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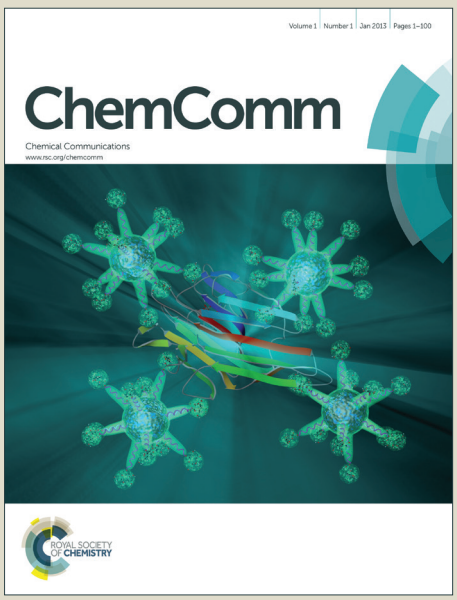





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# Protein Secondary-Shell Interactions Enhance the Photoinduced Hydrogen Production of Cobalt Protoporphyrin IX

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**Hydrogen is an attractive fuel with potential for production scalability, provided that inexpensive, efficient molecular catalysts utilizing base metals can be developed for hydrogen production. Here we show for the first time that cobalt myoglobin (CoMyo) catalyzes hydrogen production in mild aerobic conditions with turnover number of 520 over 8 hours. Compared to free Co-Protoporphyrin IX, incorporation into the myoglobin scaffold results in a 4-fold increase in photoinduced hydrogen production activity. Engineered variants in which specific histidine resides in proximity of the active site were mutated to alanine result in modulation of the catalytic activity, with the H64A/H97A mutant displaying activity 2.5-fold higher than wild type. Our results demonstrate that protein scaffolds can augment and modulate the intrinsic catalytic activity of molecular hydrogen production catalysts.**

In the quest for alternative, sustainable fuels to address increasing societal needs, molecular hydrogen has arisen as a forerunner because it is potentially carbon independent, energy-rich, and transportable.[1] At the moment, however, hydrogen production relies on steam reforming of hydrocarbons at high temperatures or on the use of precious metal catalysts, such as platinum, and can't be scaled up in a sustainable manner. Nature, in contrast, utilizes a class of enzymes called hydrogenases, which contain an unusual bimetallic active site ([FeFe] or [NiFe]) and reversibly catalyze the reduction of protons to molecular hydrogen.[2] Remarkably, hydrogenases function in weakly acidic conditions, at low overpotentials, with non-noble metals, and catalyze hydrogen production with high turnover frequencies (5000-21000 $s^{-1}$).[3] However, the application of these enzymes to scalable hydrogen production is hampered by their difficulty of overexpression and high oxygen sensitivity.[4] For these reasons, chemists have sought to develop robust organometallic catalysts as alternatives to the natural enzymes.[5] Among those, cobalt macrocycle catalysts are an attractive option, as they often exhibit structural heterogeneity, which allows for tuning of catalytic properties, low overpotentials, and high oxygen tolerance; however they function mostly in organic solvents with strong acids.[6]

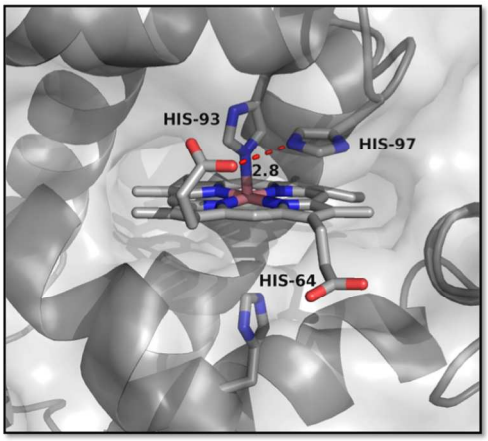


***Figure 1.*** Crystal structure (PDB 1YOI, Ref. 26) of Co-myoglobin active site, highlighting residues H93, H97 and H64.

