Abstract:

Semi-artificial metallo-enzymes for CO_2 reduction and oxygenation reactions

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Heme-containing proteins, predominantly myoglobin and cytochrome P450s, have been used as platforms to create semi-artificial enzymes, containing non-native cofactors. But how catalysis proceeds and why specific changes bring the desired outcome, has typically not been investigated. Here, we want to generate, analyze and optimize variants of the hemoprotein HTHP containing non-natural cofactors. Variants of metals incorporated into protoporphyrin-IX, as well as redesign of the active site pocket shall be used to create different stable non-native enzyme-variants. These variants will be tested against target reactions including the reduction of CO_2 and the activation of dioxygen for non-physiological hydroxylation reactions. Enzyme redesign will be guided by protein crystallography at sub-Angstrom resolution, which is also used to compare with the results of model calculations. The mechanism of generated variants will be analyzed by a combination of rapid mixing and spectrophotometry (UV/Vis, resonance Raman (RR) and EPR) as well as by following turnover in crystals by X-ray diffraction.

Extended description version of the project:

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1. Overall goal of the project

The overall goal is to generate, analyze and optimize variants of the hemoprotein HTHP containing non-natural cofactors. Variants containing various metals incorporated into protoporphyrin-IX, as well as redesign of the active site pocket shall be used to create stable non-native enzyme-variants, whose reactivity with CO_2, protons and dioxygen shall be investigated and modulated. The structural basis for reactivity differences will be analyzed by combining crystallography at sub-Angstrom resolution with spectroscopy and model calculations to gain a mechanistic understanding informing further rounds of active site design. Furthermore, we want to optimize X-ray diffraction protocols to minimize X-ray reduction during data collection. To identify reaction intermediates, the reaction kinetics will be studied by rapid mixing techniques in combination with UV/Vis absorption and vibrational spectroscopies.

2. State of the art

Heme-containing proteins, predominantly myoglobin and cytochrome P450s, have been used as platforms to generate semi-artificial enzymes containing non-native cofactors.[1-3]

The small hexameric heme-containing enzyme (HTHP) harbors an exposed heme-binding pocket with short heme-to-heme distances within a hexameric ring.[4] Native HTHP carries catalase and peroxidase reactivity. We have optimized recombinant production systems for two different native HTHPs, one from *Silicibacter pomeryoi* and from *Oligotropha carboxydovorans*, generating holo- as well as stable apo-proteins. Production of stable apo (heme-free) HTHP allowed recently incorporation of non-native heme-groups by chemical reconstitution (Co-protoporphyrin-IX) .

HTHP is highly thermostable (T_m~120 °C), facilitating large-scale production and redesign of its structure. HTHP offers several advantages for in-depth mechanistic investigations: (I) protoporphyrin-IX cofactors are sensitive to small local changes, which can be followed by optical spectroscopy as well as resonance-Raman (RR) / EPR spectroscopy in combination with rapid mixing in a stopped-flow spectrophotometer and rapid freeze quench set up,[5] respectively. (II) Crystals of HTHP are of high symmetry (*P*622) allowing to collect complete datasets with minimal X-ray exposure at resolutions around 1.0 Å, which can be also characterized by RR spectroscopy, both together allowing to resolve mechanistic details of stabilized intermediate states at chemically informative resolution.

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[2] S. N. Natoli, J. F. Hartwig, *Acc. Chem. Res.* **2019**, *52*, 326–335.
[3] K. Oohora, A. Onoda, T. Hayashi, *Acc. Chem. Res.* **2019**, *52*, 945–954.
[4] J.-H. Jeoung, D. A. Pippig, B. M. Martins, N. Wagener, H. Dobbek, *J. Mol. Biol.* **2007**, *368*, 1122–1131.
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3. Specific aims and how they may be reached

Objectives:

- Production of HTHP variants with non-native cofactors
- Analyze the reactivity of variants
- Optimize ratio of CO_2 reduction / hydrogen production of Co-HTHP in the presence of strong reductants
- Analyze the reactivity and structure of oxidizing species generated on HTHP (native and non-native HTHP)

Experimental strategy:

- **Computational design** of HTHP variants, changing charges and proton donors/acceptors in the active site
- **Production** of apo-HTHP variants by heterologous overexpression and one-step purification
- Integration of **various metals** into protoporphyrin IX according to established protocols
- Analyzing **reactivity** of HTHP variants in CO_2 and H^+ reduction
- Determine **structure** of HTHP variants with substrates/intermediates bound
- Follow **reaction kinetics** using rapid mixing techniques (stopped-flow UV/Vis investigations as well as rapid freeze quench (resonance) Raman and EPR spectroscopy on the same sample) will be used to study and trap reaction intermediates. Rapid freeze quench involves e.g. the rapid mixing of enzyme solutions with substrate containing buffer solution(s) and rapid freezing (quenching) with varying delay times for subsequent analysis of substrate conversion and intermediates by RR and EPR spectroscopy.
- Optimize conditions to **stabilize highly oxidizing intermediates** analogous to Compound I and II
- Optimize protocols for **crystallographic data collection** to minimize reduction by X-rays (e.g. by helical scans, multi-crystal data collections strategies)
- Combine X-ray diffraction at single crystals with RR spectroscopy on single crystals and **model calculations** at high-resolution structures for detailed analysis of the geometry and electronic structure of the active site

Potential collaborations:

- EPR spectroscopy on single crystals and solution samples - Christian Teutloff / Robert Bittl (FU Berlin, UniSysCat, CRC 1078)
- Low-temperature UV and Mössbauer spectroscopy – Kallol Ray (HU Berlin, UniSysCat)
- Model calculations – Maria-Andrea Mroginski (TU Berlin, UniSysCat, CRC 1078)