Artificial metallo-proteins for photocatalytic water splitting: stability and activity in artificial photosynthesis

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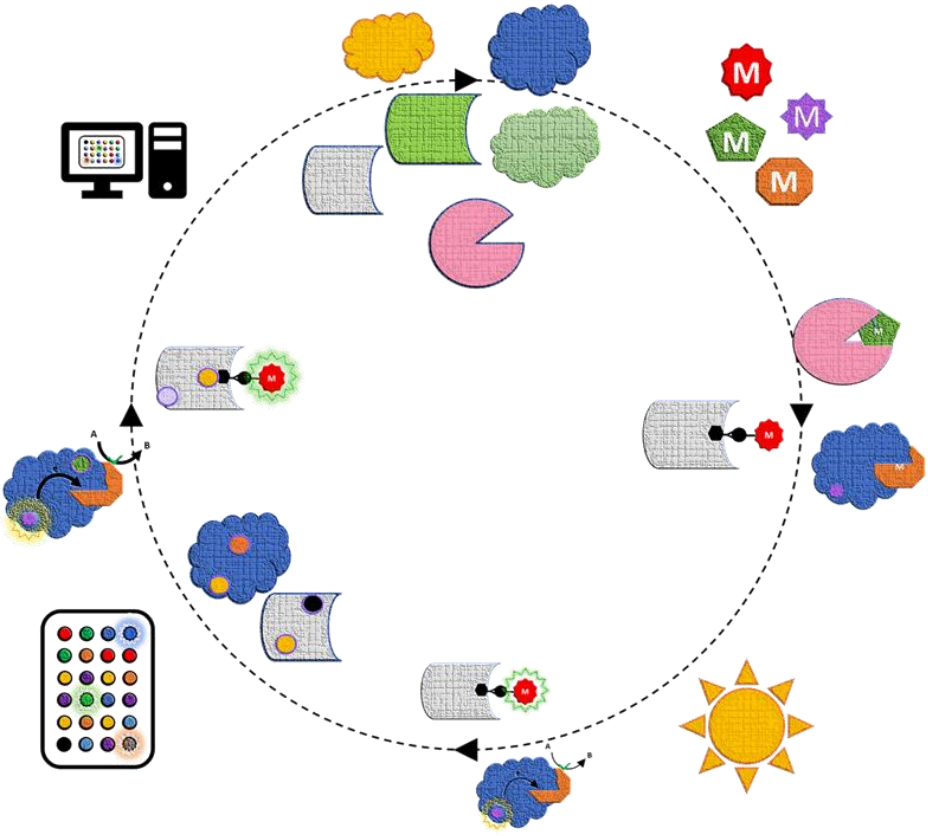
CHAPTER 6

GENERAL DISCUSSION AND OUTLOOK



Climate change as a consequence of the increasing CO_2 concentration in our atmosphere is one of the largest challenges faced by humanity at this time and if measures are not taken to address it the consequences will be severe. This increase is a consequence of the burning of fossil fuels, it is therefore of great import to find an alternative fuel. One such fuel is hydrogen, which releases no harmful gases upon its burning. This requires that a sustainable and economical means of dihydrogen production is found. To this end, research is being done into photocatalytic water splitting, with the potential to use solar light directly to produce fuel, which requires catalysts to drive the reduction of protons and the oxidation of water. Especially the latter catalysts suffer from instability under the highly oxidative conditions in which they operate [1]. Nature is capable of driving both water oxidation (WO) and dihydrogen evolution (HE) using protein-based catalysts, in which the protein scaffold offers a secondary coordination sphere capable of supporting the catalyst, permitting WO and HE under mild conditions [2]–[4]. Such a secondary coordination sphere offers a means of control over the direct environment of the catalysts, which may be of use in improving their stability and efficiency. In this thesis, we explored the effect of both HE and WO on the stability of a protein scaffold before, during, and after photocatalysis.