Recently, a number of cobalt porphyrin catalysts have been reported for their ability to both electrochemically and photochemically produce hydrogen at high turnover numbers, implicating them for use in dye-sensitized fuel cells.[7] Porphyrin and related macrocycles serve as cofactors in proteins, raising the possibility that such natural proteins could be used as scaffolds to accommodate hydrogen production catalysts.[8] Initial work utilizing

cobalt-functionalized microperoxidase 11 (CoMP11), however, showed that the catalytic activity decays after about 15 minutes due to porphyrin degradation, likely from porphyrin surface exposure to the environment.[8]

Here, we present an alternative approach by which a cobalt-derivatized porphyrin is buried into a protein scaffold, myoglobin. This well-folded, stable protein scaffold binds heme (Fe-Protoporphyrin IX) leaving the metal in a pentacoordinate state; the sixth position is available for coordination to molecular oxygen and other substrates. Myoglobin withstands mutagenesis at positions close to the vacant axial site, facilitating the engineering of catalytic sites into the scaffold.[9] In addition, myoglobin readily accommodates unnatural cofactors.[10] In the case of Co-Protoporphyrin IX, incorporation into myoglobin provides additional second sphere and long-range interactions, while protecting the cofactor from degradation. Our results show that CoMyo (Figure 1) catalyzes photoinduced production of hydrogen with high efficiency, and that activity is modulated by engineered mutations.

Preparation of CoMyo was accomplished with cobaltous protoporphyrin IX (CoPP(IX)) using established methods.[10a] The electronic spectra of the air-oxidized protein displays absorbance maxima at 425, 534, and 567 nm, typical of Co(III) porphyrins (Figure S1). These maxima are red shifted relative to other peptide-based catalytic systems, which exhibit maxima at 415, 530, and 560 nm, indicating shielding CoPP(IX) from the aqueous environment.[8a, 11] Reduction of the holo protein at pH 7.5 with a 500 molar excess of sodium dithionite resulted in shift of the Soret and *q*-bands to 394 and 557 nm, respectively, corresponding to a Co(II) species (Figure S1).

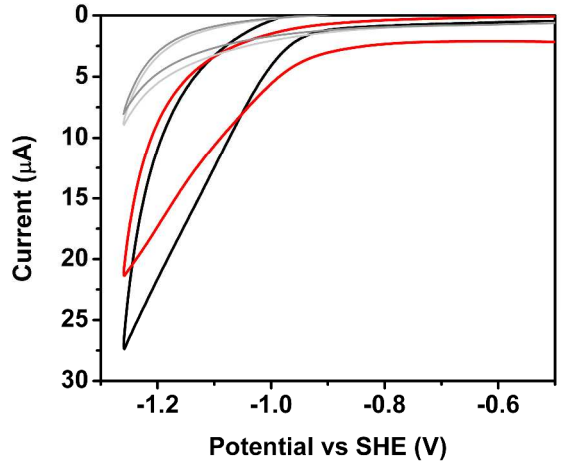


***Figure 2.*** Cyclic voltammagrams of a blank electrode (light grey), 1.5 μM apomyoglobin (dark grey), and 1.5 μM CoMyo in the absence (black) and presence (red) of oxygen in a 200 mM Tris-HCl, 100 mM NaCl, pH 7.5 solution at 100 mV/s with a 0.28 $cm^2$ glassy carbon working electrode.

We then assayed the ability of CoMyo and CoPP(IX) to catalyze the reduction of protons to hydrogen by cyclic voltammetry (CV) (Figure 2). CoPP(IX), which has low solubility in water, was studied in acetonitrile (MeCN) with 0.1 M ($n$-$Bu_4N$)($PF_6$) as the supporting electrolyte. (Figure S2) A quasi-reversible wave appears at -1.17 V vs SHE after referencing to ferrocene monocarboxylic acid, assigned to the reduction of Co(I) to Co(0). Addition of para-toluenesulfonic acid to the anhydrous acetonitrile results in the onset of a catalytic wave at -1 V, thus implicating the Co(I)/Co(0) redox couple as the catalytically active species (Figure S3).[7a] In the case of CoMyo, the onset of a strong catalytic wave is observed at -0.95 V against SHE in the CV scans in 200 mM Tris-HCl buffered solution, concealing the Co(I)/Co(0) peak (Figure 2). This wave is 200 mV positive of the catalytic wave of a bare glassy carbon electrode (Figure S4), and not seen in the presence of apomyoglobin alone (Figure 2). These values are in agreement with previous work on aqueous cobalt macrocycles, and support a mechanism in which reduction of Co(I) to Co(0) and concerted PCET result in catalytic hydrogen production.[7, 12] The catalytic onset potential observed for CoMyo is approximately 100 mV closer to the thermodynamic value compared to other water-soluble cobalt porphyrins assayed.[7b]

The peak catalytic current for scans down to -1.26 V is linearly dependent on CoMyo concentration (Figure S5). The catalytic current was found to be strongly dependent on the pH of the solution, as expected for a molecular hydrogen generating system (Figure S6). CV scans were also performed in unsealed electrochemical cells open to the ambient atmosphere; negligible catalytic current loss was observed after introduction of oxygen into the electrochemical setup (Figure 2). However, loss of activity of the CoMyo system is seen below pH 6, due to protonation of the ligating His93 in the myoglobin core and loss of the porphyrin from the hydrophobic core of myoglobin.

