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Biomimetic and Microbial Approaches to Solar Fuel Generation

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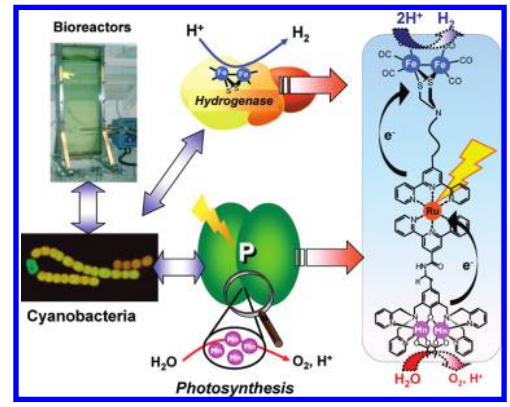

Photosynthesis is performed by a multitude of organisms, but in nearly all cases, it is variations on a common theme: absorption of light followed by energy transfer to a reaction center where charge separation takes place. This initial form of chemical energy is stabilized by the biosynthesis of carbohydrates. To produce these energy-rich products, a substrate is needed that feeds in reductive equivalents. When photosynthetic microorganisms learned to use water as a substrate some 2 billion years ago, a fundamental barrier against unlimited use of solar energy was overcome.

The possibility of solar energy use has inspired researchers to construct artificial photosynthetic systems that show analogy to parts of the intricate molecular machinery of photosynthesis. Recent years have seen a reorientation of efforts toward creating integrated light-to-fuel systems that can use solar energy for direct synthesis of energy-rich compounds, so-called solar fuels. Sustainable production of solar fuels is a long awaited development that promises extensive solar energy use combined with long-term storage.

The stoichiometry of water splitting into molecular oxygen, protons, and electrons is deceptively simple; achieving it by chemical catalysis has proven remarkably difficult. The reaction center Photosystem II couples light-induced charge separation to an efficient molecular water-splitting catalyst, a Mn_4Ca complex, and is thus an important template for biomimetic chemistry. In our aims to design biomimetic manganese complexes for light-driven water oxidation, we link photosensitizers and charge-separation motifs to potential catalysts in supramolecular assemblies.

In photosynthesis, production of carbohydrates demands the delivery of multiple reducing equivalents to CO_2. In contrast, the two-electron reduction of protons to molecular hydrogen is much less demanding. Virtually all microorganisms have enzymes called hydrogenases that convert protons to hydrogen, many of them with good catalytic efficiency. The catalytic sites of hydrogenases are now the center of attention of biomimetic efforts, providing prospects for catalytic hydrogen production with inexpensive metals. Thus, we might complete the water-to-fuel conversion:

$$\text{light} + 2H_2O \rightarrow 2H_2 + O_2$$

This reaction formula is to some extent already elegantly fulfilled by cyanobacteria and green algae, water-splitting photosynthetic microorganisms that under certain conditions also can produce hydrogen. An alternative route to hydrogen from solar energy is therefore to engineer these organisms to produce hydrogen more efficiently. This Account describes our original approach to combine research in these two fields: mimicking structural and functional principles of both Photosystem II and hydrogenases by synthetic chemistry and engineering cyanobacteria to become better hydrogen producers and ultimately developing new routes toward synthetic biology.

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Introduction: From Natural to Artificial Photosynthesis

The use of water as an electron donor in photosynthetic energy conversion has given a great evolutionary advantage to cyanobacteria, algae, and higher plants. The four-electron oxidation of water to molecular oxygen is accomplished by the Photosystem II (PSII) reaction center, with its catalytic Mn_4Ca complex (Figure 1).[1−3] The abundance and environmental benefits of water as raw material also for artificial solar energy conversion[4] makes biomimetic systems built on the principles of PSII a goal of utmost importance. Light-induced charge separation on a single-electron level has been demonstrated in numerous synthetic systems,[5−9] including manganese complexes linked to Ru^{II}-polypyridyl photosensitizers aimed to mimic photoprocesses of PSII.[10] However, efficient use of water as an electron source demands that several light-induced charge separations on the single-electron level are coupled to accumulation of redox equivalents on the catalyst. This we denote *accumulative* electron transfer. Although many details of charge separation in PSII are known, the structure and catalytic mechanism of the Mn_4Ca complex are less well-understood.[1−3] Biomimetic chemistry in this area is therefore far from straightforward, and there is no synthetic manganese complex that achieves true catalytic water oxidation to O_2 in solution.[11,12]

Cyanobacteria and green algae perform oxygenic photosynthesis and are also able to release some of their excess energy in the form of molecular hydrogen.[13,14] The organisms can also reoxidize H_2 and feed on the free energy. Both the oxidation of H_2 and the reduction of protons to H_2 are catalyzed by hydrogenases. Most hydrogenases fall into two main classes: the NiFe hydrogenases found in cyanobacteria, containing a NiFe complex in the catalytic site, and the Fe_2 hydrogenases found in green algae. In addition, in nitrogen-fixing cyanobacteria, the enzyme nitrogenase uses at least 25% of the reducing equivalents for the coupled H_2 production. Because these organisms can produce H_2 from solar energy and water, they are attractive targets for efforts to improve their productivity via genetic engineering. This requires understanding of the bioenergetic pathways and the genetic regulation in these diverse organisms.

