Description of the main results in SOLARH2.

The science in SOLARH2 has been remarkably productive and the work has been published in ca 380 scientific papers, in 100s of lectures and many 100 posters in Europe, the US, Asia and even in Africa at a few occasions. Many coworkers have been involved and there are many young scientists that have taken major steps in their early careers as Ph D students, post doctors or advanced post doctor levels (varies in different countries and different research systems).

The report that is compiled here has been written by the experts in the different fields we have covered. These experts were leaders of the scientific work packages in SOLARH2 and have overviewed the science continuously for the 4 years the project has extended.

WP1: Photochemical water-splitting by Artificial Photosynthesis: Design, synthesis, characterization and functional studies of modular, biomimetic photocatalysts. (Report by Prof Leif Hammarström, Uppsala University, Sweden.)

This WP was broken down into three tasks; the tasks and scientific approach are illustrated in Figure WP1.1.

Task 1.1. Light-driven water splitting catalysts.

Task 1.2. Light-driven hydrogen producing catalysts.

Task 1.3. Control of multiple, light-induced charge separation and transport of redox equivalents.

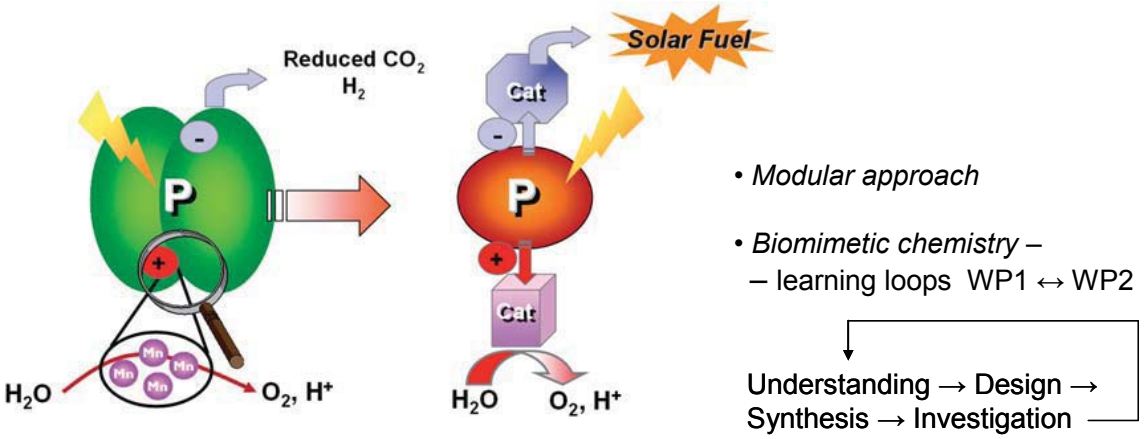


Figure WP1.1. Principles of WP1: based on principles and understanding of natural photosynthetic (left) and hydrogen producing enzymes we design and synthesize complexes carrying out the different tasks (right): A photosensitizer (P) absorbing light and initiating charge separation reactions, where the electrons and holes are transported (Task 1.3) to catalysts (Cat) for light-driven water oxidation (Task 1.1) and Solar fuel/H_2 production (Task 1.2). With our modular systems approach we can synthesize and investigate the different components separately. Our results from biomimetic systems also serve as models for understanding of the natural processes, providing the productive learning loops between WP1 and WP2 that is core to the SOLAR-H2 concept.

Task 1.1. Summary: ***We have designed a range of new, functional water oxidation catalysts and in several cases reached far in mechanistic understanding. We have adapted to results in the field on cobalt oxide catalysts and made important practical and fundamental advances in that area.***

SOLAR-H2 (2008-2012) and its predecessor SOLAR-H (2005-2007) were the first, and are still recognized as one of the major, projects in the field. When we started there were two reported molecular catalysts for water splitting to O_2, both based on ruthenium. During our project period a remarkably large number of catalysts have been designed and discovered, many of them within SOLAR-H2. We reported the first example where the catalytic mechanism of O-O bond formation could be clearly established, in this case an intramolecular radical coupling (Figure WP1.2). Our mechanistic investigations of several mono- and dinuclear ruthenium complexes have employed a range of kinetic, advanced spectroscopic and computational methods available within the collaboration of SOLAR-H2, and have provided deep insight into the different catalytic mechanisms that are manifested as well as important structure-function relationships. Several of the ruthenium catalysts have also been of sufficiently low potential to allow for light-driven water oxidation in photochemical cycles with a $[Ru(bpy)_3]^{2+}$ sensitizer and a sacrificial acceptor.

