Ruthenium– and cobalt–based artificial metalloenzymes for photocatalytic water oxidation in artificial photosynthesis
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Chapter 6

Summary, Conclusions & Outlook

6.1 Summary of this thesis

In **Chapter 2**, we reported a screening method to study the interaction between ruthenium- and cobalt–based water oxidation catalysts with two different haem proteins, Cytochrome B5 (**CB5**) and Myoglobin (**Myb**). This study aimed at the development of new artificial metalloenzymes for photocatalytic water oxidation.[1] The study showed how a new approach, called semi–native gel electrophoresis, offered the best and most practical resolution for detecting protein-cofactor interaction. Compared to the two other types of gel electrophoresis methods tested, *i.e.*, denaturing and native, the semi–native method showed clearer band separation. Among the proteins and complexes studied and according to this method, the *apo* form of Cytochrome B5, ***apo*CB5**, and the cobalt complex **Co^{II}Salen** (Salen = N,N'–Bis(salicylidene)ethylenediamine) showed the best interaction. ESI–MS corroborated the formation of an ***apo*CB5:CoSalen** adduct as possible artificial metalloenzyme candidate for water oxidation. This work provided an efficient tool to study the formation of artificial metalloenzymes, which was used in other chapters of this thesis.

In **Chapter 3**, a cobalt–based artificial metalloenzyme, ***apo*CB5:CoSalen**, was prepared and proved to promote photocatalytic water oxidation in presence of $[Ru(bpy)_3]^{2+}$ as photosensitizer and sodium persulfate as sacrificial electron acceptor. Two artificial metalloenzymes called ***apo*CB5:CoSalen 1:1** and ***apo*CB5:CoSalen 1:5** were isolated and studied, defined by the different stoichiometry of the cobalt cofactor *vs. apo* protein used during preparation. Using ICP–MS and ESI–MS it was possible to characterize the effective stoichiometry of both ArM's. In both types of samples (1:1 and 1:5 protein:complex), NMR clearly demonstrated the binding of one **CoSalen** molecule to the haem-binding pocket of ***apo*CB5**. In ***apo*CB5:CoSalen 1:5** samples, however, the binding location of the excess cobalt molecules was more difficult to establish. In photocatalytic water oxidation conditions, the ***apo*CB5:CoSalen 1:1** samples, where **CoSalen** was bound

only to the haem-binding pocket of the protein, was catalytically inactive, while the ***apo*CB5:CoSalen 1:5** samples, where ~3 additional **CoSalen** molecules were found bound to the protein exterior, promoted photocatalytic water oxidation. Unfortunately, O_2 evolution using the artificial metalloenzyme ***apo*CB5:CoSalen 1:5** as catalyst was not improved, compared to analogous photocatalytic systems containing free **CoSalen** as WO catalyst. However, the use of the ***apo*CB5** protein scaffold to support the **CoSalen** catalyst inhibited the formation of nanoparticles, which were the catalytically active species with free **CoSalen**.[2] With ***apo*CB5:CoSalen 1:5** the soluble cobalt–containing protein species that formed during photocatalysis remain to date unidentified. Overall, this work represents one of the first studies presenting the effect of coordination of an *apo* haem-binding protein to a molecular WOC on its activity and stability in photocatalytic conditions.

In **Chapter 4**, a set of ruthenium-based artificial carbonic anhydrase metalloenzymes for water oxidation was described. Two reported ruthenium-based water oxidation catalyst, [Ru(2,2'–bipyridine-6,6'–dicarboxylic acid)(pyridine)$_2$] and [Ru([2,2':6',2''–terpyridine] –6,6''-dicarboxylic acid)(pyridine)$_2$] were functionalized with two aryl-sulfonamides linkers, **L1** or **L2,** producing a series of four small molecules cofactors for the functionalization of **BCA** with a WOC: two Ru(bda)–like analogues, **Ru1** and **Ru2**, and two Ru(tpyda)–like complexes, **Ru3** and **Ru4**. These complexes were anchored *via* supramolecular interaction of the sulfonamide moiety to the zinc centre of the **BCA** protein, thus obtaining four artificial **BCA–Ru** metalloenzymes. In this series only two ArM's, **BCA–Ru1** and **BCA–Ru2**, were found active for photocatalytic O_2 evolution in presence of $[Ru(bpy)_3]^{2+}$ as photosensitizer and sodium persulfate as sacrificial electron acceptor. Binding of the Ru-complexes to **BCA** improved the stability of the complex in photocatalytic conditions, by raising its TON by 4x fold in the case of **BCA–Ru1** and allowed to promote photocatalytic water oxidation at lower ruthenium concentrations compared