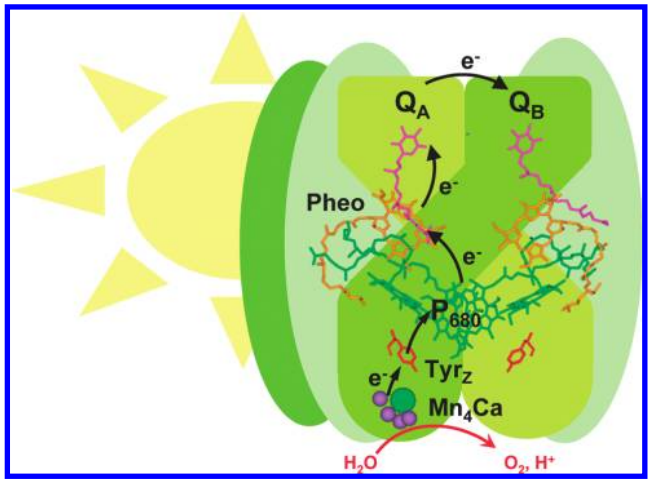

FIGURE 1. In PSII, excitation of the central chlorophylls P_{680} leads to charge separation in a series of electron-transfer reactions, generating reductive equivalents. New electrons are provided by oxidation of water at a catalytic Mn_4Ca complex, yielding molecular oxygen. This is the only known example of a rapid and efficient homogeneous water oxidation catalyst.

In both Fe_2 and NiFe hydrogenases, the active site has predominantly inorganic ligands, which makes them attractive targets for biomimetic hydrogen-producing catalysts.[3,15−17] In comparison to the very efficient hydrogenases, however, synthetic Fe_2 complexes thus far work at larger overpotentials and smaller turnover frequencies.

In this paper, we describe our joint efforts in the two fields: (1) synthetic chemistry mimicking structural and functional principles of PSII and hydrogenases and (2) engineering cyanobacteria to become better hydrogen producers. The design and realization of such systems call for concerted interdisciplinary collaboration. Both approaches are dependent upon intimate knowledge on the natural enzymes. Moreover, studies of the biomimetic systems may give information on the enzymes that are important to consider also in the cyanobacterial approach. In the Swedish Consortium for Artificial Photosynthesis, we have built up a research center including scientists and methods from all fields pertaining to our research, interacting on a daily basis. This Account highlights some scientific principles and results of our joint effort.

Ru−Mn Complexes Mimicking PSII Reactions

Coupling Charge Separation to Multi-electron Catalysts—Accumulative Electron Transfer. New challenges arise for accumulative electron transfer to or from a molecular unit, which are not relevant for charge separation on the single electron level.[18] One difficulty is thermodynamic and appears because of build-up of charge. Charge accumulation makes it increasingly difficult to add or remove the next electron, unless each electron transfer is coupled to a charge compensation reaction. The Mn_4Ca complex of PSII compensates for charge accumulation by deprotonation and ligand rearrangements, which keep its redox potential close to that of water oxidation.[1] However, the coupled reaction will influence also the rate of electron transfer. An example of this is the proton-coupled electron-transfer reactions of the Ru−tyrosine com-

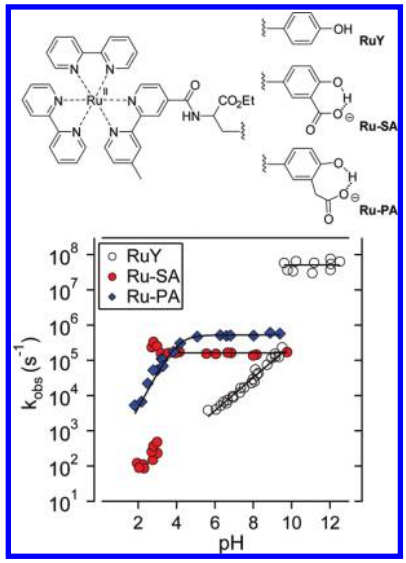

FIGURE 2. Rate constant for proton-coupled electron transfer from phenol to photogenerated Ru^{III} in a series of Ru−tyrosine complexes. At intermediate pH values, the primary proton acceptor for RuY is external water, giving a nontrivial pH dependence (slope = 0.5). For Ru−SA and Ru−PA, the proton acceptor is instead the internal carboxylate group that is hydrogen-bonded to the phenolic proton. The rate varies over 6 orders of magnitude for the same basic Ru−tyrosine structure because of variations of the proton acceptor and phenol protonation state.[19]

plexes in Figure 2.[19] A similar reaction occurs in PSII, where the intermediate electron donor $tyrosine_Z$ transfers a proton to a nearby base as it is oxidized, which lowers the potential below that of P_{680}^+.

Another difficulty in accumulative electron transfer is kinetic competition from new unproductive reactions. Because the excited sensitizer is often both very oxidizing and reducing, it may both reduce an already oxidized donor (D^+) and oxidize the reduced acceptor (A^-) in reverse reactions (Figure 3). In addition, D^+ and A^- are often radicals or metal centers with low-lying excited states that can lead to rapid energy transfer or paramagnetic quenching of the sensitizer. To avoid these reactions, the electron-transfer steps in the productive direction must be more rapid to compete kinetically. In PSII, the accumulative units, the Mn_4Ca complex and $quinone_B$, are located at some distance from the chlorophyll excited states and productive charge separation is ensured by rapid primary electron transfer to a pheophytin.

Charge Compensation in Accumulative Electron Transfer. Figure 4 illustrates one of a few synthetic systems where charge compensation has been studied in light-induced, accumulative electron transfer.[20−22] Repeated photo-oxidation of the Ru^{II}−trisbipyridine unit to Ru^{III} resulted in oxidation of the linked $Mn_2^{II,II}$ complex in three steps to $Mn_2^{III,IV}$.[20] In acetonitrile, only two electrochemical oxidation steps were found: $Mn_2^{II,II} \rightarrow Mn_2^{II,III} \rightarrow Mn_2^{III,III}$, both occurring below the $Ru^{III/II}$ potential. A third oxidation step was achieved in aqueous solution, where charge compensating ligand exchange of the acetates for water-derived ligands could take place.