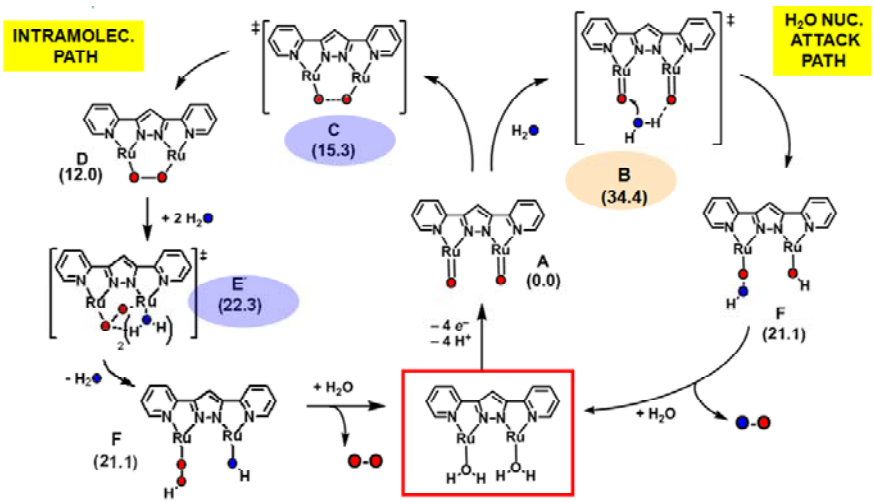
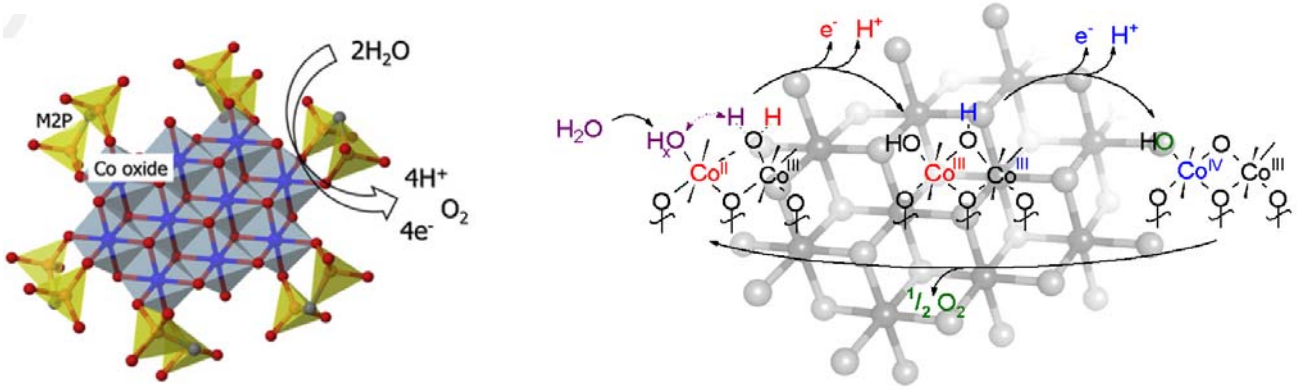


Figure WP1.2 Mechanistic cycles of water oxidation (left): intramolecular radical coupling; (right) water nucleophilic attck.

