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# Photocatalytic hydrogen production and carbon dioxide reduction catalyzed by an artificial cobalt hemoprotein

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**Abstract:** The covalent insertion of a Cobalt heme into the cavity of an artificial protein named alpha Rep ( $\alpha$ Rep) leads to an artificial cobalt hemoprotein that is active as a catalyst not only for the photo-induced production of H<sub>2</sub> but also for the reduction of CO<sub>2</sub> in a neutral aqueous solution. This new artificial metalloenzyme has been purified and characterized by Maldi-TOF MS, circular dichroism and UV-visible spectroscopy. Using theoretical experiments, the structure of this biohybrid and the positioning of the residues near the metal complex were examined, which made it possible to complete the coordination of the cobalt ion by an axial glutamine Gln283 ligand. While the Co(III)-porphyrin catalyst alone showed a weak catalytic activity for both reactions, 10 times more H<sub>2</sub> and 4 times more CO<sub>2</sub> were produced when the Co(III)-porphyrin complex was buried in the hydrophobic cavity of the protein. This study thus provides a solid basis for further improvement of these biohybrids, using well-designed modifications of the second and outer coordination sphere, by site-directed mutagenesis of the host protein.

**Keywords:** artificial metalloenzymes, cobalt porphyrin, proton reduction, CO<sub>2</sub> reduction, catalysis

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## 1. Introduction

One of the greatest challenges facing our society is to replace current fuels with new, abundant and renewable sources of energy. Hydrogen (H<sub>2</sub>) seems to be the ideal energy carrier because, in addition to its highly energetic H–H bond, it's environmentally friendly combustion releases only water as product. H<sub>2</sub> production processes are numerous (thermal, photochemical, biological, electrolytic, etc.) and the resources used in these processes are equally as abundant (water, solid waste, cellulose, biomass, etc.). To meet the fast-growing global demand for H<sub>2</sub>, the development of catalysts for the production of hydrogen is highly sought after. Specifically, new original molecular complexes, most often bioinspired, based on non-noble metals such as nickel, cobalt or molybdenum, have been synthesized and studied to replace platinum, a catalyst that is certainly effective but scarce and expensive [1-4]. Molecular catalysts have been shown to be proficient in the hydrogen evolution reaction (HER) showing increased efficiency and stability. In particular, a variety of molecular cobalt catalysts have been developed for the production of hydrogen, such as: glyoxime and the vitamin B12, which have the disadvantage of being poorly soluble and unstable in water [5]; Mononuclear and dinuclear cobalt polypyridines [6,7]; macrocyclic ligands like cobalt corroles [8]; cationic cobalt porphyrins [9]; anionic cobalt porphyrins [10]; cobalt heme-peptides [11]; and more recently a synthetic mini-

enzyme known as mimochrome VI<sup>a</sup> [12]. These cobalt complexes underwent light-induced proton reduction in water when combined with a photosensitizer and a sacrificial electron donor. However, many of these complexes are sparingly soluble in water and their chemical stability is not optimal. To improve the catalytic performance of these systems, their outer coordination sphere can be designed to promote the efficient transfer of electrons, H<sup>+</sup>, or H<sub>2</sub>. To overcome this limitation, efforts have been made to develop artificial metalloenzymes with an unnatural active site integrated into a protein. This strategy has been shown to improve the production of H<sub>2</sub> [13] and different artificial Co-artificial metalloenzymes have been obtained by non-covalent insertion of cobaloxime derivatives into apo-hemoproteins, such as sperm whale myoglobin or heme oxygenase [14,15] or in a protein carrying out electron transfers such as ferredoxin [16]. In order to improve the catalytic efficiency of those artificial metalloenzymes, another cofactor like the cobalt protoporphyrin IX was incorporated into a sperm whale myoglobin scaffold by the group of Ghirlanda [17]. In a recent publication, the same group successfully reported the light-driven reduction of CO<sub>2</sub> and the generation of H<sub>2</sub> using an artificial hemoprotein made from the Cytochrome b562 apoprotein that included Co-Protoporphyrin IX as a cofactor [18]. Inspired by these results, we decided to assemble new artificial metalloenzymes by a covalent-grafting strategy, involving the synthesis of various cobalt cofactors, bearing a moiety that would be able to react with the SH group of a cysteinyl residue that could permanently graft them to the protein. Recently, our group has shown that a new class of thermostable artificial proteins named  $\alpha$ Reps have the potential to be transformed into artificial metalloenzymes by covalent grafting of a synthetic metal complex [19]. These  $\alpha$ Rep proteins are easily produced in *E. coli*, are very stable within a large pH range, and exhibit a large hydrophobic core. We have thus previously reported a proof of concept by creating artificial Diels-Alderases by covalent attachment of phenanthroline- or terpyridine-Cu(II) complexes in a specific  $\alpha$ Rep single-chain asymmetric bidomain variant named (A3A3')Y26C [20]. This protein has been shown to adopt a closed bivalve shell-like conformation and molecular modeling identified that this cavity was large enough for the anchoring of even larger metal complexes such as *meso*-tetraphenylporphyrin [21]. Thus, from an enzyme designer's point of view, the artificial  $\alpha$ Rep proteins bring together the properties required for the development of artificial metalloenzymes dedicated to H<sub>2</sub> production. Accordingly, we here report the production and characterization of a new artificial metalloenzyme obtained by covalent attachment of a cobalt porphyrin derivative into the  $\alpha$ Rep (A3A3')Y26C variant and further show its remarkable activity to catalyze the photoinduced production of H<sub>2</sub> and CO<sub>2</sub> reduction.