Studies by Fourier transform infrared (FTIR) spectroelectrochemistry and electrospray ionization (ESI)−mass spectrometry,[21] and extended X-ray absorption fine structure (EXAFS)[22] led us to the following, simplified scheme (Figure 4): at low water concentration (≤10%), ligand exchange occurs predominantly in the $Mn_2^{III,III}$ state. In 90% water instead, essentially all acetates have dissociated already in the $Mn_2^{II,II}$ state. Even though the charge is increased upon ligand exchange, oxidation is facilitated because water ligands allows for a proton-coupled electron transfer. The $Mn_2^{III,IV}$ complex formed had two fully deprotonated, water-derived oxo ligands and, thus, the same charge (2+) as the bis-acetato $Mn_2^{II,III}$ complex. In addition, the dioxo $Mn_2^{III,IV}$ complex could be produced in the presence of 10% water at only somewhat higher electrochemical potential than that required to generate the bis-acetato $Mn_2^{II,III}$ in neat acetonitrile. Thus, three oxidation steps can be reached within a narrow potential range of ca. 0.2 V, thanks to charge compensating ligand exchange and proton-coupled electron transfer. These reactions mimic the stepwise oxidation of the Mn_4Ca complex of PSII, which is coupled to charge-compensating deprotonation and changes in bridging ligands.[1−3]

Competing Kinetics in Accumulative Electron Transfer. The Ru^{II} excited state of the Ru^{II}−$Mn_2^{II,II}$ complex in Figure 4 has a lifetime of 110 ns, which allows for efficient electron transfer to the Co^{III} acceptor. In the $Mn_2^{II,III}$ and $Mn_2^{III,III}$ states, the manganese complex quenches the excited state to much shorter lifetimes (unpublished), cf. Figure 3, which reduces the electron-transfer yield.

For an isostructural Ru−Ru_2 complex, we examined the quenching reactions in different oxidation states: Ru^{II}−$Ru_2^{II,II}$, Ru^{II}−$Ru_2^{II,III}$, and Ru^{II}−$Ru_2^{III,III}$.[23] In all states, the excited state lifetime was quenched to below 1 ns by exchange energy transfer and/or electron transfer to the Ru_2 unit. Neither of the mechanisms is productive for photo-oxidation of the Ru dimer, and the short excited-state lifetime makes it difficult to obtain efficient electron transfer to an acceptor. By substitution on the remote bipyridines of the Ru^{II}−trisbipyridine unit, we could localize the excited metal-to-ligand charge-transfer state away from the appended dimer and, thus, reduce quenching by an order of magnitude. Moreover, we used the substituents to attach the complex to TiO_2 nanoparticles (Figure 5). The ultrafast electron injection into TiO_2 from the excited state outcompeted the other quenching reactions, and we obtained the photo-oxidized $TiO_2^{(-)}/Ru^{II}Ru_2^{II,III}$ state. This study neverthe-

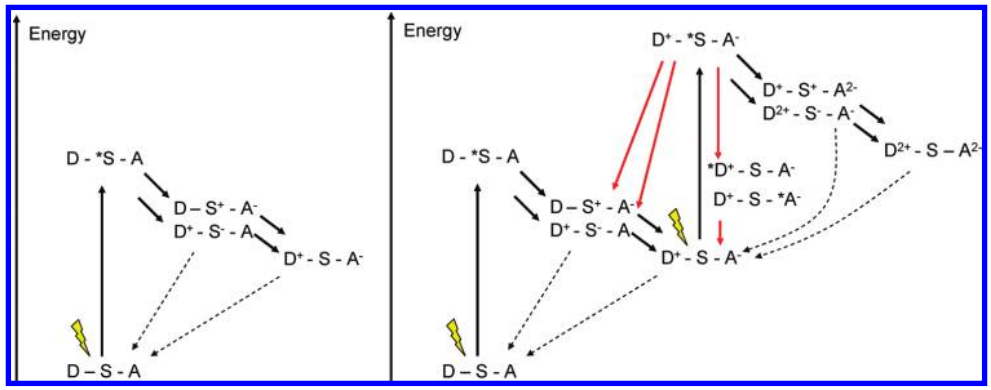

FIGURE 3. Reaction scheme for accumulative electron transfer in a donor−sensitizer−acceptor (D−S−A) triad upon successive absorption of two photons, leading to the final state D^{2+}−S−A^{2-}. The left side shows the reactions induced after single-photon absorption for comparison. Dashed arrows denote recombination reactions that lower the charge separation yield. Red arrows show new competing pathways that exist after the second excitation: photo-induced reverse electron transfer and energy transfer to the D^+ and A^- intermediates.