to the free ruthenium catalyst. The difference in activity between **BCA–Ru1** and **BCA–Ru2** indicated that the second coordination sphere provided by **BCA** influenced the O_2 production of the ruthenium catalyst, which was corroborated by studying the activity of both proteins at different pH. This pH and structural variations in the rate of O_2 production indicated that an acid–base mechanism might be involved in the photocatalytic cycle of water oxidation. Overall, **Chapter 4** proved that it is possible to use commercially available proteins for the development of artificial systems for water oxidation. It also confirmed the excellent properties of **BCA** as a potential support for the development of artificial metalloenzymes for artificial photosynthesis.

In **Chapter 5**, a system for photocatalytic water oxidation based on two ruthenium-containing artificial metalloenzymes, **BCA–Ru$_{WOC}$** and **BSA–Ru$_{PS}$**, was described. A $[Ru(bpy)_3]^{2+}$-like complex functionalized with a maleimide group was synthetized, **Ru$_{PS}$**. This precursor was reacted with free **BSA** to covalently bind the ruthenium photosensitizer to the protein by reaction with its free Cys34 residue, thereby producing an artificial light–harvesting metalloenzyme, **BSA–Ru$_{PS}$**. The protein scaffold was not affected by the attachment of the artificial complex, but the photochemical properties of **BSA–Ru$_{PS}$** changed compared to the native $[Ru(bpy)_3]^{2+}$ complex, with for example a doubled excited state lifetime. The photosensitizing properties of **BSA–Ru$_{PS}$** for photocatalytic water oxidation were evaluated in presence of the artificial metalloenzyme **BCA–Ru$_{WOC}$** reported in **Chapter 4**, using sodium persulfate as sacrificial electron acceptor and blue light. In such conditions, photocatalytic activity was observed towards water oxidation even at low concentrations of both the artificial water-oxidizing and light-harvesting enzymes. These results suggested some form of interaction between **BCA–Ru$_{WOC}$** and **BSA–Ru$_{PS}$** which allowed electron transfer between both active metal centers to occur, thus driving the formation of O_2. The presented work shows an alternative, biomimetic approach towards artificial photosynthesis.

6.2 General conclusions and outlook

Coordination of a cobalt and/or ruthenium water oxidation catalyst to a protein proved to alter the capacity of the complex to catalyse the water oxidation reaction under photocatalytic conditions. This catalytic activity depends significantly on the interaction between the protein scaffold and the metal complex, i.e., its location within the protein backbone, and the type of binding of the protein to the metal complex. In **Chapter 3**, the activity of the adduct ***apo*CB5:Cosalen** depended on the coordination of **CoSalen** to **CB5** scaffold *via* histidines. The hexacoordinated **CoSalen** molecule bound to the hydrophobic pocket of ***apo*CB5** was inactive for water oxidation, while the coordination of **CoSalen** to outer residues of ***apo*CB5** provided water oxidation catalytic activity under photocatalytic conditions. In both cases, ***apo*CB5:Cosalen 1:1 and 1:5,** an increase in the photocatalytic activity compared the free **CoSalen** complex was not observed, but the coordination and environment of each **CoSalen** molecule in **CB5** was different. Conjugation to the protein backbone improved the water solubility of the cobalt complex and the stability of the active specie formed during the photocatalytic reactions in aqueous solutions. A similar effect was observed in **Chapter 4**, where the coordination of a Ru catalyst to **BCA** scaffold increased the solubility of the metal complexes in aqueous solutions, thereby avoiding the use of organic solvents.

Given the difficulties to bind *via* coordination ruthenium complexes to ***apo*CB5** in the studied conditions, which were discussed in **Chapter 2**, a more successful supramolecular approach using sulfonamide linkers was chosen to promote the **BCA**–ruthenium adducts formation (**Chapter 4**). Unlike **CB5** on **CoSalen**, using **BCA** as a host for a Ru(bda)–like WO complex was found to have a positive effect on the catalytic properties of the ruthenium catalyst. Indeed, the photocatalytic activity of a water oxidation system comprising **BCA**–**Ru1** as catalyst was increased, in particular at low catalyst concentration, compared with the system comprising the protein-free metal complex as catalyst, which also required organic solvent in the

medium. For **Ru2**, no activity was observed for the free complex, while the **BCA–Ru2** artificial enzyme did show some activity. In contrast, for ArM containing the Ru(tpyda)–like complexes, **BCA–Ru3** and **BCA–Ru4**, the protein scaffold did not increase the WO activity of the complexes, as photocatalytic systems based on either the free complexes or the ArM's, were inactive. The modulation of the activity of ArM's as WO catalyst, which also depended on the pH and linker length (**L1** *vs.* **L2**), proved that the protein environment, also called second coordination sphere, generated around the metal-based catalytic center, tunes the mechanism of water oxidation in ArM's.