While many molecular water oxidation catalysts (WOC) of ruthenium, iridium and other rarer elements have been discovered, there were until 2010 no clear reports of molecular WOCs of earth-abundant elements like the first-row transition metals. Yet, the WOC of natural Photosystem II is a $CaMn_4$ cluster. Within SOLAR-H2 we have made important fundamental studies of new manganese complexes. We were able to refute claims of WOC activity for some Mn_2-complexes made by other workers. Also, spectroscopic studies by XAS, XES and IR are very important for advancing our current understanding of the WOC mechanism of Photosystem II. We have made analogous studies on Mn-model complexes that have provided a basis for interpretation of the results for Photosystem II (synergies with WP2). More recently, we have studied mononuclear Mn-aquo complexes with a pendant base to understand how water is activated in the proton-coupled oxidations of manganese. We were partially successful in water splitting with molecular manganese complexes. In a study with chemical oxidants and isotopically labeled water we could show one Mn_2-complex that actually oxidized water to O_2 in an initial rapid reaction phase, albeit sub-stoichiometrically, but only with two-electron oxidants. We also linked a Mn-salen complex to a Ru(II)-

photosensitizer and were able to photochemically catalyze epoxidation reactions, where the manganese complex transferred an oxygen for water to form the epoxide.

Much attention has recently been given to cobalt oxides as WOCs. In SOLAR-H2 we developed a simple and robust method to stabilize Co-oxide nanoparticles (NPs) by diphosphonate ligands. They were shown to be quite active WOCs in photochemical experiments, showing turnover frequencies (TOFs) up to 0.5 s^{-1} per cobalt atom present (although the majority of cobalt is not on the NP surface). We have made extensive XAS and XES investigations of these NPs as



well as a range of cobalt-, nickel- and manganese oxides (Figure WP1.3). Notably, all these oxides are structurally similar; and they all share structural motifs with the Mn_4CaO_5 core of the biological catalyst bound to the proteins of Photosystem II (Risch et al, 2011, Chem. Commun.). An electrodeposited Co-oxide WOC on a conducting anode was shown to undergo oxidation-state changes from 40% Co^{II} (0.8 V vs. NHE, pH 7) via pure Co^{III} to 20% Co^{IV} (1.35 V vs. NHE) which are coupled to µ-oxo bridging-type changes at the periphery of oxide fragments (Risch et al, unpublished, *Fig. WP1.3b*). Similar oxidation state changes were detectable in the Ni- and Mn-based electrocatalysts. Analogous changes upon proton-coupled oxidation are know to occur in molecular catalysts. This means that the amorphous oxides accumulate oxidizing equivalents by means of chemical changes previously observed only in molecular complexes. Both the NPs and surface films have can be incorporated in a biomimetic system for complete solar fuels production, as represented in Figure WP1.1.

Figure WP1.3 (left) Structure of catalytic cobalt oxide nanoparticles stabilized by diphosphonate ligands; (right) Mechanistic steps of the electro-deposited cobalt oxide catalyst.

Task 1.2 Summary: ***Our studies of synthetic FeFe-complexes as active site mimics [FeFe] hydrogenases have led to improved catalysts. We have also developed new structures and shown mononuclear Fe-catalysts with favorable properties. Also, the interactive feedback loops between WP1 and WP2 have been particularly strong.***

SOLAR-H2 partners were early in the field of FeFe-mimics and made the first azadithiolate catalyst as well as the first covalently linked sensitizer-Fe_2 complexes for light-driven charge separation (2003, 2004). During SOLAR-H2 we could demonstrate the first light-induced H_2 production with multiple-turnovers (>200) in near-neutral aqueous solution (Streich *et al*, 2010). The result showed this class of complexes as viable catalysts and not mere model complexes. Light-driven catalysis also opens up for time-resolved laser spectroscopic

techniques, such as transient IR, which is currently pursued to resolve intermediates in the catalytic cycles.

These successful results were based on our investigation of Fe_2-complexes with aromatic dithiolate bridging ligands. We found that such hexacarbonyl complexes were stable towards CO-loss and CO bridging coordination upon reduction, in contrast to their aliphatic counter parts. This leads to good electrochemical reversibility. Moreover, the aromatic bridges gave other very different effects of reactivity in ligand exchange reaction that allowed us to prepare, for the first time., mononuclear Fe-ditiolate complexes as H_2 production catalysts.