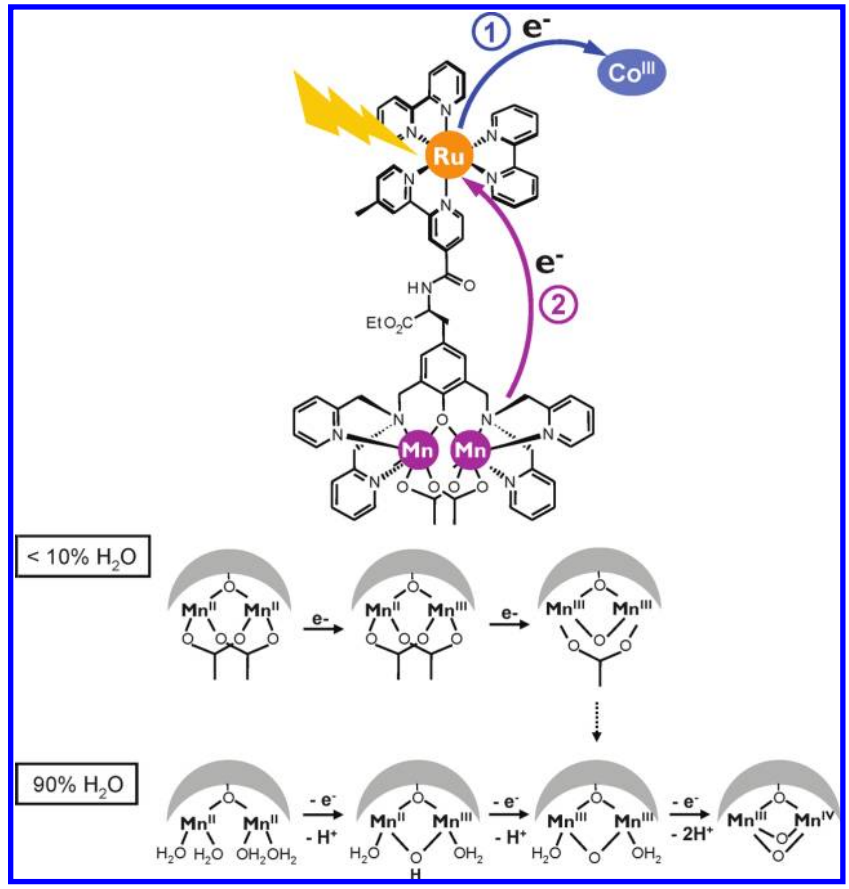

FIGURE 4. Ru^{II}−$Mn_2^{II,II}$ complex partially mimicking light-induced stepwise oxidation of the Mn_4Ca cluster in PSII. Repeated photo-induced electron transfer to the acceptor $[Co(NH_3)_5Cl]^{2+}$ led to oxidation from $Mn_2^{II,II}$ to $Mn_2^{III,IV}$. The lower panel is a simplified scheme of the charge-compensating reactions in aqueous solution that facilitate oxidation of the Mn_2 complex. The assignment of H_2O and OH ligands is tentative.[20]

less illustrates that unwanted quenching reactions may represent a serious problem for accumulative electron transfer, even when the units are at ca. 15 Å distance.

Long-Lived Charge Separation in a Manganese-Based Triad. By attaching two naphthalene diimide (NDI) electron-acceptor units to the Ru^{II}−$Mn_2^{II,II}$ complex of Figure 4, we obtained the manganese-based triad shown in Figure 6.[24] Excitation of the Ru−trisbipyridine unit led to rapid ($\tau = 40$ ns) electron transfer to the acceptor and oxidation of $Mn_2^{II,II}$. The charge-separated state had an average lifetime of 600 μs at room temperature, much longer than for any other Ru^{II}−trisbipyridine-based triad system.[7] In liquid butyronitrile at 140 K, it increased substantially, to ca. 0.5 s, which is on the same time scale as charge recombination in photosyn-

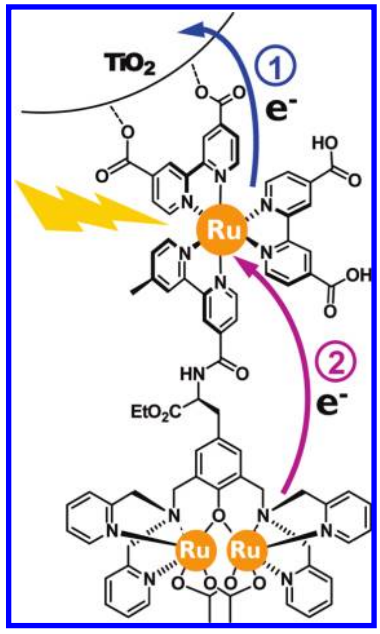

FIGURE 5. $Ru^{II}-Ru_2^{II,II}$ complex attached to a TiO_2 nanoparticle being photo-oxidized to the $Ru^{II}-Ru_2^{II,III}$ state.[23]

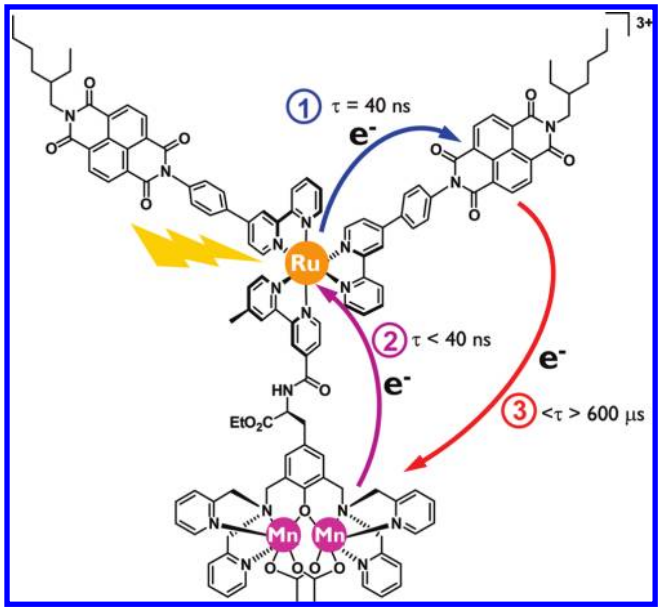

FIGURE 6. $Mn_2^{II,II}-Ru-NDI_2$ triad showing long-lived charge separation.[24]

thetic reaction centers. We could detect both the oxidized donor and the reduced acceptor, and the reaction was reversible, which proves that we achieved a genuinely intramolecular charge-separated state.

The strong temperature dependence of charge recombination was analyzed by the Marcus theory,[25] and we derived an unusually large reorganization energy of $\lambda \approx 2.0$ eV, where the inner reorganization energy contribution was as large as $\lambda_{in} \approx 1.0$ eV. This was supported by analysis of the published crystal structures of the corresponding $Mn_2^{II,II}$ and $Mn_2^{II,III}$ complexes that reveal a shortening of all of the metal−ligand

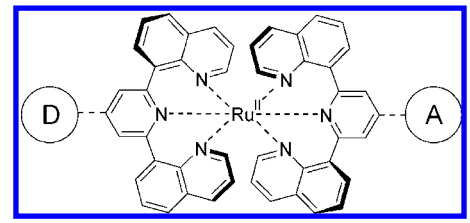

FIGURE 7. Bis-tridentate Ru^{II} complex with a 3 μs excited-state lifetime for vectorial electron transfer. The long lifetime is attributed to the large bite angle (178°) of the ligands, destabilizing the ligand field states.[27,28]

bonds of the oxidized manganese on the average of 0.2 Å/bond. The high activation energy leads to a rate retardation of about 3 orders of magnitude at room temperature, which is sufficient in itself to explain the comparatively long charge separation lifetime.