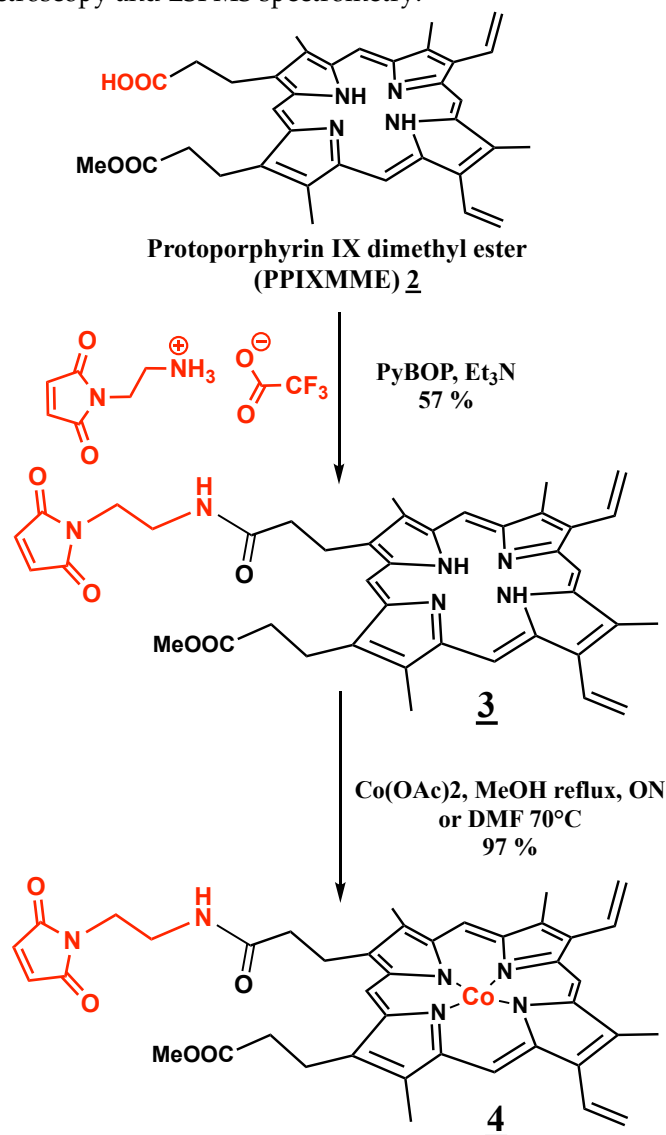
## 2. Results and discussion

### 2.1. Synthesis of Co(III)Mal-PPIXMME **4**

The Co(III)Mal-PPIXMME cofactor **4** was prepared in 3 steps. In a first step, the hydrolysis of protoporphyrin IX dimethyl ester (PPIXDME) by 6N HCl during 15 min. at room temperature (RT) following the reaction conditions described by Ellsworth R.K. [22] (see Materials and Methods). Protoporphyrin IX monomethyl ester (PPIXMME) **2** was then obtained in a 28 % yield, and was characterized by NMR and UV-visible spectroscopies as well as by ESI MS (see Materials and Methods).

The two following steps of the synthesis are shown in figure 1. Reaction of **2** with a slight excess of *N*-(2-aminoethyl)-maleimide trifluoroacetate salt (1.1 equiv.), in the presence of triethylamine and PyBOP as a coupling agent, in dimethylformamide (DMF) for 30 min. at RT afforded the maleimido-ethylamido-protoporphyrin IX monomethyl ester Mal-PPIXMME **3** as a brown solid in a 45 % yield. **3** was characterized by NMR and UV-visible spectroscopies and ESI MS (see Materials and Methods). These characteristics were in good agreement with those of a very similar product maleimido-ethylamido-protoporphyrin IX monoethyl ester Mal-PPIXMEE already reported by the group of Y. Watanabe [23].

Finally, the addition of 10 equiv. of  $\text{Co}(\text{OAc})_2 \cdot 4 \text{H}_2\text{O}$  to the obtained compounds **3** in anhydrous DMF at 70 °C led, after removal of the excess of cobalt salt by extraction in a dichloromethane (DCM)/water mixture and removal of the solvent under reduced pressure, to Co(III)-Mal-PPIXMME as a dark green solid in a 97 % yield. **4** was characterized by UV-visible spectroscopy and ESI MS spectrometry.



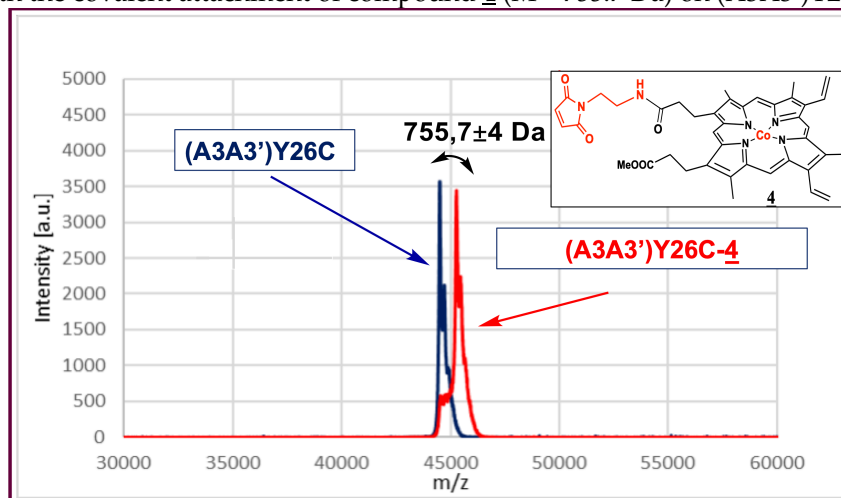
**Figure 1.** Synthesis of the Co(III)Mal-PPIXMME cofactor **4** from Protoporphyrin IX Monomethyl ester (PPIX MME) **1**.

## 2.2. Biohybrid preparation

The (A3A3')Y26C-Co(III)Mal-PPIXMME (A3A3')Y26C-**4**) biohybrid was prepared in three steps. The single-chain bidomain protein in which a cysteine-free domain was linked with a domain bearing the Y26C mutation ((A3A3')Y26C) was first produced and purified as recently described [24]. Compound **4** was then synthesized from protoporphyrin IX in three steps as described above. The biohybrid was finally assembled by incubation of the (A3A3')Y26C protein with 10 eq. of compound **4** for 3 days at 4 °C, in a 50 mM Na-phosphate pH 7.75 coupling buffer containing 150 mM NaCl and 25% dimethyl sulfoxide (DMSO). After removal of the excess of compound **4** by extraction with butan-2-one, the (A3A3')Y26C-**4** biohybrid was recovered in the aqueous phase.

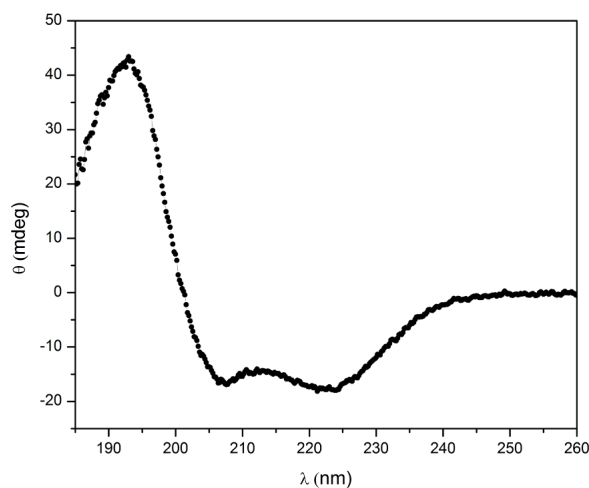
The (A3A3')Y26C-**4** biohybrid was then characterized by MALDI-ToF mass spectrometry analysis (Fig. 2). Whereas (A3A3')Y26C showed a major peak at  $M = 44502 \text{ Da}$ , a

peak at 45262 Da was observed for the (A3A3')Y26C-**4** biohybrid, which was in agreement with the covalent attachment of compound **4** ( $M = 755.7$  Da) on (A3A3')Y26C.