The current mechanistic model for the function of [FeFe] hydrogenase enzymes postulates that all the catalytic steps occurs at *one* Fe center distal to the [4Fe4S] cluster (Fe_d), despite of the *di*nuclear nature of the active site (Figure WP1.4). This observation prompted us to investigate *mono*nuclear Fe complexes that resemble Fe_d. We were the first to prepare such a mononuclear complex and to show that they do not only catalyze electrochemical proton reduction, but even rival the best *di*nuclear catalysts in terms of required overpotential (Kaur et al., Angew. Chem. 2010). A crucial step in the catalytic cycle of the complex reported in 2010 is the dissociation of one ligand that liberates a free coordination site for substrate binding, thereby enabling catalytic turnover. This deligation, however, also compromises catalyst stability. Thus, we designed and incorporated a new ligand set that creates a free coordination site already in the ground state (Figure 4b; Beyler et al., Chem. Commun. 2011). Destabilizing ligand losses are therefore not necessary any longer in the resulting *penta*-coordinated Fe(II) complexes. In addition, with the incorporation of a basic site in the second coordination sphere to the Fe center, another crucial feature of the enzymatic active site could be incorporated into the complex (Figure WP1.4). The basic site is capable of shuttling substrate protons into the active site, giving rise to increased turnover numbers and rates. In electrochemistry experiments we could show that this is a very rare species that catalyzes proton reduction *both* at low overpotential (ca. 100 mV) *and* with high rates (TOF = 500 s^{-1}) under the same conditions.

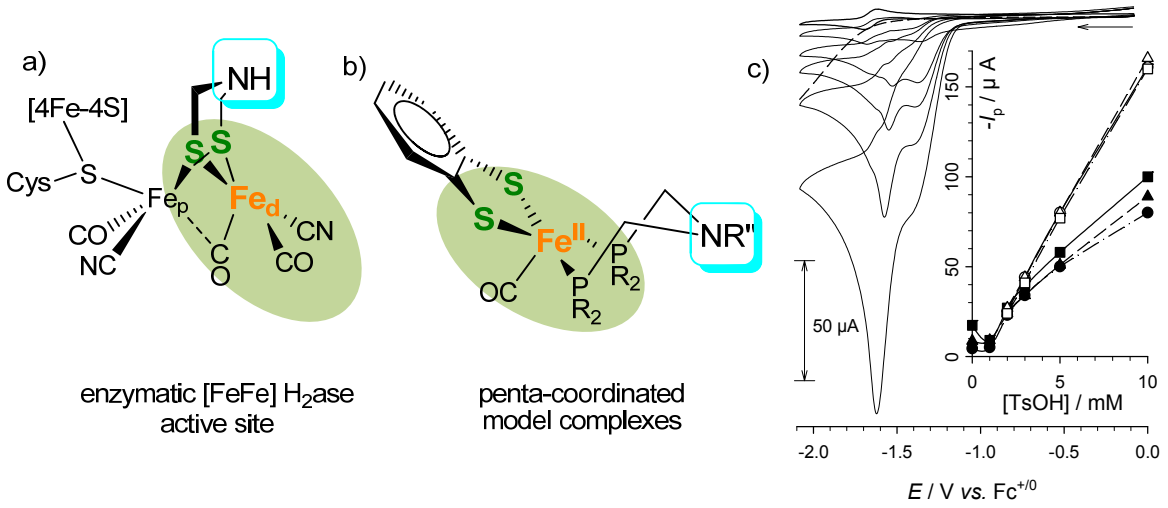


Figure WP1.4: a) Active site of the [FeFe] hydrogenase enzyme. b) Penta-coordinated model complex of the distal Iron Fed in the enzyme active site. Highlighted are common structural features (green) and the basic site for proton shuttling (blue) c) Cyclic voltammetry (v = 0.100 Vs^{-1}) (0.25 mM in CH_3CN solution with $[(C_4H_9)_4N][PF_6]$ (0.10 M) as supporting electrolyte) with 0, 1, 2, 3, 5, 10 mM TsOH and background current on the glassy carbon electrode for 10 mM TsOH without catalyst (---).