1 SELECTION AND SCREENING

The first challenge in preparing such a system is selecting an appropriate protein scaffold. In this thesis, the aim was axial coordination of metal catalysts with planar ligands, therefore haem-binding proteins were selected as these are the same requirements as for coordinating haem. Three haem-binding proteins were chosen, with different haem-binding properties: cytochrome b_5 (CB5), a small electron transfer protein that unfolds when its cofactor is removed, myoglobin (Mb), an oxygen-transporting protein with a more stable fold in absence of its cofactor, and haem acquisition system A from *Pseudomonas aeruginosa* (HasAp), a transient haem binding protein with an opening and closing mechanism. After selecting one or multiple protein scaffolds a screening with different potential catalysts is required to identify potential ArMs. To this end, we introduced semi-native (SN) gel electrophoresis, a screening method for the uptake of non-native metal cofactors by haem proteins, in **Chapter 2**. We found that the size and axial ligands of the introduced metallo-complex are the primary determining

factors in its binding to the protein. We further observed that the greater ligand binding strengths of cobalt and ruthenium-based complexes often led to binding to the protein exterior, which could be disadvantageous for the characterization of the properties of the binding site and with that for optimization of the ArM. Overbinding to the protein exterior can further lead to destabilization of the protein fold as well as reduced solubility and aggregation. Therefore, this potential for overbinding should be considered when selecting a protein scaffold, for the complexes employed in this thesis histidine formed a good potential binding site. We observed that a scaffold with a lot of solvent-exposed histidine on the protein exterior led to significant overbinding and could even lead to the precipitation of the protein in the presence of the catalyst.

We primarily observed binding of small cobalt catalysts to our protein scaffolds. Consequently, we found that bi-coordination was not optimal for catalyst function. Smaller cobalt catalysts are not often able to coordinate their substrate as a 7th planar ligand, leaving no site for catalysis to take place. One could instead screen for the binding of catalysts that can perform ligand-based catalysis or select or mutate a scaffold that coordinates the catalyst mono-axially. It should be further noted that smaller catalysts may be more buried inside the binding pocket which could limit interaction with *e.g.,* the photosensitizer (PS) and sacrificial reagent (SR). For a photosensitizer ArM, however, bi-coordination was not a problem and our two PS ArMs showed good activity and stability.

2 CATALYSIS IN AND STABILITY OF AN ARTIFICIAL METALLOENZYME (ARM)

Incorporation of a catalyst in a protein scaffold to create an artificial metalloenzyme (ArM) offers a 2nd coordination sphere to the catalyst with which its properties and environment can be directly controlled, but the impact it has by simply improving aqueous solubility should not be underestimated. Improved aqueous solubility removes the need for mixing with (environmentally unfriendly) apolar solvents and allows a HE or WO catalyst to be dissolved directly in its substrate. It further prevents aggregation and in the case of photosensitizers prevents the resulting self-quenching at higher concentrations.

The importance of selecting the right protein scaffold lies not only in its ability to coordinate catalyst complexes but also in its stability. Firstly, chemical stability in the presence of the PS and SR. Both could have unwanted side reactions with the protein scaffold. We observed both dark and light crosslinking of the protein scaffold in presence of the PS and SA as well as the binding of the PS to the protein under irradiation. Secondly, the stability of the protein in presence of the active catalysts. ArMs involved in water splitting reactions will be exposed to potential reduction or oxidation of their amino acids. Particularly during water oxidation reactive oxygen species (ROS) can be formed, which can lead to the oxidation of the protein. We indeed observed signs of ligand oxidation in our water oxidation ArM. The protein scaffold was, however, still soluble, showing only a limited increase in its multimerization, and was still partially folded. This indicates some resistance against ROS even without further optimization. The conditions of a HE half reaction appeared to impact the surrounding proteins less, the HasAp-based PS and HEC ArMs were active for at least 42 h, showing only some minimal protein cleaving after ~42 h of activity. The stability of the Mb-based HEC ArM was limited by the lacking stability of the protein scaffold at lower concentrations and therefore cannot be commented on directly.