**Figure 2.** Mass spectrometry analysis of the (A3A3')Y26C-**4** biohybrid : Maldi-ToF spectrum of (A3A3')Y26C (blue) and (A3A3')Y26C-**4** (red).

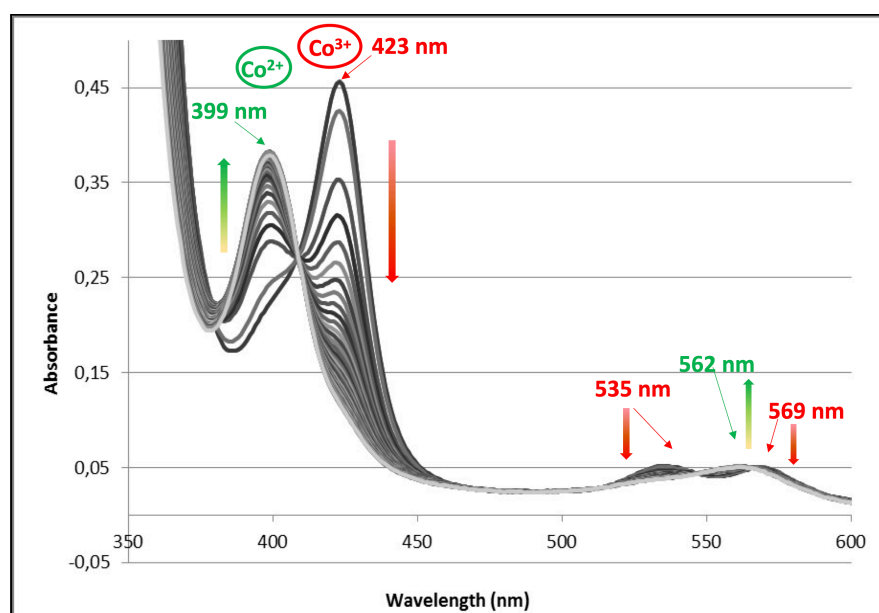
To analyze the effect of the binding of **4** into the cavity of (A3A3')Y26C on the folding of the protein circular dichroism (CD) measurements were carried out. The far UV circular dichroism spectrum of the (A3A3')Y26C-**4** biohybrid (Fig. 3) showed a signal with two negative bands at 222 and 208 nm and a positive band around 190 nm that was characteristic of proteins containing mainly  $\alpha$  helices as secondary structure. This signal was very similar to that of the non-modified (A3A3')Y26C protein [19, 21] indicating that its 3D structure was not altered after covalent attachment of the porphyrin cofactor in its hydrophobic pocket, even in the presence of 25% DMSO, and further treatment with butan-2-one.



**Figure 3.** Far-UV CD spectrum of a 2.2 mM solution of the (A3A3')Y26C-**4** biohybrid in 20 mM sodium phosphate buffer pH 7.5.

### 2.3. UV-visible characteristics of the (A3A3')Y26C-**4** biohybrid

The addition, under anaerobic conditions, of 1.5 mM sodium dithionite to a 7.5  $\mu$ M solution of (A3A3')Y26C-**4** in 10 mM HEPES, NaCl 150 mM buffer, pH 8.0 led to the gradual replacement, with an isosbestic point at 408 nm, of the spectrum characteristic of Co(III)-Mal-PPIXMME **4** with peaks at 423, 535 and 569 nm by a new one with peaks at 399 and 562 nm that was characteristic of the reduced form of the biohybrid Co(II)-Mal-PPIXMME **4** (Fig. 4).



**Figure 4.** Evolution of the UV-Vis spectrum of (A3A3')Y26C-4 7.5  $\mu$ M in 10 mM HEPES, NaCl 150 mM buffer, pH 8.0 after the addition of a 1.5 mM anaerobic solution of sodium dithionite. Spectra were recorded every 5 min during 2h.

The characteristics of these spectra were compared in Table 1 with those obtained after incorporation of Co(III)- and Co(II)-protoporphyrin IX into the apoprotein of various hemoproteins including myoglobin (Mb) [24] and its H64V-V68A double mutant [25], 2 cytochromes P450; P450<sub>Cam</sub> [26] and CYP119 [27] two peroxidases; Horseradish peroxidase (HRP) [24] and dye decolorizing peroxidase (DYP) [28], Aldoxime deshydrogenase (Oxd) [28], and Catalase (Cat) [28].

**Table 1.** Comparison of the maxima of absorption of (A3A3')Y26C-4 with those of various Co(II)- and Co(III)-PPIX-substituted hemoproteins produced either *in vivo* or *in vitro* through reconstitution with analogous proteins with CoPPIX from ref 28.

Protein	Co(III) $\lambda_{Max}$ (nm)			Co(II) $\lambda_{Max}$ (nm)			Ref.	
	Ligands	Soret	$\beta$	$\alpha$	Ligand	Soret		$\alpha/\beta$
Mb	His/H <sub>2</sub> O	424	535	572	His	406	558	[24]
MbH64VV68A	His/H <sub>2</sub> O	428	540	572	His	406	555	[25]
P450 <sub>Cam</sub>	Cys/H <sub>2</sub> O	422	538	570	Cys	404	556	[26]
CYP119	Cys/H <sub>2</sub> O	422	539	566	Cys	410	558	[27]
Oxd	His/H <sub>2</sub> O	429	542	574	His	402	559	[28]
HRP	His/H <sub>2</sub> O	421	533	565	His	401	553	[24]
DYP	His/H <sub>2</sub> O	422	534	567	His	403	564	[28]
Cat	His/?	427	537	570	His	403	561	[28]
<b>(A3A3')Y26C-4</b>	<b>?</b>	<b>423</b>	<b>535</b>	<b>569</b>	<b>?</b>	<b>399</b>	<b>562</b>	<b>This work</b>

As shown in Table 1, the electronic absorption spectrum of the oxidized Co(III)-(A3A3')Y26C-4 biohybrid is similar to those obtained by incorporation of Co(III)PPIX not only into apoproteins which bind the cobalt cation through an axial thiolate ligand such as P450 cam [26] and CYP 119 [27] but also to those of some apoproteins which bind the cobalt cation through an axial histidine ligand such as Mb, HRP [24] and DYP [28]. Such a comparison does thus not allow the identification the of cobalt axial ligand of cobalt in (A3A3')Y26C-4. However, when comparing the electronic absorption spectra of the