*Inset: Peak/plateau current as function of [TsOH] with 0.25 mM **5** (●), 0.50 mM **5** (▲), and 1.0 mM **5** (■) at -1.4 V (-1.69 V for [TsOH] = 0) and at -1.53 to -1.63 V (open symbols).*

Comparative studies of synthetic model complexes and the FeFe-hydrogenase active site by advanced EPR and X-ray spectroscopic methods have led to important results. This is described in WP2. Most importantly, we could finally demonstrate that the central atom of the dithiolate bridge is indeed a nitrogen (and not oxygen or carbon). This is crucial to mechanistic understanding as protonation of the nitrogen is believd to be a key catalytic step.

Task 1.3 Summary: We have developed new structural motifs of Ru(II)-polypyridine photosensitizers to simultaneously obtain long excited state lifetimes, favorable redox properties and directional control over charge separation. Redox active links have been incorporated to control electron transfer and proton-coupled electron transfer. Photo-induced accumulation of redox equivalents have been obtained, for the first time in a reversible manner, providing a link between light-induced charge separation and multi-electron catalysis. Functional connection to semiconductor electrodes have been made, paving the way towards an integrated system for water splitting and solar fuels production in a two-cell arrangement.

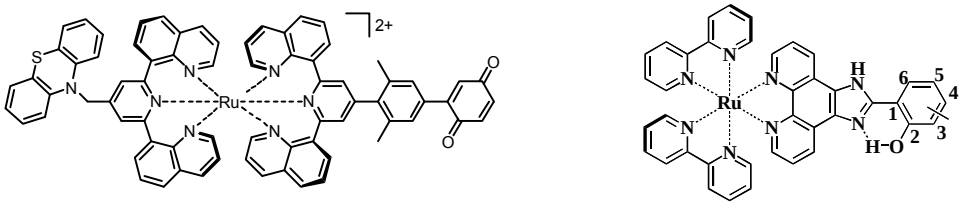


Figure WP1.5 (a, left): A donor-Ru(II)-acceptor triad based on a bis-tridentate Ru(II) complex; thanks to a nearly perfect octahedral coordination geometry of the latter it shows an intrinsic MLCT lifetime of 3 us as compared to < 1ns for analogous bis-terpyridine complexes. (b, right): a sensitizer with a redox-active link, inspired by the proton-coupled reactions of the TyrosineZ-Histidine190 pair of Photosystem II. The PCET potentials have been tuned by substituents to allow tailoring of the redox gradient towards the potential catalyst to be oxidized.

Ruthenium(II)-polypyridines is a major class of photosensitizers, yet we designed a new biquinoline-pyridine ligand that for the first time gave bis-tridentate complexes with microsecond excited state lifetimes. Thus a rod-like donor-Ru-acceptor triad could be made showing efficient and fairly long-lived charge separation (Figure WP1.5a). In a different approach, we have recently used click chemistry as a synthetically less time consuming way to assemble donor-Ru(II)-acceptor complexes. Rapid and efficient charge separation was demonstrated over the resulting triazole unit of the link.