Although in this thesis the development of artificial metalloenzymes for water oxidation was achieved, these ArMs shared a common fate during photocatalytic WO: they oxidized and denatured. Like in nature, the O_2 produced in our ArM for water oxidation can interact with the excited state of the photosensitizer formed during photocatalysis (*i.e.* $[Ru(bpy)_3]^{2+\bullet}$) to produce a highly oxidant specie, $^1O_2$, as well as the oxidized state of the photosensitizer, $[Ru(bpy)_3]^{3+}$. Both species have enough oxidizing potential to denature the protein by oxidizing e.g. the metal center or nearby tyrosine residues. Tyrosine oxidation is known to form radicals that can further dimerize by radical coupling, leading to protein oligomerization.[3] To avoid this oxidation process and denaturation of the ArM, it may be possible to use a singlet oxygen trapping molecule such as 9,10-dimethylanthracene (DMA).[4] An example of such strategy was reported by Sato *et al.* who used N'–acyl–N,N–dimethylphenylenediamine as a tyrosyl radical trapper (**TRT**) to prevent protein dimerization *via* tyrosine oxidation. Additionally, **TRT** acts in such systems as a scavenger of $^1O_2$.[5] The main disadvantage is that this strategy may induce the coupling of the trapping agent to the protein scaffold.

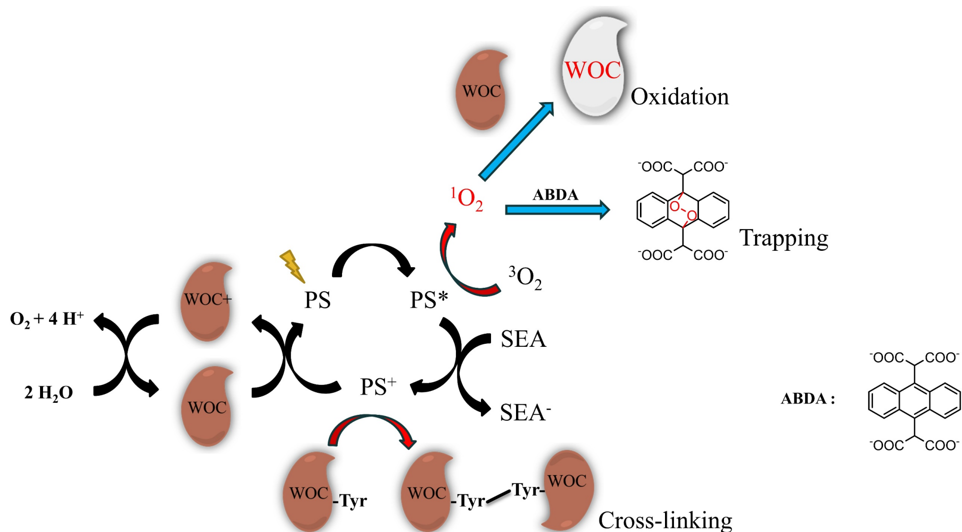

Figure 6.1. Proposal scheme of sides reaction of singlet oxygen ($^1O_2$) generated from the water oxidation reaction by the artificial metalloenzyme.

An alternative strategy would be to use a singlet oxygen trapper such as 9,10–Anthracenediyl–bis(methylene)dimalonic acid (**ABDA**), which has been reported to have a high selectivity for this specie (Figure 6.1).[6,7] Another well-used molecule is 1,3–diphenylisobenzofuran (**DPBF**), which is not selective for singlet oxygen but also traps other oxidative species such as H_2O_2.[8] These molecules might not only prevent the oxidation of the protein scaffold but also protein cross–linking as results of the tyrosine residues oxidation (Figure 6.1). Despite the accuracy and sensibility of the Clark electrode to measure the O_2 formed during photocatalysis, these single oxygen trapper molecules may also help to quantify traces of solubilized O_2 in solution that could not be detected by the electrode, by simply following formation of the oxidized singlet oxygen trapper.