Triads showing long-lived charge separation typically have their recombination reaction in the Marcus inverted region, where the driving force is larger than the reorganization energy. This combines a high-energy content of the charge-separated state with a somewhat activated and, thus, slow recombination. Our results show long-lived charge separation with high-energy content ($\Delta G^0 = 1.07$ eV relative to the ground state) but with recombination in the Marcus normal region ($\lambda \approx 2.0$ eV). Because electron transfer from Mn^{II} complexes is often accompanied by a large reorganization energy, they are slow donors,[26] but once oxidized, this intrinsic property helps maintain a long-lived charge separation. The long-lived charge-separated state may allow for further light-induced charge separation and accumulative electron transfer, which we are currently exploring.

The multi-exponential charge recombination in the triad was attributed to the mixture of geometrical isomers with different Mn_2-NDI distances (cf. ref 8). To control the geometry of similar assemblies, we have developed a new family of bistridentate Ru^{II} polypyridyl-type complexes, which allow for substitution of electron donor−acceptor units along a C_2 axis for vectorial electron transfer (Figure 7). Importantly, the photophysical properties including microsecond luminescent lifetimes are superior compared to the related $Ru^{II}-$bisterpyridine.[27,28]

Water Oxidation by Synthetic Manganese Complexes. Despite numerous reports of manganese complexes aimed to function as water oxidation catalysts, studies of their oxidizing capabilities are rare. In the few cases where oxygen evolution has been reported, experimental conditions have varied, making comparisons difficult. We carried out a systematic study, screening a series of manganese complexes (Figure 8) at comparable reaction conditions.[29] Complex **1** is the Mn_2

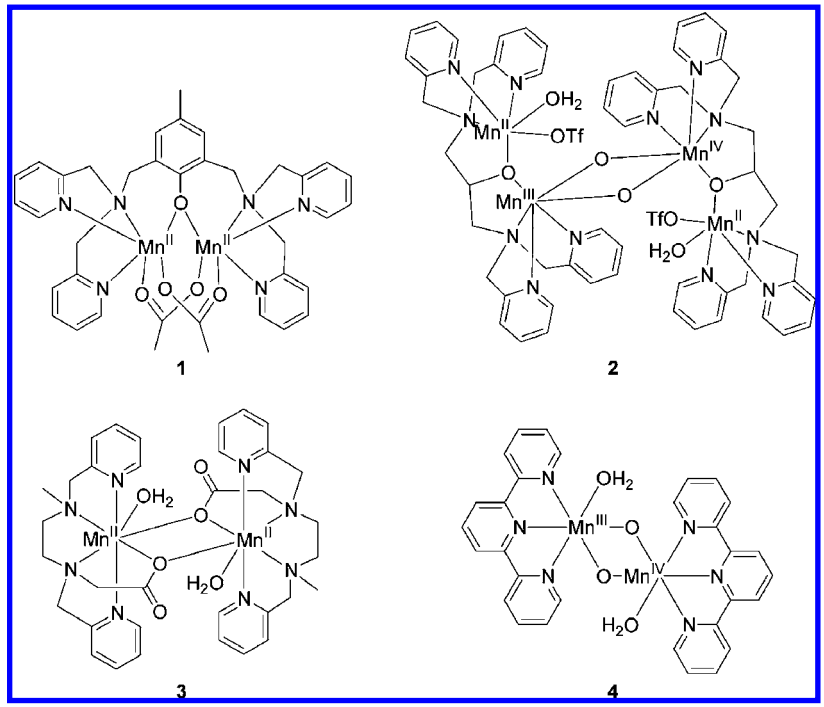

FIGURE 8. Four complexes that promoted oxygen evolution in the presence of oxone. Only complex **1** was found to yield O_2 where both oxygen atoms were derived from water.[29]

unit of the $Ru^{II}-Mn_2^{II,II}$ complex in Figure 4 and complex **2** is from ref 30. Oxygen-evolving reactions have previously been reported for complexes **3** and **4**.[31,32] We tested the reactivity of all complexes using one-electron oxidants, such as Ce^{4+} and ClO^-, and oxone (HSO_5^-), which is both an oxygen-transfer agent and a two-electron oxidant. For the first time, we observed oxygen evolution from complexes **1** and **2**, using oxone as an oxidant. We also obtained oxygen evolution with complexes **3** and **4**, in partial agreement with previous results.

To identify the origin of the released oxygen, we used a partially ^{18}O-labeled water/acetonitrile mixture and mass spectrometry.[33] With complexes **2**–**4**, molecular oxygen was evolved with a distribution of $^{16}O^{18}O$ and $^{16}O_2$, consistent with one oxygen atom, at most, originating from the bulk water and one from the oxidant. With complex **1**, however, addition of oxone resulted in an initial burst of oxygen completely derived from the bulk water, according to the percentage of O^{18} labeling (Figure 9). The initial reaction could not be replicated by adding more oxone to the same reaction mixture because of degradation of the Mn_2 complex, and the reaction was not catalytic. Nevertheless, this is possibly the only reported solution reaction mediated by a synthetic Mn complex where two water molecules are oxidized to form O_2.

