Abstract:

Exploring Multifunctional Hydrogenases by Advanced Vibrational Spectroscopy: Reaction Site Coupling in Complex Biocatalytic Systems

Co-supervisor 1: Dr. Marius Horch; FUB, UniSysCat

Co-supervisor 2: Dr. Ingo Zebger; TUB, UniSysCat, PP1927

[NiFe] hydrogenases are metalloenzymes that catalyse the reversible cleavage of H_2 – an ideally clean fuel – by using a sophisticated bimetallic active site. Some of these enzymes also catalyse additional reactions, e.g. the reduction of O_2 or the redox conversion of biochemical cofactors like NAD(H) or coenzyme F_{420}. These reactions, which are coupled to H_2 conversion, involve additional organic and inorganic cofactors as well as a complex network of proton, electron, and gas transport pathways. Consequently, multifunctional hydrogenases are complex biocatalytic systems that represent valuable model targets for understanding how catalytic reaction networks are realized in Nature. The current project aims to explore this aspect by using advanced vibrational spectroscopic techniques together with complementary theoretical, biochemical (with O. Lenz), crystallographic (with H. Dobbek & P. Scheerer), and model chemistry (with C. Limberg) studies. This approach will provide insights into the complex interplay between the structure, function, and dynamics of biocatalytic systems, thereby providing valuable inspiration for coupling catalytic reactions.

Extended description version of the project:

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1. Overall goal of the project

The project aims to reveal the principles of reaction-site coupling in multifunctional [NiFe] hydrogenases that connect the reversible cleavage of H_2 to other biocatalytic reactions. This involves elucidating (1) catalytic mechanisms at individual reaction sites, (2) the flow of educts, products, and intermediates, and (3) possible regulatory interactions between catalytic sites. Besides facilitating the rational utilization of these metalloenzymes, e.g. in bioinspired catalyst design, this approach promises to reveal how Nature creates synergy in biocatalytic reaction networks by orchestrating different reactivities within modular catalytic machineries. Emphasis will be placed on reaction and equilibrium dynamics, thereby expanding the static picture of the structure-to-function paradigm. Moreover, we aim to understand what defines a catalytic unit by going beyond the idea of localized reactive (metal) sites. Questions to be addressed in this context include: What is the minimum set of reaction sites required to accomplish a certain catalytic function? How do outer coordination layers tune the function of (biological) metal sites? Which principles underly regulation and the coupling of remote catalytic sites in bioinorganic systems?

2. State of the art

Hydrogenases have been studied for decades by spectroscopic, biochemical, electrochemical, and crystallographic methods. Nevertheless, the principles of biological H_2 conversion are discussed controversially, and even generally accepted aspects often lack experimental proof. In addition, most studies have been performed on 'canonical' [NiFe] hydrogenases composed of two subunits harboring the [NiFe] catalytic site and three FeS clusters. More complex [NiFe] hydrogenases that catalyze additional reactions are less well explored, and the interplay between different reactivities has been barely addressed – despite its relevance for understanding complex catalytic systems.

We have previously studied three such modular and multifunctional [NiFe] hydrogenases. All these Soluble Hydrogenases (SH) couple the reversible cleavage of H_2 to the redox conversion of other substrates by connecting the [NiFe] active site with a flavin cofactor through a chain of five FeS clusters. In the case of SH from *R. eutropha* (*Re*) and *H. thermoluteolus* (*Ht*), the second substrate is NAD(H). *Re*SH is highly O_2-tolerant due to its additional (per)oxidase activity. Our results indicate that this reactivity involves electron donation from NADH, reverse electron transfer *via* the FeS clusters, a second flavin cofactor, and reversible sulfoxygenation at the [NiFe] active site, but direct proof and mechanistic insights are missing so far.[1] *Ht*SH is also O_2-tolerant – although less than *Re*SH – and thermostable. In line with crystal structure data, a recent study from our groups indicates that *Ht*SH protects itself and other cellular constituents from O_2 damage and reactive oxygen species by a sophisticated mechanism involving large-scale structural rearrangement, remote redox sensing, and intramolecular signaling.[2] Details remain to be elucidated, though, and the enzyme's thermostability is not understood. In total, *Re*SH and *Ht*SH are complimentary model systems for