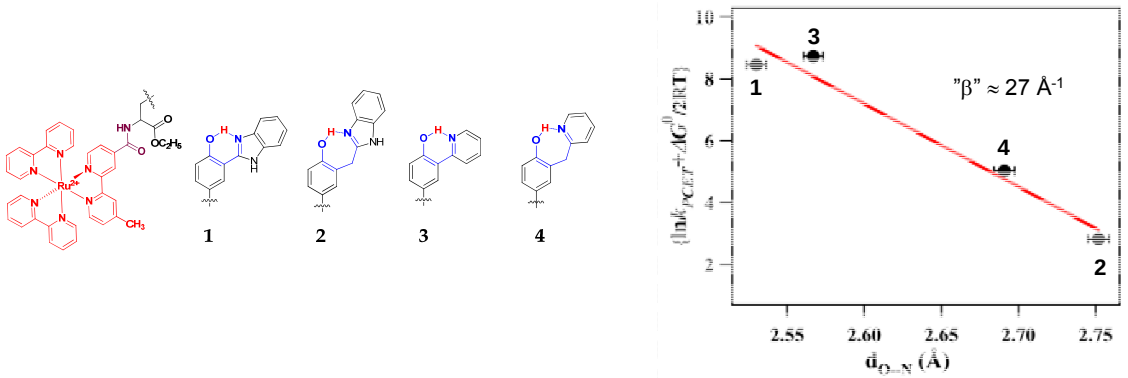


Figure WP1.6 Ru(II)-tyrosine model complexes with internal bases for intramolecular PCET. The plot shows the large impact of the O-N distance on the PCET rate, with a nearly 1000-fold increase for just a 0.2 Å decrease in O-N distance.

Sensitizers with redox active links towards catalysts have been developed. With the phenantroline-imidazole motif (*cf.* Figure WP1.5b), several structures have been made. A Ru(II)-Mn(III)-(u-O)$_2$Mn(IV)-Ru(II) complex was prepared and investigated, where the Mn-dimer unit is that of the "Brudvig" terpyridine dimer. Detailed insight into the proton-coupled electron transfer (PCET) of Ru(II)-tyrosine model complexes have been obtained. In particular, we have demonstrated a remarkable sensitivity to the PCET rate on the proton tunneling distance (Figure WP1.6). Solar fuels catalysts also operate by PCET reactions. We have suggested that this requires careful design of proton-shuttling bases in solar fuels catalysts to enhance the catalytic rates.

Finally, the first example of an accumulative charge separation in a molecular system was demonstrated. In contrast to previous examples, no sacrificial agents were used, so that electrons and holes were accumulated simultaneously on the TiO_2 particle and the oligoamine, respectively. Thus, this is a genuinely energy storing reaction that bridges light-indced charge separation on a single –electron level and multi-electron catalysis. This proof-of-principle experiment will be extended to incorporate catalytic units for water oxidation. The same principle is also pursued on the reducing side, using our expertise on dye-sensitized p-type materials (NiO). These two halves can be connected (electronic circuit and proton conduction), which points towards an integrated, two-cell system for water splitting and solar fuels production, using molecular catalysts.

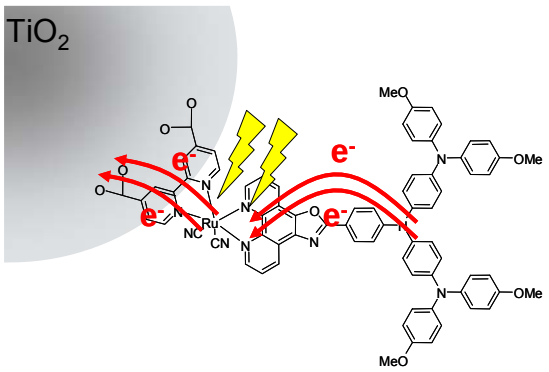


Figure WP1.7: Molecular system for accumulative charge separation. With two laser flashes delivered 1 us after one another, two holes were accumulated on the oligoamine and two electrons in the TiO_2 particle with ca. 100% yield. Recombination was slow (100 us timescale) which shows that rapid catalysts would have time to utilize the charges being separated.

WP1 Milestones and expected results

M 1.1 Evaluation of the water splitting capacity of synthetic Ru_2, Mn_2 and RuMn catalysts, by photochemical as well as "dark" methods. This will provide new mechanistic insight and identify obstacles, which we will use in designing the next generation of water-oxidizing catalysts. [18 months]

M 1.2 Demonstration of the first system for photochemical reduction of protons to molecular hydrogen with Fe_2- or NiFe-complexes coupled to photoactive Ru. This will provide new

mechanistic insight and identify obstacles, which we will use in designing the next generation of hydrogen-producing catalysts. [30 months]

M 1.3 Evaluation of the first systems for accumulation of several light-induced redox equivalents in a molecular array, controlled by our new designs of photoactive centres and redox-active links. This will pave the way for coupling of the catalytic O_2- and H_2-generating sides of the artificial systems. [24 months]