To confirm the loss of CoPP(IX) from the active site, rinse tests were performed (Figure S7). Consecutive scans on a 1.5 μM sample of CoMyo at a pH of 7.5 showed negligible loss of catalytic current. Removing the electrode from the CoMyo solution, rinsing, and placing into fresh buffer without CoMyo yielded no catalytic current, indicating no degradation of the catalytic system at the electrode surface to form an electroactive film. Upon lowering of the pH to 4.5, consecutive scans on CoMyo in solution yielded a catalytic wave, as expected. When the electrode was rinsed and placed into buffer containing no CoMyo, the catalytic wave observed persisted, indicating deposition of CoPP(IX) onto the electrode surface. UV-Vis spectra of CoMyo at pH 4 revealed a red shift of the Soret band to 427 nm, consistent with the loss of coordination by the axial His93 (Figure S8).[13] Because of this loss of coordination, we chose to characterize the CoMyo system at pH levels above 6.5, avoiding mixtures of myoglobin-bound and unbound CoPP(IX).

We investigated whether incorporation of catalysts into hydrophobic, peptide-rich environments could modulate the intrinsic activity of CoPP(IX) by assessing hydrogen production in the presence of a photosensitizer and sacrificial electron donor. In a typical experiment, samples were irradiated with 1100 $W/m^2$ of visible light (>400) in the presence of 1 mM $Ru(Bpy)_3^{2+}$ and 100 mM sodium ascorbate, a sacrificial electron donor, over the course of 12 hours. Hydrogen evolution was quantified by analyzing aliquots of headspace volume of the anaerobic cuvette via gas chromatography as a function of time.

We found that the CoMyo system efficiently produces hydrogen under these conditions, achieving turnover numbers (TON) ranging from 230-520. Direct comparison of photoinduced catalysis of the CoMyo system to free-CoPP(IX) showed that incorporation of the porphyrin into the protein scaffold increased the TON by a factor of 3 on average (Table 1). In the same conditions, ferric myoglobin produces only a nominal amount of $H_2$, comparable to controls lacking any catalyst at all (Figure 3).

The activity of porphyrin based catalysts, expressed as TON, have strong inverse relationship with concentration, because activity is lost when dimers form.[7b] In contrast, CoMyo proved to be resilient to changes in catalyst concentration, yielding the same TON for all