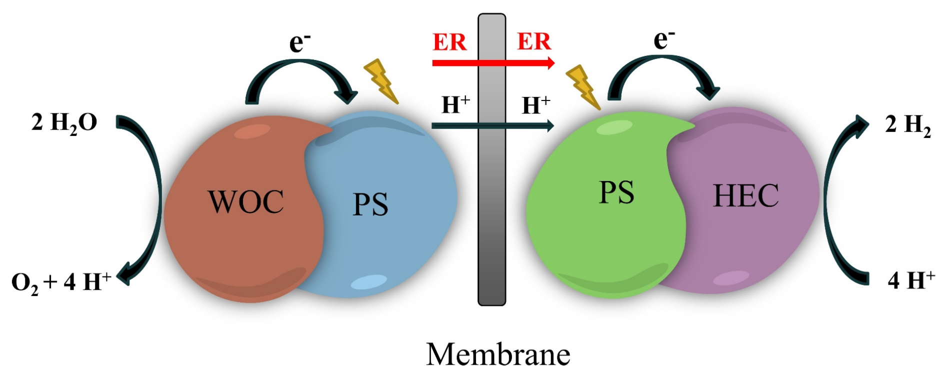

Figure 6.2. Proposal scheme for an artificial metalloenzyme system for water splitting. Each coloured blob represents a different artificial metalloenzyme: WOC: water oxidation catalyst (orange), PS: photosensitizer (blue and green), HEC: Hydrogen evolution catalyst (purple), ER: electron relay.

In this thesis, we also addressed the question of protein–protein interactions between the artificial metalloenzymes **BCA–Ru$_{WOC}$** and **BSA–Ru$_{PS}$**, which drive the formation of O_2 from water in photocatalytic conditions. An interesting approach to study artificial photosynthesis in conditions closer to nature could be the study of a system for water splitting using two compartments containing ArM's: one for O_2 evolution using the system established in **Chapter 5**, and another one for H_2 evolution, using artificial metalloenzymes for hydrogen evolution. For the later, the interaction between a protein capable to drive H_2 formation,[9] and a protein bearing a photosensitizer capable of activating the catalyst, should be studied (Figure 6.2). In addition, to ensure electron transfer and maximize the activity of the whole system, these compartments should be separated by a membrane that would control the flow of protons and of electron relay used to replace the electron acceptors and electron donors used in most work. Preliminary studies of a system using **BSA– Ru$_{PS}$** and a reported Co–based ArM for H_2 evolution, **Myb – Co^{II}PPtIX**,[10] using sodium ascorbate as electron donor, showed no activity (Table 6.1). These results may be related to the electrostatic repulsion between proteins. Electron relays such as benzoquinone (BQ), methyl viologen (MV), hemin, or iron(III) chloride, have been

used in the literature to promote electron transfer between the photosensitizer and the cobalt catalytic center, but not catalysis was observed. More studies needs to be done using different protein couples, or proteins that bear both actives molecules, *i.e.*, the catalyst and the photosensitizer.[11]

Table 6.1. Study of H_2 evolution with two artificial metalloenzymes.[a]

Entry	BSA–Ru_{PS} (µM)	Sodium ascorbate (mM)	Electron relay	H_2 (µmol)
1	300	50	-	-
2	20	50	-	-
3	300	50	BQ (2 mM)	-
4	300	50	MV (0.1 mM)	-
5	300	50	Hemin (0.1 mM)	-
6	300	50	Hemin (1 mM)	-
7	300	-	$FeCl_3$ (2 mM)	-
8	300	50	$FeCl_3$, MV (2 mM)	-
9	300	50	$FeCl_3$ (2 mM)	-

[a] **Conditions:** [**Myb – Co^{II}PPtIX**] = 5 µM, pH 7.0 phosphate buffer (20 mM), 450 nm (19 mW), 25°C.

In conclusion, binding molecular water oxidation catalyst based on heavy metals as ruthenium and earth–abundant metal as cobalt to well–studied and accessible proteins, allowed us to produce interesting ArM for photocatalytic water oxidation. Important knowledge was acquired on the interaction between small–molecule metal complexes and proteins, and on the fate of such hybrid systems in

the harsh conditions or photocatalytic water oxidation. It is still unclear whether this strategy may one day become an attractive method to develop upscalable and environmentally friendly systems for artificial photosynthesis. However, this approach may one day provide convincing evidence that it is possible to be inspired from nature to split water efficiently without using (photo)active compounds based on rare metals.

6.3. References

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