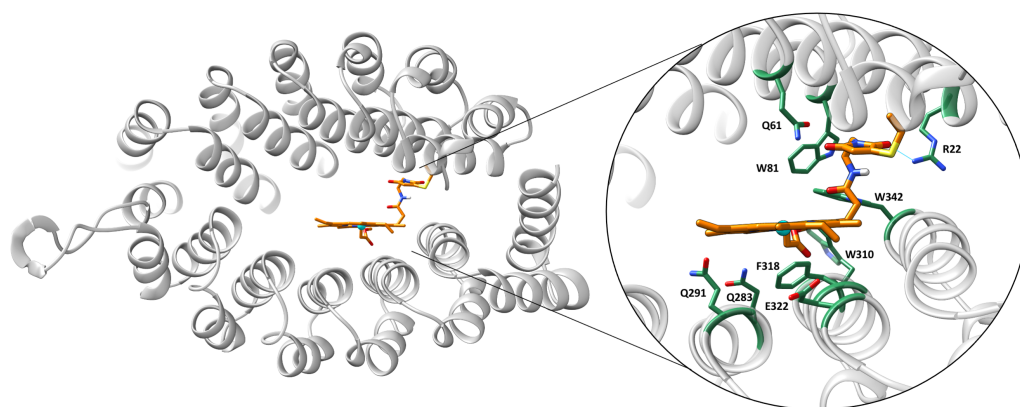
various reduced complexes, it appears that the spectral characteristics of the Co(II)-(A3A3')Y26C-4 biohybrid are very different from those observed with P450 cam [26] and CYP 119 [27] whereas they are closer from those observed in the case of proteins bearing an axial histidine ligand with, in particular, a very strong similarity with those of the Co(II) dye decolorizing peroxidase (DYP) [28]. This latter comparison tends to suggest axial coordination of the cobalt ion by a histidine ligand in the Co(II)-(A3A3')Y26C-4 biohybrid. However, in order to clarify the environment of the cobalt in the distal pocket of this artificial metalloenzyme, and in particular the nature of its axial ligand, computational modeling studies were engaged.

#### 2.4. Computational modeling studies

In the absence of X-ray structure of the (A3A3')Y26C-4 biohybrid, we embarked on a molecular modeling study. It aimed primarily at elucidating the most stable orientation of the non-natural porphyrin into (A3A3')Y26C and assessing relevant interactions between both subsystems including possible coordination bonds between nearby laying amino acids and the metal.

First, protein-ligand dockings were carried out. As protein structure, we use the representative geometry of the most populated cluster of conformations of a 300 ns molecular dynamics (MD) performed with AMBER [29] of the wild type A3A3' for which Tyr26 was mutated to cysteine using the UCSF Chimera plug-in for amino acid substitution [30]. The artificial cofactor was followingly docked using the covalent protocol available in Gold and the GoldScore scoring function that we updated for metalloligand recently [31].

A first docking run was performed with a rigid protein scaffold. From the 50 solutions obtained, the lowest energy solution reached 55.1 GoldScore units and can be considered a good interaction between both molecular partners. The porphyrin stands very well into the inter-dominial pocket and displays good interactions with amino acids of the cavity like Phe318 with the macrocycle, the methyl group of the methyl propionate ester side chain interacts with a hydrophobic patch constituted by Trp81, Trp310 and Trp342 and the cyclic moiety of the linker interacting polarity with Arg22 (Fig. 5). **Close-lying residues that could coordinate are Gln 61,283 and 291 or Asp322.**

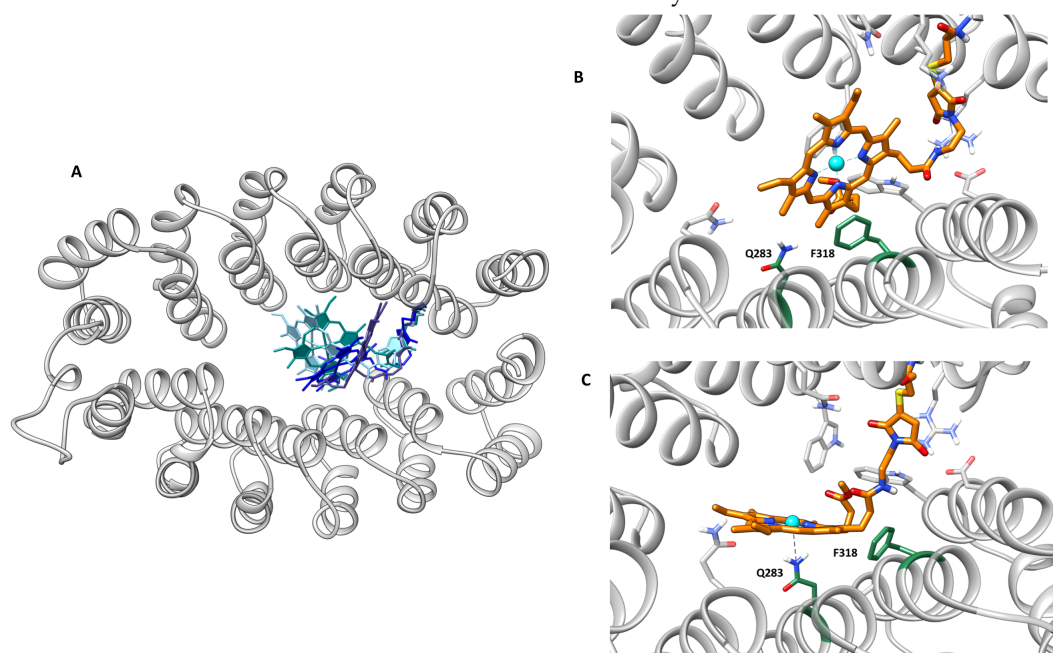


**Figure 5.** Representation of the lowest energy geometry of the (A3A3')Y26C-4 biohybrid obtained with Gold and the Goldscore scoring function.

Based on this structure, further dockings were carried out to see if nearby laying residues could eventually interact with the metal. This has been performed by giving rotational flexibility to the side chain of amino-acid Glu322 and Phe318 as well as Gln283. From those calculations, it appears that Glu322 and Gln291 may both reach convenient distances for coordination though the former needs Phe318 to be out of the way to the iron. Additionally, these docking solutions show different degrees of rotation of the porphyrin around the linker. This highlights a certain amount of flexibility of the porphyrin

that could be related to transient interactions between the metal and the protein. To further analyze this hypothesis, classical molecular dynamics (MD) were performed.