understanding how Nature couples multiple redox reactions under challenging conditions. Finally, we have also studied SH from *M. barkeri* (*Mb*), whose second substrate is coenzyme F_{420}. Using a combined approach of spectroscopy and crystallography, we have shown that *Mb*SH forms multimers with extended pathways for H_2 transport and electron redistribution between individual protomers.[3] Thus, this model enzyme provides the exceptional opportunity to explore 'hardwired' biocatalytic reaction networks. Moreover, *Mb*SH can be enriched in the H_2-binding intermediate of the [NiFe] site (both in the solute and crystal phase), thereby allowing to study the unexplored catalytic key step of H_2 activation.

1. Horch et al. *J. Am. Chem. Soc.* **2015**, 137, 2555–2564.
2. (a) Preissler et al. *BBA Bioenergetics* **2018**, 1859, 8–18. (b) Kulka et al. *in preparation*.
3. Ilina et al. *Angew. Chem. Int. Ed.* **2019**, 58, 18710–18714.

3. Specific aims and how they may be reached

Utilizing the three model systems listed above, the project will address the following key objectives:

Objective 1: Understand how multifunctional hydrogenases accomplish and orchestrate multiple catalytic reactions through several cofactors and over tens of ångstroms, respectively.

Objective 2: Reveal the principles and additional catalytic reactions that allow performing these tasks under challenging conditions, e.g. ambient O_2 levels or high temperature.

Objective 3: Explore the interplay of catalytic and non-catalytic reaction sites in the model hydrogenases and possible regulatory interactions within these intramolecular reaction networks.

Objective 4: Elucidate how the protein matrix facilitates all these aspects by (1) tuning reactive metal sites and (2) mediating intramolecular communication through structural rearrangement.

To this end, we will use a multi-spectroscopic approach with a focus on vibrational methods that allow studying hydrogenases in solution, on electrodes, in crystallized form, at low-temperatures, or in living cells. This approach will provide insights under biologically relevant conditions and yield data that can be compared with those from other techniques, e.g. electrochemistry, EPR spectroscopy (R. Bittl & C. Teutloff; FUB, UniSysCat), crystallography (H. Dobbek & P. Scheerer; HUB/Charite, UniSysCat / PP1927 & UniSysCat / SFB1078), and biochemical assays (O. Lenz; TUB, UniSysCat / PP1927). Besides well-established methods like resonance Raman and conventional infrared (IR) spectroscopy, we will also employ ultrafast and two-dimensional (2D) IR spectroscopy, thereby gaining insights into dynamics, energy transfer, and unexplored structural aspects. Spectroscopic studies will be supported by model chemistry (C. Limberg; HUB, UniSysCat) and theory.

Besides addressing consensus features of reaction coupling in all three targets systems, we will study the following key aspects by focusing on each one of the three model hydrogenases.

Work Package 1 will focus on *Re*SH and its exceptional O_2 tolerance. Specifically, we aim to elucidate the catalytic mechanism of sulfur-centered O_2 detoxification, the structure of involved [NiFe] intermediates, the role of other cofactors and substrates (H_2 and NADH), and the relevance of structural plasticity in the protein matrix.

Work Package 2 is dedicated to *Ht*SH, its thermostability, and the unique O_2 protection strategy. Here, we will focus on protein dynamics, large-scale structural rearrangement, and energy transfer

to elucidate how these aspects may be involved in long-range intramolecular communication of remote sites and the increased resistance towards elevated temperatures.

Work Package 3 aims to understand the complex flow of electrons and H_2 within individual protomers and superordinate multimeric assemblies of *Mb*SH. In addition, we intent to tackle the unresolved question of how H_2 is activated in biology by utilizing (ultrafast) vibrational spectroscopy and the unique features of *Mb*SH.

Experimental and computational resources are available at the supervisors' laboratories and/or their host institutions. A new 2D-IR set-up will be available for the PhD project. Application for access to the STFC Central Laser Facility will allow advanced IR studies that go beyond resources available in Berlin. Further experiments of this type can be performed with N. Hunt (University of York, UK).