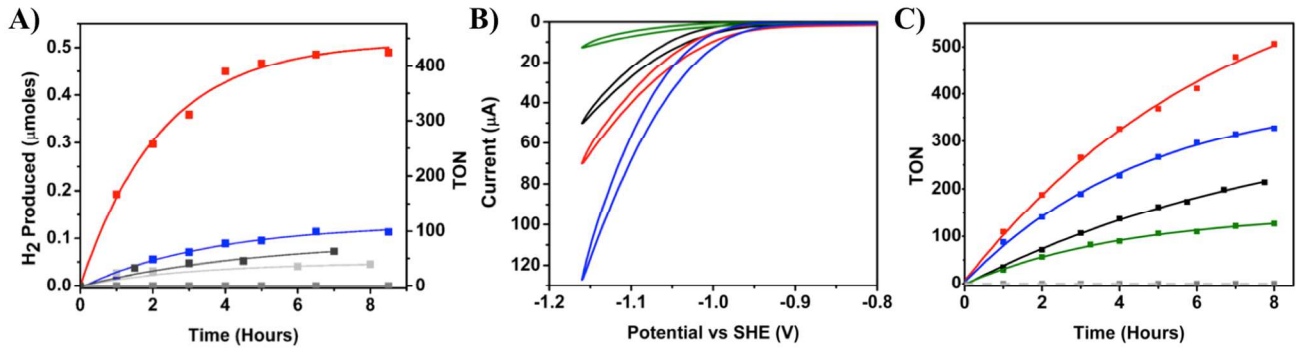


***Figure 3.*** A) Photoinduced (410-770 nm) hydrogen production in 1M KPi with 0.1 M sodium ascorbate and 1 mM $Ru(bpy)_3^{2+}$ at pH 7. Traces correspond to WT CoMyo (Red), CoPP(IX) (Blue), Ferric WTMyo (Dark Grey), No Catalyst (Light Grey), and WT CoMyo lacking $Ru(bpy)_3^{2+}$ (Grey). B) Cyclic voltammagrams of 1.5 μM myoglobin mutants in 200 mM Tris-HCl, 100 mM NaCl, at pH 7 at a scan rate of 100 mV/s with a 0.28 $cm^2$ glassy carbon working electrode (WT (Black), H97A (Green), H64A (Blue), H64/97A (Red)). C) Photoinduced hydrogen production of CoMyo variants, traces same as in (B) and conditions same as in (A).

**Table 1. pH dependence of photoinduced hydrogen production of WT CoMyo**

| pH | TON | TOF ($min^{-1}$) | Enhancement Factor[a] |
|---|---|---|---|
| 6.5 | 234 | 0.47 | 2.13 |
| 7 | 518 | 1.47 | 4.32 |
| 7.5 | 454 | 0.85 | 2.84 |

[a] Conditions: 1 M potassium phosphate; [a]Enhancement over CoPP(IX) assayed under the same conditions.

concentrations tested (1 μM – 5 μM). This behavior suggests that individual CoPP(IX) molecules are efficiently sequestered inside the myoglobin core. It has been proposed that the rate limiting step for monometallic species is the reduction of Co(I) to Co(0), in agreement with our electrochemical analysis.[7b, 14] The reaction then proceeds through a Co(II) hydride intermediate, resulting in the activity of CoMyo being pH dependent, with maximal photoinduced hydrogen production observed at pH 7, and being lower as pH either increased or decreased (Table 1). The loss of activity observed at higher pH is easily justified, as lower concentrations of protons will result in lower TON. However, protonation of the axial histidine and of the two distal histidines in the active site (His64 and His97) with pKa value of ~5 and 5.6, respectively,[15] also results in loss of activity.

To verify this hypothesis, we generated three mutants of myoglobin, in which histidines were exchanged for non-ionizable alanine: H64A, H97A, and H64/97A (Figure 1). We found that the H64A and H97/64A point mutants increased catalytic activity of CoMyo at pH 6.5 to 331 and 512 TON, respectively, compared to the 231 TON observed for WT (Figure 4). However, the H97A mutant reduced the TON to 120, only slightly higher than the porphyrin alone (TON 100). Electrochemical analysis of the mutants at pH 7 revealed an increase of current at -1.26 V vs. SHE for both the H64A and double mutant and a reduction of catalytic current of H97A, but no change of the onset of catalytic hydrogen reduction (Figure 4). Two factors could play a role in the change in the catalytic peak height upon removal of the two active site His. First, removing His64 from the proximity of the CoPP(IX) catalysts removes a slight positive charge at physiological pH levels, as well as the potential for His64 to compete for proton binding. The loss of this positive charge will shift the redox potential of the catalyst more positive, allowing for easier reduction of protons at the catalytic site. Characterization of the electrochemical pH dependence of the H64A mutant shows a stronger dependence of catalytic activity on pH, a direct result of these two changes. (Figure S9).

Secondly, closer inspection of the active site of CoMyo reveals a hydrogen bond forming between the His97 residue and one of the propionic acid groups of CoPP(IX). Removing this residue destabilizes the binding of the porphyrin, altering steric constraints on the porphyrin and increasing the degrees of freedom between the scaffold and the catalyst, lowering its catalytic ability by diminishing enhancing contacts with the scaffold. It has been shown that with heme in the myoglobin active site, a H97D mutation increases lability of the cofactor by a factor of 38.[16] In CoMyo, increased cofactor lability results in loss of scaffold-mediated enhancement of catalytic activity, as the H97A mutant is nearly indistinguishable from the free porphyrin. During photoinduced hydrogen production, removal of this residue also increases the solvent-accessibility of the active site, and may allow for increased interaction between the photosensitizer and the catalyst, increasing the TON of the H97/64A mutant relative to the H64A mutant.