Classical MDs were carried out with the AMBER suite of programs using the AMBER20 force field [29]. The porphyrin linker was set up by defining a non-standard amino acid and using of the MCPB.py algorithm for the parameterization of the metallic center and its first coordination sphere (see ESI for further details on the computational section) [32, 33]. Calculations started from the best solution for the initial series of dockings and run for 400 ns. The trajectory shows the stability and convergence of the system after this time (Fig. S1). The calculations agree with the flexible docking and show a flexible cofactor in the binding site (Fig. 6A). However, some orientations clearly dominate. The most populated one is similar to the best-docked structure described at the beginning of this section (Fig. 6B). One can observe a very stable  $\pi$ -stacking between Phe318 and the porphyrin. Interestingly, the Phe318 side chain lays most of the simulation just below the metal with a position that would be occupied by an axial ligand of the metal in natural hemoenzymes. This shows that only one face of the porphyrin is accessible for catalysis and has a well-defined and asymmetric distal environment. Alternative orientations correspond to geometries in which the interaction Phe318-4 is lost. When such interaction is lost, compound **4** behaves very freely in the interface between A3 and A3' subdomains. Importantly, one of the most populated geometries of these alternative orientations shows short distances between Gln283 and the cobalt (Fig. 6C), some consistent with direct Co-Gln283 coordination and others with a bridged water molecule between the amino acid and the metal. Such interaction is only possible because the absence of the interaction Phe318-4 is associated with an increase in the accessibility of the metal.



**Figure 6.** A. Overlap structures of the main clusters extracted from the 400ns MD trajectory for the (A3A3')Y26C-**4** systems. B. MD snapshot of the most populated cluster of the trajectory. C. snapshot with the closest distance between the metal and the nitrogen side chain of Gln283.

In conclusion, the molecular modeling study shows that: 1). The linkage of compound **4** to (A3A3')Y26C leads to a macrocycle located at the interface between both subdomains and excluded from the solvent, 2) the main orientation of **4** in A3A3' presents a strong contact between Phe318 and the macrocycle that aids the packing of the porphyrin hydrophobically to one domain and leads to an asymmetric environment on the distal side, and 3) that possible coordination of the metal would only appear on transient structures with Gln283.

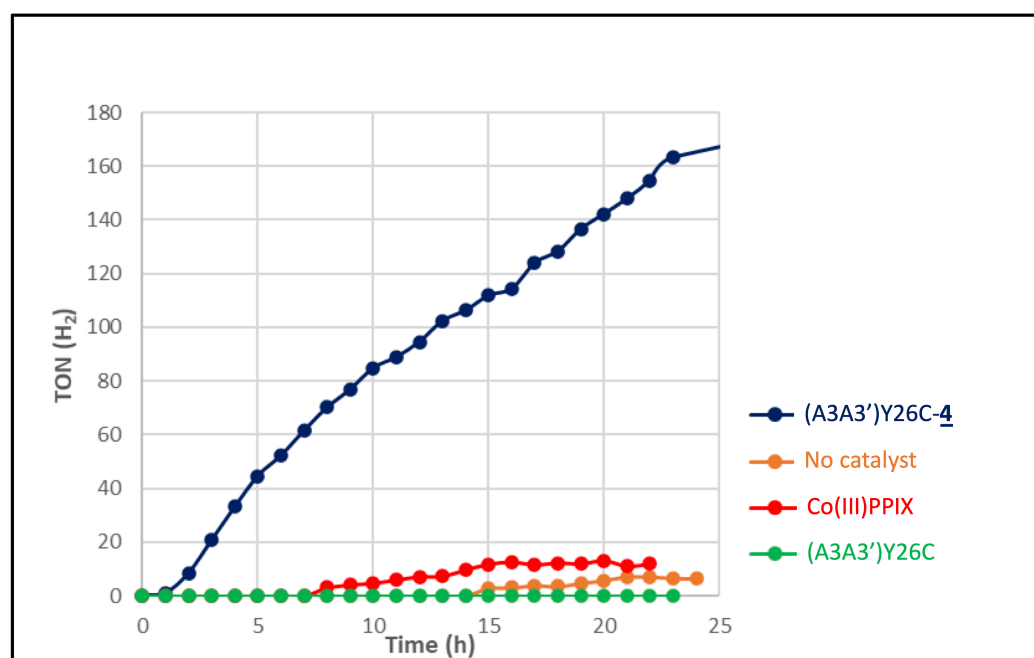


### 2.5. Photocatalytic activity of the (A3A3')Y26C-4 biohybrid

The ability of the (A3A3')Y26C-4 biohybrid to catalyze not only the production of H<sub>2</sub>, but also to reduce CO<sub>2</sub> under photoinduced conditions. [Ru(bpy)<sub>3</sub>]<sup>2+</sup> (bpy = 2,2'-bipyridine) was chosen as the photosensitizer and ascorbic acid as the sacrificial electron donor agent, since it was shown previously that under irradiation at 405 nm the excited state of [Ru(bpy)<sub>3</sub>]<sup>2+</sup> is reduced by ascorbic acid to [Ru(bpy)<sub>3</sub>]<sup>+</sup>, which in turn transfers electrons to the catalyst generating its active form capable of reducing substrates such as protons [34] or CO<sub>2</sub> [35].

