

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21. The structure of the molybdenum sites of nitrogenase

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Key Words: Molybdenum Nitrogenase, Metalloenzymes, MoFe Cluster, X-ray Absorption Spectroscopy

Abstract content

The biogeochemical nitrogen cycle is one key to life on earth, and an essential component of this is the enzyme nitrogenase, which converts relatively unreactive atmospheric nitrogen to bio-available ammonia. This reaction is perhaps the most chemically challenging in biology and is catalyzed by a novel molybdenum-iron-carbide-sulfur cluster which is at the heart of the nitrogenase enzyme. Despite being among the most intensively studied of metalloenzymes, there is still controversy about the catalytic mechanism of nitrogenase. New structural proposals involving a different form of nitrogenase have generated significant controversy but provide tantalizing new ideas about how nitrogenase might work. Our study uses X-ray absorption spectroscopy to compare the molybdenum site of the two forms of the enzyme.

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