The variation of photoinduced catalysis of hydrogen production by these mutants confirms that the protein environment plays a major role in the overall increase of catalysis of the CoPP(IX), affecting not only the stability of the catalyst but also the competing processes and redox activity in the active site. These effects are reminiscent of the role of the protein matrix in [FeFe] hydrogenases, in which it augments and modulates the intrinsic activity of the diiron center through secondary shell and long range interactions.[2b, 17] This concept was exploited by burying biomimetic inorganic complexes into simple hydrophobic environments, such as chitosan,[16a] metal-organic frameworks,[16b] micelles,[16c] peptides,[16d,e] and dendrimers,[16f] which also resulted in increased stability of the inorganic complexes and higher turnover in photocatalytic experiments. Compared to CoMP11,[8a] which is essentially a helical peptide covalently linked to a porphyrin, the myoglobin scaffold presents several advantages. First, the scaffold efficiently protects the porphyrin cofactor from degradation, resulting in sustained photoinduced catalytic activity over 480 minutes. In contrast, the electrocatalytic activity of CoMP11 ceased after 15 minutes. Second, the increased complexity of the protein scaffold surrounding the porphyrin allowed us to introduce specific mutations in the second coordination sphere, thus modulating the activity of the protein. Further optimization of the system via directed mutagenesis may be possible. Third, the scaffold is robust and can be functionalized on the surface for attachment to a number of photosensitizers or solid-support materials, allowing for development of a heterogeneous

catalytic system. Finally, the non-covalent nature of the interaction between the porphyrin and myoglobin allows for the identity of the catalyst studied to be altered from the native protoporphyrin IX.

In conclusion, we show here that incorporation of macrocycle catalysts through non-covalent interactions into a protein environment allows studying the effect of secondary shell interactions on catalysis. This strategy also facilitates catalysis in aqueous environments using catalysts that would otherwise be limited to organic solvents. The CoMyo system shows significant enhancement of photoinduced hydrogen production for a simple macrocyclic complex, CoPP(IX), due to stabilization of the catalyst by incorporation into a hydrophobic, protein scaffold. Along with the ability to stabilize the catalyst, point mutants of myoglobin affected the catalysis of the CoPP(IX), suggesting that *in vitro* selection techniques could be used to enhance the catalytic activity of CoMyo. Future studies will aim to optimize both the catalyst, as well as the protein environment solubilizing the catalyst, to achieve efficient photoinduced hydrogen production.

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Electronic Supplementary Information (ESI) available: Further electrochemical characterization, UV-Vis traces, and photoinduced $H_2$ production controls. See DOI: 10.1039/c000000x/

## Notes and references

1 (a) J. O. Bockris, *Science*, 1972, **176**, 1323; (b) N. S. Lewis and D. G. Nocera, *P. Natl. Acad. Sci. USA*, 2006, **103**, 15729.

2 (a) M. Faiella, A. Roy, D. Sommer and G. Ghirlanda, *Biopolymers*, 2013, **100**, 558; (b) W. Lubitz, H. Ogata, O. Rudiger and E. Reijerse, *Chem. Rev.*, 2014, **114**, 4081; (c) J. W. Peters, W. N. Lanzilotta, B. J. Lemon and L. C. Seefeldt, *Science*, 1998, **282**, 1853; (d) P. M. Vignais, B. Billoud and J. Meyer, *FEMS Microbiol. Rev.*, 2001, **25**, 455.

3 (a) F. A. Armstrong and J. Hirst, *P. Natl. Acad. Sci. USA*, 2011, **108**, 14049; (b) C. Madden, M. D. Vaughn, I. Diez-Perez, K. A. Brown, P. W. King, D. Gust, A. L. Moore and T. A. Moore, *J. Am. Chem. Soc.*, 2012, **134**, 1577.

4 (a) F. A. Armstrong and J. C. Fontecilla-Camps, *Science*, 2008, **321**, 498; (b) J. C. Fontecilla-Camps, A. Volbeda, C. Cavazza and Y. Nicolet, *Chem. Rev.*, 2007, **107**, 4273.

5 (a) J. M. Camara and T. B. Rauchfuss, *Nat. Chem.*, 2012, **4**, 26; (b) M. Y. Darensbourg, E. J. Lyon, X. Zhao and I. P. Georgakaki, *P. Natl. Acad. Sci. USA*, 2003, **100**, 3683; (c) M. L. Helm, M. P. Stewart, R. M. Bullock, M. R. DuBois and D. L. DuBois, *Science*, 2011, **333**, 863; (d) C. Tard and C. J. Pickett, *Chem. Rev.*, 2009, **109**, 2245.