#### 2.5.1. Light-driven H<sup>+</sup> reduction

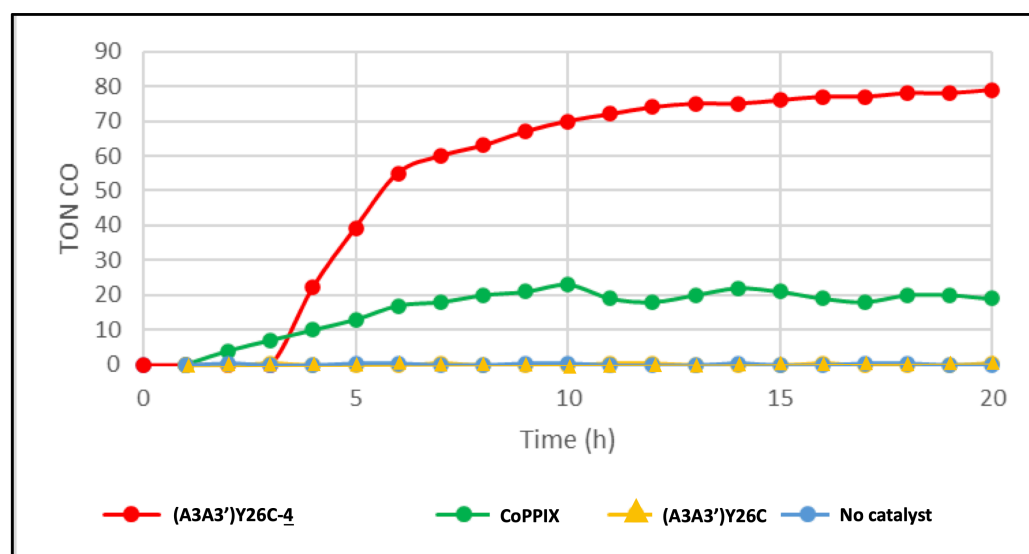
To assay the production of H<sub>2</sub> upon photoinduced reduction of protons, experiments were carried out in argon degassed 100 mM ascorbic acid in the presence of 1 mM [Ru(bpy)<sub>3</sub>]<sup>2+</sup> as a photosensitizer and of either 7 μM (A3A3')Y26C-4 or 7 μM Co(III)PPIX as catalyst, in 100 mM potassium phosphate buffer at pH 7.0, under irradiation with a white LED light at λ > 405 nm. Figure 7 shows the evolution of the turnover number (TON) related to H<sub>2</sub> production over time for 25 h. It appears that in the presence of the A3A3'Y26C-4 biohybrid the production of H<sub>2</sub> increases over time to reach a value of 163 TON after 25 hours. On the contrary, when the metalloporphyrin CoPPIX was used as a catalyst, only a low production of H<sub>2</sub> not exceeding 13 TON was observed after 25 h whereas a negligible H<sub>2</sub> production was observed in the presence of the non-modified (A3A3')Y26C protein or in the absence of a catalyst. These results clearly show that the production of H<sub>2</sub> catalyzed by the cobalt (III)-porphyrin was greatly enhanced, by a factor of at least 10, when the latter was buried in the hydrophobic cavity of the (A3A3')Y26C protein. In addition, it is noteworthy that the TON value obtained with the biohybrid (A3A3')Y26C-4 as catalyst was similar to that described by the Ghirlanda's group using Co-cytochrome b 562 as the catalyst [30]. It is also important to note that, even after 25h of irradiation time, the A3A3'Y26C-4 did not show any sign of degradation and continued to show steady production of H<sub>2</sub>.



**Figure 7.** H<sub>2</sub> Produced over time from the photoinduced reduction of protons by (A3A3')Y26C-4 and CoPPIX at pH 7.0 under argon. The experiments were carried out in 100 mM ascorbic acid, 100 mM potassium phosphate buffer in the presence of 1 mM [Ru(bpy)<sub>3</sub>]<sup>2+</sup> as photosensitizer and of 7 μM (A3A3')Y26C-4 (●), Co(III)PPIX (●), (A3A3')Y26C (●) as catalyst or without catalyst (●) under white LED light, λ > 405 nm irradiation. All experiments were carried out at least two times.

## 2.5.2. Light-driven CO<sub>2</sub> Reduction

The photoinduced reduction of CO<sub>2</sub> catalyzed by (A3A3')Y26C-4 (7 μM) was also studied at pH 7.0 and 20 °C in a CO<sub>2</sub> saturated potassium phosphate buffer containing 100 mM ascorbic acid as a sacrificial electron donor and 1 mM [Ru(bpy)<sub>3</sub>]<sup>2+</sup> as a photosensitizer. Figure 8 shows that, under irradiation with a white LED light at λ > 405 nm, a significant amount of CO can be produced reaching a plateau with a TON of 80 in about 12 hours. The appearance of this plateau is probably due to the deactivation of the cobalt porphyrin through a carbonyl transfer to one of the meso positions of the porphyrin rim as it was reported by Jiang *et al.* [37]. The time lag of 3h observed before CO production was detected by gas chromatography (GC) can probably be rationalized by an initial accumulation of the CO inside the hydrophobic pocket of the protein before being released when its concentration increases. This CO characteristic was reported to be essential for its transport from CO dehydrogenase where it is produced to the acetyl CoA synthase via direct hydrophobic gas channels before being used in a carbonylation reaction [37]. When (A3A3')Y26C-4 is replaced by the CoPPIX metalloporphyrin, a plateau at TON = 20 is reached after 7 h. In control experiments, no CO production was observed in the presence of the non-modified (A3A3')Y26C protein as a catalyst or in the absence of a catalyst. Here also, our results clearly show that the production of CO catalyzed by the cobalt porphyrin was greatly enhanced by a factor of 4 when the latter was inserted in the hydrophobic cavity of the (A3A3')Y26C protein. Interestingly, the TON value obtained with the biohybrid (A3A3')Y26C-4 as catalyst was twice higher than that described by the Ghirlanda's group using Co-cytochrome b 562 as the catalyst (42 TON) under similar reaction conditions [18].



**Figure 8.** Production of CO over time from the photoinduced reduction of CO<sub>2</sub> by (A3A3')Y26C-4 and CoPPIX at pH 7.0 in a CO<sub>2</sub> saturated solution. The experiments were carried out in 100 mM ascorbic acid, 100 mM potassium phosphate saturated with CO<sub>2</sub> in the presence of 1 mM [Ru(bpy)<sub>3</sub>]<sup>2+</sup> as photosensitizer and of 7 μM (A3A3')Y26C-4 (●), Co(III)PPIX (●), (A3A3')Y26C (▲) as catalyst or without catalyst (●) under white LED light, λ > 405 nm irradiation. The experiments were carried out in duplicate.

## 3. Materials and methods

### 3.1. Synthesis of Co(III)Mal-PPIX 4

The Co(III)Mal-PPIX cofactor 4 was prepared in 3 steps.

#### 3.1.1. Protoporphyrin IX dimethyl ester (PPIX DME) hydrolysis [23].

66.8 mg of Protoporphyrin IX dimethyl ester PPIX DME 1 were dissolved in 6 N HCl and the solution was allowed to stand for 15 minutes at room temperature. This solution

was then added to 200 ml chloroform overlaid with 200 ml of 10% (w/v) aqueous sodium acetate. The resulting mixture was then completely neutralized with solid NaHCO<sub>3</sub> to transfer all of the porphyrin into the chloroform organic layer. This layer was then evaporated to dryness and the obtained solid was redissolved in 2 ml of 2,6 lutidine for purification *via* silica gel column chromatography with dichloromethane/methanol 99/1 as eluent. PPIX monomethyl ester (PPIX MME) **2** was obtained as a purple solid (18.4 mg, 28 % yield).

<sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz): δ 9.85-9.98 (m, 4H, meso-H), 8.00-8.16 (m, 2H, -CH=), 6.30, 6.26, 6.15, 6.11 (s each, 4H, =CH<sub>2</sub>), 4.23 (m, 4H, -CH<sub>2</sub>-), 3.52-3.69 (m, 15H, 2, 7, 12, 18,-CH<sub>3</sub>, -OCH<sub>3</sub>), 3.23 (m, 2H, CH<sub>2</sub>-C<sub>acide</sub>), 3.14 (m, 2H, CH<sub>2</sub>-C<sub>ester</sub>), -4.14 (bs, 2H, pyrrole, NH).