M 1.4 Coupling of the oxidizing and reducing sides via one photoactive centre, a surface, a membrane and/or a macromolecular matrix. [48 months]

Comments: we have passed the first three milestones, with substantial success. On all points we have also modified our approach and direction based on the results and insight we have obtained during the project. For the last milestone we have passed parts of these aspects; we have coupled non-catalytic systems to the same sensitizer for single-and multiple charge separation, and we have had sues in utilizing semiconductor surfaces. We can now see better how we could, in the near future, construct an integrated system for complete water splitting and solar fuels production.

Table WP1.1: Deliverables brief description and month of delivery

1.1. WP1-specific young investigator meeting, with group leaders [18 months]
This meeting was held for a full day Friday April 3, 2009 in Cambrils (Spain), organized by Partner 4 and the WP1 leader. 30 participants from Partners 1, 2, 3, 4 and 6, including group leaders. All ongoing collaborative work in WP1 was discussed, and future work was planned. The minutes from the meeting were attached to the period report.
Status: Delivered.

1.2. A photochemical water-splitting system on the small lab scale using molecular Ru- and/or Mn-based catalysts. [48 months]
The first complete systems were reported already in the second periodical report (18-36 months). SOLAR-H2 has now several publications describing such systems.
Status: Delivered.

1.3. A photochemical hydrogen production system on the small lab scale using molecular Fe2- or FeNi-based catalysts [36 months]
The first complete system was reported already in the second periodical report (18-36 months). SOLAR-H2 has now several publications describing such systems.
Status: Delivered.

1.4. At least 40 scientific papers in international peer-reviewed journals will be published based on the results of WP1. [48 months]
We have delivered ca 92 scientific papers during the contract period.
Status: Delivered

1.5 State-of-the-art report on the scientific field of WP1 at the end of the project. [48 months]
These were published in special issues of two leading journals in the field, guest-edited by the WP1 leader. Partners 1 – 6 contributed articles and reviews to these issues.

- "Artificial Photosynthesis and Solar Fuels", *Accounts of Chemical Research* (2009) issue 12 (December). *[Together with Chemical Reviews the leading review journal in chemistry]*
- "Biomimetic approaches to artificial photosynthesis" *Energy & Environmental Science* (2011) issue 7 (July). *[A field-leading journal with impact factor >9]*.

Status: Delivered.

Work Package 2 – The bio-molecular foundation for photochemical water-splitting and photobiological hydrogen formation (Report by Prof Bill Rutherford, Imperial College, London, UK; earlier with CEA Saclay, Partner 2, France.)

This WP was broken down into three tasks.

Task 2.1 Molecular enzymology of the water oxidising enzyme

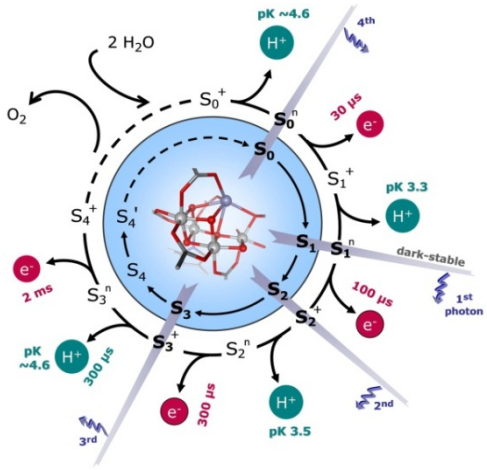
Task 2.2. Molecular enzymology of hydrogenases: understanding the structure and catalytic mechanism and transfer of knowledge to WP1 and WP3.