A further consideration is the stability of the interaction of the protein with the catalyst complex, oxidation of coordinating ligands can lead to the release of the coordinated complex as was observed in our ArM system in **Chapter 3** (WOC ArM CB5:CoSalen 1:5 in **Chapter 3**). Lacking ArM4 stability in presence of PS and SA can also lead to the release of its cofactor as was observed with HEC ArM ArM3 in **Chapter 4**. It may therefore be advisable to introduce the catalyst to the protein via a more stable, oxidation-resistant covalent linking.

3 Recommendations for ArMs with WO and HE activity

As mentioned above analysis of two of our ArM systems revealed that the catalyst does not always remain bound in its initial location on the protein scaffold, this can make optimization challenging. It may be advisable to attempt to link the catalyst to the protein with a stronger covalent interaction where possible.

Two paths for improving its catalytic activity and stability may be explored for our current ArMs: Firstly, protein stability may be improved by creating a direct path for electron transfer in the photocatalytic system as this prevents side reactions and can improve catalytic efficiency [5]. Linking the PS to the ArM catalyst protein directly can be a good first step, though when catalyst migration takes place, it will be challenging to determine the optimal binding site for the photosensitizer. From **Chapter 5** we can infer that the mutation should point outward from the protein to promote interaction with the SR. In a system containing both a dihydrogen evolution and a water oxidation ArM this may be even more important and a covalent link/wire or support to create a direct path between the two may be required for both catalytic efficiency and system stability. It is further possible to incorporate the HEC and WOC ArMs in a liposome, in mimic of the natural photocatalytic system, to promote electron transfer between them. Our CB5 protein scaffold is the soluble portion of a protein with a membrane tail, if an ArM is prepared from its complete form, it could be incorporated into a system of this nature. Secondly, random mutagenesis of the protein scaffold, followed by testing of the activity. This requires a means for screening for catalytic activity more efficiently, preferably in a 96 well-plate. Some potential options for such a screen are: 1.) A pH indicator to detect the formation/uptake of H^+ during the WO or HE reaction. This does require a careful selection of the PS and SA, as catalyst/photosensitizer degradation may impact the pH or lead to a significant colour change that could result in false positives (*e.g.,* $[Ru(bpy)_3]^{2+}$ degradation involves attack of a bpy ligand by an OH^- and effects the pH [6]). 2.) Formed dioxygen in a WO reaction can be detected more directly by a fluorescent or phosphorescent oxygen sensor as is often done for cell viability essays [7]–[9].

More specifically from this thesis: 1.) In **chapter 5** we observed that the CB5 L75C mutant bound to a $[Ru(bpy)_3]^{2+}$-derived PS showed very efficient electron transfer to haem in its binding pocket. This suggests that it could make a good scaffold for a HEC. The selected HEC would ideally need to be structurally similar to haem to improve CB5 folding upon its coordination, to this end, CoPPIX, which has been previously shown to catalyse HE while biaxially coordinated [10] could be tested. Other candidates that can be considered are NiPPIX. A nickel hangman porphyrin was previously reported to show good dihydrogen evolution activity, with the hangman ligand

providing protons during the catalysis [11]. When NiPPIX is introduced into a protein scaffold, site directed mutagenesis could be employed to introduce a ligand with this same hangman functionality. Both zinc and copper diacetyl-bis(N-4-methyl-3-thiosemicarbazide) [12], [13] display good ligand-based dihydrogen evolution activity, though they are limited by low aqueous solubility. Both are sufficiently small that they should coordinate well to CB5, which would resolve their solubility challenges and allow for the optimization of their activity via site directed mutagenesis. 2.) One of the histidines in the CB5 binding pocket could be mutated to introduce an open axial site on the coordinated catalyst to permit metal-based catalysis. This may be interesting to attempt with CB5:CoSalen 1:1, it will be interesting to see if CoSalen remains inside the binding pocket during catalysis. 3.) HasAp has shown great potential as a protein scaffold, both in terms of its stability and its ability to coordinate non-native cofactors (**chapter 4** and [14], [15]). In **chapter 4** we showed HasAp to be an effective and stabilizing scaffold for both a HEC and PS and produced a HEC/PS ArM. It would be interesting to use this system as a starting point for further optimization.

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