6 (a) C. Baffert, V. Artero and M. Fontecave, *Inorg. Chem.*, 2007, **46**, 1817; (b) P. Connolly and J. H. Espenson, *Inorg. Chem.*, 1986, **25**, 2684; (c) R. M. Kellett and T. G. Spiro, *Inorg. Chem.*, 1985, **24**, 2378; (d) S. Losse, J. G. Vos and S. Rau, *Coord. Chem. Rev.*, 2010, **254**, 2492; (e) S. C. Marinescu, J. R. Winkler and H. B. Gray, *P. Natl. Acad. Sci. USA*, 2012, **109**, 15127; (f) W. R. McNamara, Z. J. Han, C. J. Yin, W. W. Brennessel, P. L. Holland and R. Eisenberg, *P. Natl. Acad. Sci. USA*, 2012, **109**, 15594; (g) V. S. Thoi, Y. Sun, J. R. Long and C. J. Chang, *Chem. Soc. Rev.*, 2013, **42**, 2388.

7 (a) C. H. Lee, D. K. Dogutan and D. G. Nocera, *J. Am. Chem. Soc.*, 2011, **133**, 8775; (b) M. Natali, A. Luisa, E. Lengo and F. Scandola, *Chem. Commun.*, 2014, **50**, 1842.

8 (a) J. G. Kleingardner, B. Kandemir and K. L. Bren, *J. Am. Chem. Soc.*, 2014, **136**, 4; (b) W. D. Robertson, A. M. Bovell and K. Warncke, *J. Biol. Inorg. Chem.*, 2013, **18**, 701.

9 (a) Y. Lu, N. Yeung, N. Sieracki and N. M. Marshall, *Nature*, 2009, **460**, 855; (b) N. Yeung, Y. W. Lin, Y. G. Gao, X. Zhao, B. S. Russell, L. Lei, K. D. Miner, H. Robinson and Y. Lu, *Nature*, 2009, **462**, 1079.

10 (a) E. A. Brucker, J. S. Olson, G. N. Phillips, Y. Dou and M. Ikeda-Saito, *J. Biol. Chem.*, 1996, **271**, 25419; (b) C. Z. Li, K. Nishiyama and I. Taniguchi, *Electrochim. Acta*, 2000, **45**, 2883; (c) T. Ueno, T. Koshiyama, M. Ohashi, K. Kondo, M. Kono, A. Suzuki, T. Yamane and Y. Watanabe, *J. Am. Chem. Soc.*, 2005, **127**, 6556; (d) T. Ueno, N. Yokoi, S. Abe and Y. Watanabe, *J. Inorg. Biochem.*, 2007, **101**, 1667.

11 S. Mathura, D. Sannasy, A. S. de Sousa, C. B. Perry, I. Navizet and H. M. Marques, *J. Inorg. Biochem.*, 2013, **123**, 66.

12 J. T. Muckerman and E. Fujita, *Chem Commun (Camb)*, 2011, **47**, 12456.

13 W. H. Wang, J. X. Lu, P. Yao, Y. Xie and Z. X. Huang, *Protein Eng.*, 2003, **16**, 1047.

14 J. L. Dempsey, B. S. Brunschwig, J. R. Winkler and H. B. Gray, *Accounts of chemical research*, 2009, **42**, 1995.

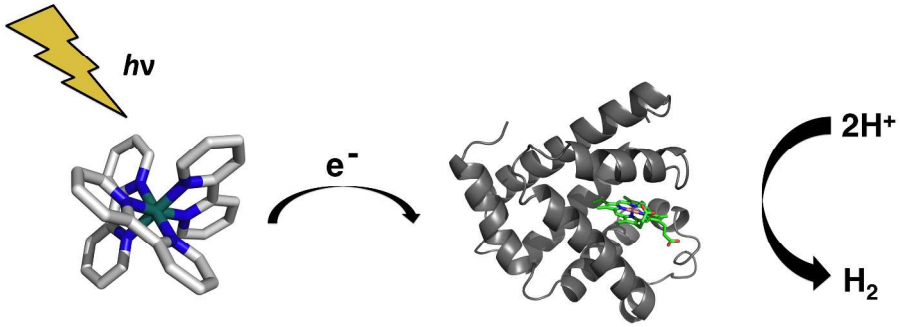
15 D. Bashford, D. A. Case, C. Dalvit, L. Tennant and P. E. Wright, *Biochemistry-Us*, 1993, **32**, 8045.

16 M. S. Hargrove and J. S. Olson, *Biochemistry-Us*, 1996, **35**, 11310.

17 P. Knorzer, A. Silakov, C. E. Foster, F. A. Armstrong, W. Lubitz and T. Happe, *J. Biol. Chem.*, 2012, **287**, 1489.



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