It has been suggested that oxygen transfer is essential for water oxidation to occur in complexes **3** and **4**.[31,32] However, using lead tetraacetate ($Pb(AcO)_4$) as an oxidant,

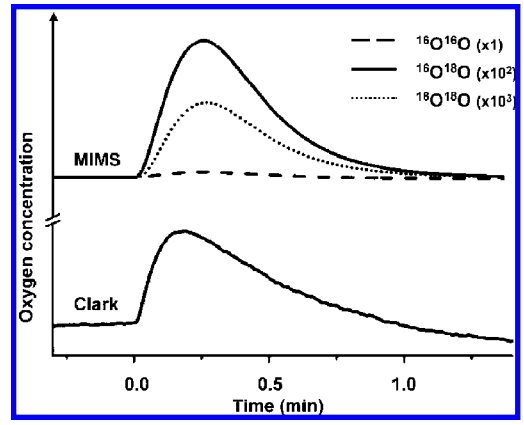

FIGURE 9. Oxygen evolution traces for complex **1** recorded by mass spectrometry (MIMS, top) and by a Clark electrode (bottom). The water in the aqueous phase contained 10% ^{18}O-labeled water and the evolved oxygen of $^{18}O_2$, $^{18}O^{18}O$, and $^{16}O_2$, at ratios that can be expected if all oxygen atoms originated from the solvent water.[29,32]

nearly the same amounts of isotopically labeled oxygen was evolved from complex **1** as when oxone was used. This demonstrated that a two-electron donor might be critical for the observed reaction, explaining why oxone worked well for this purpose.

Although none of the Mn complexes performed catalytic water oxidation, it is encouraging to find that the few oxygen-evolving reactions reported in the literature are far from unique and there is still more to learn from existing systems.

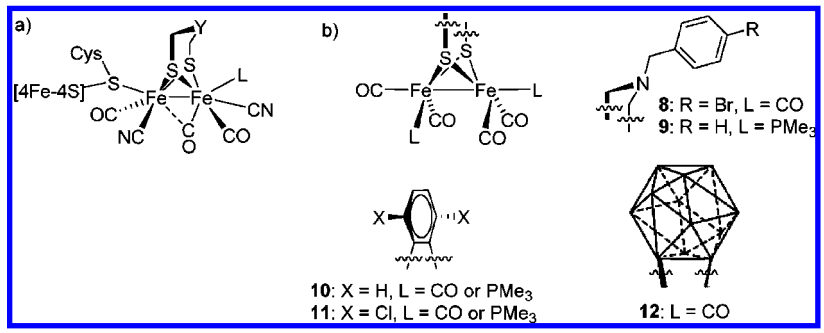

FIGURE 10. Examples of photosensitizer−diiron dyads.

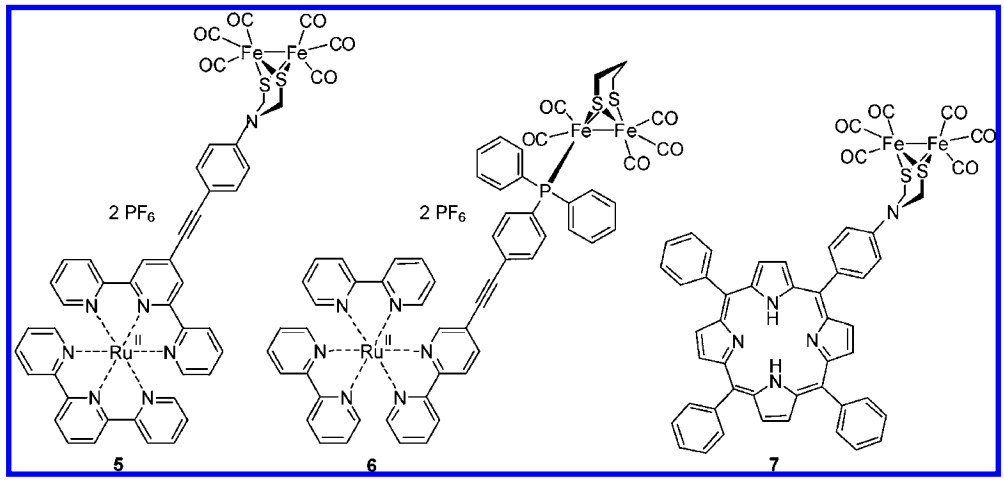

FIGURE 11. Selected $[(\mu\text{-SRS})Fe_2(CO)_4(L)_2]$ complexes.

Biomimetic Fe_2 and Ru−Fe_2 Chemistry for Hydrogen Production

In our biomimetic approach to artificial photosynthesis, the water-to-fuel conversion is complemented with Fe_2 hydrogenase mimics as proton reduction catalysts. The overall goal is to use reduction equivalents from excited photosensitizers to generate molecular hydrogen in a supramolecular array. The active site of Fe_2 hydrogenases are very efficient proton reduction catalysts. The constitution of the active site is crystallographically determined to a large extent[3,15,16] and can be modeled by relatively well-established iron coordination chemistry (Figure 10).[17]

There are a few examples in the literature where a diiron dithiolate model complex is covalently linked to a photosensitizer with the aim to realize light-driven reduction of protons (complexes **5**−**7**, Figure 11).[34−36] However, in none of these dyads could electron transfer or hydrogen production be established unambiguously. Typical Ru polypyridyl sensitizers have excited-state reduction potentials of ca. −1.2 V versus ferrocene, which is far from sufficient for the reduction of the diiron units in Figure 11. Note that protonation of the azadithiolate (adt) nitrogen in complex **5**, to increase the Fe_2 potential, was impeded by its extraordinarily low basicity. Instead, reductive quenching of the excited photosensitizer may be accomplished by an external electron donor. The resulting photoreduced sensitizer is a stronger reducing agent than the excited state and is thus capable of electron transfer to the diiron site in a subsequent dark reaction. This approach has recently been used in a bimolecular system where hydrogen was generated with 4 turnovers using a diiron complex as a catalyst, Ru^{II}−trisbipyridine as a photosensitizer, and ascorbic acid as a sacrificial electron donor.[37] The need for easily oxidized donors in the reductive quenching step would, however, preclude the oxidation of water at the donor terminal of a complete molecular water-splitting system. We are therefore in the first line aiming for systems where proton reduction can occur by oxidative quenching of the photosensitizer.