MS-ESI [M + H<sup>+</sup>]: found. 577.278, calc. 577.280.

UV-vis (acetonitrile) λ<sub>max</sub> (nm) = 401, 503, 537, 573, 628.

### 3.1.2. Synthesis of maleimido-ethylamido-protoporphyrin IX monomethyl ester Mal-PPIX MME **3**.

PPIX MME **2** (28.5 mg, 49.42 μmol) was dissolved in 1 mL of anhydrous DMF and 15 μL of triethylamine were added. PyBOB (36 mg, 69.18 μmol) dissolved in 1mL anhydrous DMF was added to this solution and the reaction medium was left under stirring for 30 minutes at room temperature (RT). To this solution a solution of *N*-(2-aminoethyl)maleimide trifluoroacetate salt (13.8 mg, 54.36 μmol) in anhydrous DMF (1mL) was added quickly. The resulting mixture was stirred at room temperature overnight and protected from light. The solvent was removed and the crude product was purified by silica gel chromatography (eluted with 1% MeOH in CHCl<sub>3</sub>) to give maleimido-ethylamido-protoporphyrin IX monomethyl ester Mal-PPIX MME **3** as a brown solid (15.7 mg, 45 %).

<sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz): δ 9.89-10.12 (m, 4H, meso-H), 8.17-8.29 (m, 2H, -CH=), 6.36, 6.32, 6.18, 6.12 (s each, 4H, =CH<sub>2</sub>), 6.18 (br, 1H, -NH), 4.52, 4.50 (s, 2H, maleimide), 4.27-4.39 (4H, -CH<sub>2</sub>-), 2.97-3.03 (M, 2H, CH<sub>2</sub>-C<sub>ester</sub>) 3.23-3.27 (m, 6H, CH<sub>2</sub>N<sub>maleimide</sub>, CH<sub>2</sub>NH<sub>amide</sub>, CH<sub>2</sub>C<sub>amide</sub>), 3.52-3.66 (m, 15H, 2, 7, 12, 18,-CH<sub>3</sub>; -OCH<sub>3</sub>), -4.14 (bs, 2H, pyrrole, NH).

MS-ESI [M + H<sup>+</sup>] = found. 699.327, calc. 699.326.

UV-vis (DCM), λ<sub>max</sub> (nm) = 400, 503, 536, 570, 628, 662.

### 3.1.3. Insertion of cobalt into maleimido-ethylamido-protoporphyrin IX monomethyl ester Mal-PPIX MME.

In a 10 mL round bottom flask, 15.7 mg (22.5 μmol) of compounds **3** was dissolved in 3 mL anhydrous DMF and a solution of Co(OAc)<sub>2</sub> · 4 H<sub>2</sub>O (55.96 mg, 225 μmol) in 3 mL anhydrous DMF was added dropwise. The solution was heated at 70 °C until the metalation was completed as proven by UV-visible spectroscopy. After the solvent was removed under reduced pressure, the residue was dissolved in 3 mL DCM. The organic layer was then washed 3 times with 100 mL of water, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and the solvent was removed under reduced pressure, to obtain Co(III)-Mal-PPIX MME **4** as a dark green solid (16.5 mg, 97 %).

MS-ESI [M + H<sup>+</sup>] = found: 755.235, calc. 755.239.

UV-vis (H<sub>2</sub>O), λ<sub>Max</sub> (nm) = 411, 527, 561.

## 3.2. Production and purification of αRep proteins

The production and the purification of the αRep proteins were carried out essentially as reported in a previous paper [24].

### 3.2.1. Protein production

The BL21-Gold(DE3)pLys bacteria strain was transformed with the plasmid encoding for the αRep protein and grown on 2YT-agar plates supplemented with ampicillin (100 μg/mL) and 1% glucose. One clone was picked and grown overnight at 37 °C in 2YT media supplemented with ampicillin (100 μg/mL) and 1% glucose. The overnight preculture was used to inoculate 1 L of fresh 2YT medium containing 100 μg/mL ampicillin and

distributed into two 2 L flasks. Cells were grown at 37 °C until the optical density at 600 nm reached 0.8 Protein expression was then induced by the addition of 500 µM IPTG and cultures were further incubated at 37 °C for 4 h. Cells were harvested by centrifugation for 20 min at 5000 g at 4 °C and resuspended into 40 mL of washing buffer (50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole pH 8) supplemented with a tablet of EDTA-free anti-protease cocktail (Roche). Suspended cells were stored at - 80°C.

### 3.2.1. Protein purification

Frozen cells from 1 L of bacterial culture were defrosted and a tablet of EDTA-free anti-protease cocktail (Roche) and 2 µL of DNase I were added. After 30 minutes of incubation at 4°C, cells were lysed by five 30 s cycles of sonication on ice and centrifuged 1 hour at 10000 g at 4 °C. His-tagged proteins were purified from the crude supernatant using nickel-affinity chromatography on a 5 mL Ni-NTA column. The column was washed with 150 mL of washing buffer (50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole pH 8) and proteins were eluted in six 1 mL fractions in the elution buffer (50 mM sodium phosphate, 300 mM NaCl, 300 mM imidazole pH 8). A typical yield of about 100 mg of protein per liter of culture was obtained. Eluted fractions were analyzed by SDS-PAGE and pure fractions of proteins were pooled. αRep protein was recovered and concentrated resulting in a 5 mL solution that was loaded on a gel filtration column (Hi-Load 16/600 Superdex 75, GE Healthcare) equilibrated with coupling buffer (10 mM HEPES 150 mM NaCl pH 7.75). Elution fractions after gel filtration were pooled and stored at 4 °C until chemical coupling. Purity was checked by SDS-PAGE and concentrations were obtained from the absorbance at 280 nm measured on a Nanodrop spectrophotometer (Thermo Scientific) using an extinction coefficient at 280 nm of 58900 M<sup>-1</sup>.cm<sup>-1</sup> calculated from the protein sequence (<https://web.expasy.org/protparam/>).

### 3.3. Covalent anchoring of compound 3 into (A3A3')Y26C

In a 50 mL conical tube, 6.25 mL of a 200 µM Co(III)-mal cofactor solution in DMSO were added (final concentration 50 µM) to a solution of (A3A3')Y26C in 10 mM HEPES 150 mM NaCl pH 7.75 at a final concentration of 5 µM, in the presence of DMSO (25 % in the final coupling solution) for a final volume of 25 mL. The solution was stirred for 3 days at 4 °C. The mixture was then extracted with 25 mL of 2-butanone at 0 °C, to remove the excess unreacted Co(III) -mal cofactor, and the remaining solution was cooled in an ice bath. It was then centrifuged at 5000 g for 5 min. The pellet containing the (A3A3')Y26C-Co(III)Mal-PPIXMME biohybrid, (A3A3')Y26C-4, was resuspended in 10 mM HEPES, 150 mM NaCl pH 8. The obtained protein solution was then loaded on a gel filtration column (HiLoad 16/600 Superdex 75, GE Healthcare), which was equilibrated with 10 mM HEPES 150 mM NaCl pH 8 and (A3A3')Y26C-4 cofactor was eluted in a single peak. The purified biohybrid was quantified from its absorbance at 280 nm measured on a Nanodrop spectrophotometer.

### 3.4. Photocatalysis Experiments

To a degassed solution of 1 mM [Ru<sup>II</sup>(bpy)<sub>3</sub>]Cl<sub>2</sub> and 100 mM NaAsc, the biohybrid protein was added to lead to a final concentration of 7 µM in a total volume of 3 mL of 0.1 M phosphate buffer pH 7. The solution was further degassed with Ar and then irradiated with a SugarCube white LED (200 Wm<sup>-2</sup>; filtered at λ>420 nm) for 25 h. During irradiation, the solution was stirred and thermostated at 20 °C with the RM6 Lauda cooling system. Gas samples from the overhead space were automatically injected into MicroGC Fusion Gas Analyser (Inficon) every hour. For CO<sub>2</sub> reduction, the cell was saturated with CO<sub>2</sub> gas. All experiments were made at least in duplicate.

### 3.5. Spectroscopic characterization of the (A3A3')Y26C-4 biohybrid

Electronic absorption spectra of the oxidized and reduced (A3A3')Y26C-4 biohybrid were recorded and compared to the spectra of other Co-PPIX-protein complexes

previously reported in the literature. 1 mL samples of a 7.5  $\mu$ M solution of purified (A3A3')Y26C-**4** were prepared in argon-sparged 10 mM HEPES, NaCl 150 mM buffer, pH 8.0 and placed in a septum-sealed cuvette to ensure anaerobic conditions. The reduction was initiated via addition of 1.5 mM sodium dithionite. Spectra were recorded every five minutes at RT until absorbance changes were no longer observed.

### 3.6. Spectroscopic characterization of the (A3A3')Y26C-**4** biohybrid

Circular dichroism (CD) spectra were recorded from 185 nm to 260 nm with a data pitch of 0.2 nm, a scan speed of 50 nm min<sup>-1</sup>, a response time of 0.5 s and a bandwidth of 2 nm using quartz cells with a 1 mm path length, on a Jasco J810 dichrograph. Each spectrum was recorded five times and averaged. The CD signal was corrected with buffer subtraction resulting in the ellipticity ( $\theta$ ) in mdeg. Data were acquired from a 2.2  $\mu$ M sample of biohybrid previously desalted on a Zeba spin desalting column (Thermo) equilibrated with 20 mM sodium phosphate buffer, pH 7.5.

### 3.7. Computational methods

The coordinates from modelled A3A3'-Y26C-MnTPP were obtained from a previous study [21]. Covalent MnTPP cofactor was removed and MD simulation was performed to obtain the most representative structure of the native form of A3A3'. Cys26 was mutated using the Dunbrack library available at UCSF Chimera [30]. Cofactor **4** structure was optimized with Gaussian16 [38] via QM calculations in water solvent (SMD) [39] using B3LYP functional and Grimme's dispersion D3 with 6-31G(d,p) for CHONS atoms and SDD + F for Co [40]. The oxidation state of Co was set to III and spin state was singlet.

Covalent dockings were performed using GOLD5.8.0 [31] using as score function Goldscore with parameters adapted from for metals [41]. Covalent linkage was performed with CA-Cys26 Radius search was set to 15 Å and 50 runs were performed. Flexibility to certain residues was given by allowing free rotation of side chains.

MD simulations were prepared using xleap [42], using as the force field FF19SB for proteins, TIP3P for water and GAFF for non-standard residues. MCPB.py was used to parametrize cofactor **4** using the Seminario method [43] for force constants and parameters and RESP for charge and RESP method for charges [44]. Optimization, frequency and RESP calculations were performed at the same level of theory specified before. The system was solvated using a cubic water box and Na<sup>+</sup> as counter ions were added to neutralize. MD simulations were performed using AMBER20 software [32]. Three different energy minimizations were performed to relax the system followed by a heating step in which the system equilibrated from 0 K to 300 K. Finally, NVT and NPT equilibrations were performed, followed by a 400 ns production run. Detailed computational can be found in SI.

## 4. Conclusion

In summary, an artificial metalloenzyme was obtained by covalent coupling of Co(III)Mal-PPIXMME **4** to the mutant (A3A3')Y26C of an artificial bidomain protein named alpha Rep ( $\alpha$ Rep). This new artificial metalloenzyme (A3A3')Y26C-**4** was characterized by Maldi-TOF MS, circular dichroism and UV-visible spectrophotometry. The Comparison of the maxima of absorption of (A3A3')Y26C-**4** with those of various Co(II)- and Co(III)-PPIX-substituted hemoproteins (Table 1) suggested the possible coordination of the cobalt ion by an axial histidine. However, investigation of the structure of this biohybrid and the positioning of the residues near the metal complex using theoretical experiments suggested the possible coordination of the metal by an axial glutamine Gln283 although it would be transient in nature and mutagenic efforts may be needed to improve axial coordination.

The (A3A3')Y26C-4 biohybrid was then shown to be able to catalyze, not only the photoinduced production of H<sub>2</sub>, but also the reduction of CO<sub>2</sub> into CO. While the Co(III)porphyrin catalyst alone showed a low activity with respect to these two reactions, more than 10 times more H<sub>2</sub> and 4 times more CO<sub>2</sub> were produced in the reaction whereby the Co(III)porphyrin was buried within the hydrophobic cavity of the protein. Based on these results, we will thus be able in the future to optimize our biohybrid by carrying out different mutants for axial coordination and also to introduce positive charges on the proximal face of the biohybrid, which should increase the local concentration of CO<sub>2</sub> in the enzymatic cavity to enhance its conversion into CO.

**Supplementary Materials:** The following supporting information can be downloaded at: [www.mdpi.com/xxx/s1](http://www.mdpi.com/xxx/s1), **Figure S1:** MD convergence analysis: RMSD, all-to-all RMSD, PCA and cluster counting.

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