Iron hydrogenases and their functional mimics catalyze hydrogen formation in a proton hydride reaction. The hydride intermediate is formed by protonation of the low valent iron sites in an oxidative addition. If the hydride is sufficiently nucleophilic, because of either the electron-donating properties of the ligand set or reduction, it can form hydrogen by reaction with a proton from the bulk or a protonation site on the catalyst. Redox and acid/base properties of the catalyst are

correlated because reduction of the metal centers increases their basicity or the nucleophilicity of a hydride ligand and, on the other hand, protonation renders reduction potentials less negative. The order of reduction and protonation events in the catalytic cycle therefore depends upon the ligand set of the diiron site, the proton activity of the medium, and the reduction potential available.

The least negative potential at which proton reduction is thermodynamically feasible is given by the proton activity in the medium. Catalytic reduction will however occur with a certain overpotential given by the most negative reduction step in the catalytic cycle. Under turnover conditions, this potential is affected by the kinetics of the coupled chemical reaction and therefore not identical to the equilibrium reduction potential of the redox couple. The potentials where catalytic peaks are observed in voltammetry indicate what reduction potential will be required from the photosensitizer for light-induced hydrogen formation.

Two strategies have been used to tune the reduction potential to be in reach of the excited state of the sensitizer. The introduction of electron-withdrawing dithiolate ligands that shift the reduction to more positive potentials has been achieved by N-protonation of the adt ligand in complex **8** (Figure 10), which renders the reduction milder by ca. 400 mV.[38] Rapid electrocatalytic hydrogen evolution is however only detected at more negative potential. Further electronic tuning of the dithiolate ligand was achieved by the introduction of electron-withdrawing substituents on an arene bridge (complexes **10** and **11**)[39] and even more so by a carborane-linking motif (complex **12**).[40]

The second strategy is based in the introduction of electron-donating phosphine or cyanide ligands at the diiron site to facilitate a mechanism that starts with the formation of a hydride species prior to the reduction event. A combination of the two strategies has led to the synthesis of a complex (**9**) that can simultaneously carry a proton at the adt nitrogen and a hydride at the diiron site.[41] As a result, the reduction potential is shifted by more than 1.2 V toward milder values compared to the nonprotonated state, demonstrating the dramatic effect that protonation has on the electronic properties of the complexes.[42] Thus, we have diiron complexes where the first reduction potential is in reach of the excited state of the photosensitizer and that exhibit catalysis at a potential close to that of the excited state of typical ruthenium-based photosensitizers. This is a promising starting point, but for efficient photocatalysis, other issues need to be addressed, such as competing quenching pathways, analogous to those in Figure 3, as well as the oxygen sensitivity and photostability of the diiron complex. The latter aspects are important also in hydrogenases. An increased understanding of biomimetic systems may therefore aid in the development of photobiological hydrogen production with hydrogenases.

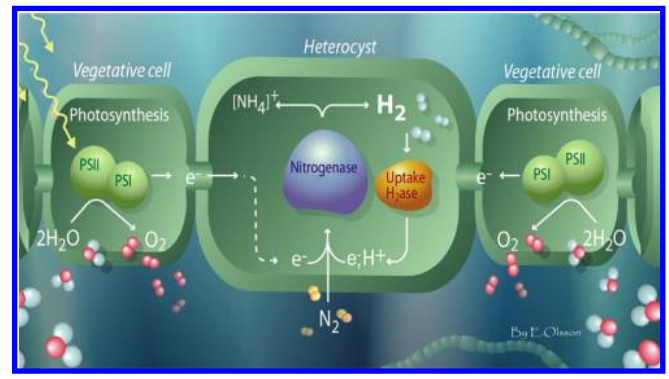

FIGURE 12. Filamentous cyanobacterium *N. punctiforme* viewed in the mind of an artist. Oxygenic photosynthesis takes place in the vegetative cells (yellow, left), separated from the micro-oxic, nitrogen-fixing heterocysts. Heterocyst-specific transcriptional regulation was studied using the green fluorescent protein (bright green).

Photobiological Hydrogen Production

Green algae and cyanobacteria are able to combine oxygenic photosynthesis with the production of H_2, an attractive pathway for renewable production of H_2 from solar energy and water.[13] Here, we restrict our discussion to cyanobacteria and outline possibilities and challenges for hydrogen production.[14] Cyanobacteria is a large and very diverse group of bacteria, with the capacity to use sunlight as an energy source, water as an electron source, and air as a carbon (CO_2) and nitrogen (N_2) source. Therefore, minimal and inexpensive growth media can be used for the cultivation of cyanobacteria, and theoretically, the overall energy conversion efficiency may become very high.

Hydrogen Production from Dinitrogen-Fixing Cyanobacteria. Many cyanobacterial strains are N_2-fixing under certain conditions. This occurs in an anaerobic environment achieved by separating N_2 fixation and oxygenic photosynthesis (Figure 12) and is accomplished in specialized N_2-fixing cells called heterocysts. Reduction of N_2 to NH_4^+ is catalyzed by nitrogenase and leads to the formation of H_2 as a byproduct. The evolved H_2 is rapidly consumed by the uptake hydrogenase, a NiFe enzyme exclusively found in N_2-fixing cyanobacteria. Because any produced H_2 is reoxidized by the uptake hydrogenase, no net production is detected.

The uptake hydrogenase is encoded by the *hup* (hydrogen uptake) genes and consists of at least a large protein sub-

unit of about 60 kDa (HupL, encoded by *hupL*) and a small subunit of approximately 35 kDa (HupS, encoded by *hupS*). A protein anchoring the uptake hydrogenase to the membrane has also been predicted. The physiological function of the uptake hydrogenase is to recover reducing equivalents from H_2.[14] Understanding the regulation and control of the uptake hydrogenase is important not only for photobiological hydrogen evolution but also for the production of genetically altered enzymes that provide molecular understanding for synthetic chemistry. In *Nostoc punctiforme* PCC73102/ATCC29133, we found that *hupS* and *hupL* form an operon together with novel and to date unexplored, so-called tandem repeat sequences with putative secondary loop structures. These secondary structures of the DNA are potentially important for gene regulation. Promoter analyses indicated existence of binding sites for the nitrogen-responsive transcriptional regulator NtcA. However, in our recent studies of the *hupSL* promoter, we observed a complex regulation mechanism, with no clear effect of the NtcA binding site, and a retained heterocyst-specific expression in all promoter constructs.[43]

The first obvious step to design a H_2-evolving cyanobacterial strain is to engineer a mutant without the capacity to recycle H_2. We constructed such a mutant in *N. punctiforme* lacking the uptake hydrogenase. The mutant showed increased, nitrogenase-based H_2 production. In gas-exchange experiments, we showed that the H_2 production was strongly dependent upon light.[44] The amount of H_2 produced per molecule of N_2 fixed under low and high light was 1.4 and 6.1, respectively. This demonstrated that the photosynthetic energy flow may be directed toward H_2 production rather than N_2 fixation during high-light conditions.

Hydrogen Production Directly from Bidirectional Hydrogenases. Cyanobacteria may contain a bidirectional hydrogenase, another NiFe enzyme that exists in both N_2- and non-N_2-fixing strains.[14] Under anaerobic conditions, it can either produce and/or oxidize significant amounts of H_2. The bidirectional hydrogenase is encoded by the *hoxEFUYH* operon, forming an enzyme with a hydrogenase part (HoxYH) and an electron-transfer partner protein (HoxEFU). Because the activity of the bidirectional hydrogenase is not directly dependent upon ATP, it might potentially be energetically more efficient than the nitrogenase. On the other hand, the native enzyme is not localized to an anoxic environment, and because it operates close to chemical equilibrium, H_2 production is inhibited above a certain H_2 partial pressure. Therefore, continuous removal of both O_2 and H_2 is necessary to improve the efficiency. Furthermore, an accumulation of ATP might impede the reaction, because ATP is produced by photosynthetic electron transport but not consumed by the hydrogenase.

The biological function of the bidirectional hydrogenase is not fully understood, but three main functions have been suggested. It may remove excess reducing equivalents during either photoautotrophic growth or anaerobic fermentation, or it may deliver electrons to the respiratory electron-transport chain by oxidizing H_2. However, because the presence of the bidirectional hydrogenase is not universal and hydrogenase-deficient mutants can be made, it does not seem essential for cell survival. Genetic modifications are thus an open possibility.

The organization of the *hox* genes vary in different strains. By analysis of the *hox* operon in the unicellular cyanobacterium *Synechocystis* PCC 6803, we and others[14] discovered that several regions in the operon are essential for regulation of gene transcription. We could show the existence of an interaction between a LexA-related protein and the *hox* promoter. Recently, we also demonstrated that an additional transcription factor, an AbrB-like protein, interacts with the promoter of the *hox* operon and that it positively regulates its transcription, increasing the hydrogenase activity.[45]

The biosynthesis and metal insertion of NiFe hydrogenases is highly complex, requiring several proteins. Genes encoding proteins with a supposedly universal maturation function for all hydrogenases (*hyp*) as well as the hydrogenase-specific proteases (*hupW* and *hoxW*) have been identified. Recently, we showed that the hydrogenase-specific proteases are under similar regulatory control as the hydrogenases that they cleave.[46] In addition, specific cleavage motifs for the Hox hydrogenase proteases have been identified that will help us understand the specificity for different hydrogenases.

Besides the specific challenges connected to the H_2-evolving enzymes, there are unsolved issues for photoautotrophical H_2 production in general. These include electron-consuming pathways in direct competition with H_2-producing reactions. Further exploration of these pathways and regulations of hydrogen metabolism is needed to accomplish efficient genetic and metabolic engineering. One strategy that we use is quantitative shotgun proteomics.[47]

Synthetic Biology. An obstacle in biological H_2 production is the high oxygen sensitivity of the H_2-evolving enzymes, and some attempts have been made to introduce less oxygen-sensitive hydrogenases into cyanobacteria. An elegant strategy for the creation of an efficient H_2 producer would therefore be the expression of an FeFe hydrogenase in the micro-oxic environment of the heterocysts of a filamentous cyanobacterium. Because protons are abundant within the cell,

the main limitation for H_2 production is the amount of reducing equivalents. The primary electron donors for the H_2-producing enzymes are ferredoxin (nitrogenase and FeFe hydrogenases) and NAD(P)H (NiFe hydrogenases). However, these reductants are also used by competing pathways, e.g., respiration. Therefore, one strategy is to direct the electron flow toward the H_2-producing enzymes and away from competing pathways. As the cyanobacterial bidirectional hydrogenase evolves H_2 at relatively high levels of reduced NAD(P), the construction of mutants with blocked electron transfer in selected key pathways may be a promising route to an increased H_2 production. Answers to questions like how much of the maturation system needs to be introduced and translated into functional units and what kind of regulation is needed are crucial for success.

The development of synthetic biology opens up new possibilities for the construction of efficient H_2-evolving cyanobacterial strains. The first attempts have been initiated to use this new concept, aiming at designing standardized molecular building blocks for making a synthetic photosynthetic bacterium containing engineered chemical pathways for competitive, clean, and sustainable H_2 production.

Outlook

For the purpose of solar fuels production, photobiological H_2 production has the potential to be more efficient than current biomass production, because it is an ongoing process in the stock culture and does not require harvesting the organism itself. Development of efficient new strains of microorganisms may provide a valuable addition to the biofuel industry in the near future. Biomimetic systems on the other hand offer a shortcut to taking advantage of the efficient principles of the primary photosynthetic reactions without dealing with life-sustaining processes. The freedom in molecular design is greater, and the components will be much smaller than their natural counterparts. Thus, integrated biomimetic systems have higher potential for direct solar fuel production, although their success lies in a more distant future.

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FOOTNOTES

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