Task 2.3 Regulation of ET associated with H_2 production in cyanos and algae

Introduction

This work package covered the biophysical studies in SolarH_2. It had two main aims. i) the WP1/WP2 interface: the molecular enzymology of the water oxidising enzyme and hydrogenases as the inspiration for catalyst design for WP1 and ii) the WP2/WP3 interface: biophysical studies aimed at contributing to WP3: understanding features of photosynthesis and hydrogenase function in photosynthetic systems that are relevant to the production of hydrogen by photosynthetic microbes. Both have been very successful, but the WP1/WP2 part has been remarkable in terms of its output, its important results and the advances made. This is a reflects the original intention the two major tasks of the work package being on the WP1/WP2 interface and only one task was aimed at the WP2/WP3 interface. In addition the WP1/WP2 interface benefited from a) the close association between the WP1 and WP2 projects (most of the WP2 groups are also directly involved in WP1), b) several new collaborations inter- and intra- work package, and not least c) major advances occurring in field. Inevitably then the results from this part of the project dominate the landscape when looking back over the last 4 years. The fact that this work package produced ca 157 published articles means that this short report covering the whole grant period will ignore large numbers of excellent and important findings.

Task 2.1 Molecular enzymology of the water oxidising enzyme



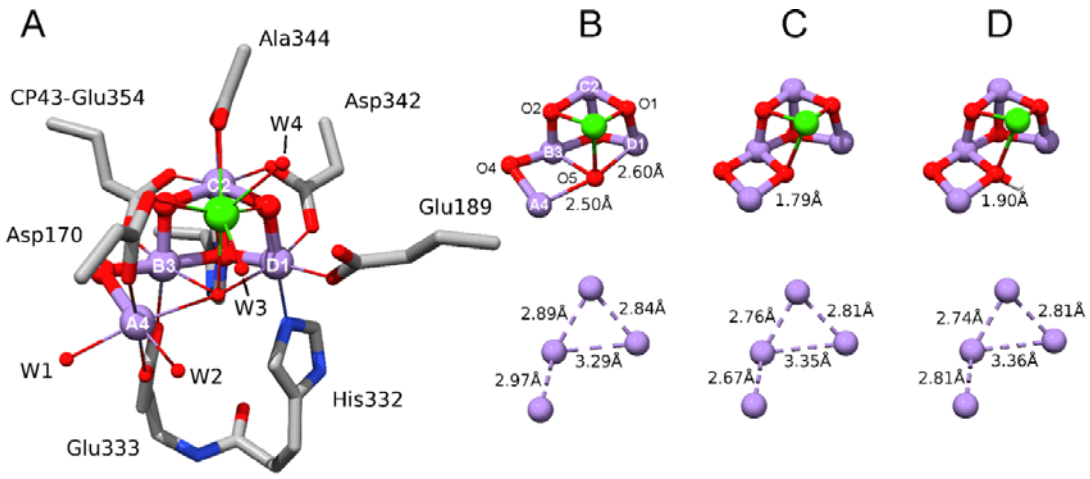
A significant body of work was done moving forward understanding of the enzyme already during the first half of the project. New concepts were developed clarifying aspects of the thermodynamics of the system (Dau and Haumann 2008, Krivanek et al. 2008; Klauss et al. 2009, Sjöholm et al. 2010). The importance of proton-coupled electron transfer became more evident, with important studies on pH, mutants, temperatures, intermediate states and cofactor exchange all pointing to the importance of hydrogen bonding networks. The

roles of the chloride and calcium ions were further elucidated (e.g. Ishida et al 2008, Cox et al. 2011), and the chloride ions were located (Murray et al 2008).

The S-state cycle of water oxidation showing charge accumulation and compensation in the four univalent oxidative steps required before water is oxidized releasing oxygen.

New states were discovered and new approaches applied using clever biochemical and spectroscopic innovations (Rappaport et al 2009). In terms of the deliverables detailed spectroscopic studies were done aimed at obtaining a good picture of the electronic structure of the MnCa complex (e.g. Pantazis et al 2009). A first step was to determine a good atomic structure and at that time crystallography had not provided a good enough structure and for the MnCa complex had not greatly advanced since 2004. Several geometries existed but ambiguity dominated. An in-depth EPR study from the SolarH2 team provided a preferred geometry: the geometry corresponded with that put forward by Siegbahn (2007) and the important feature was the presence of a single 5 coordinate MnIII in the S2 state (Cox et al 2011).

Before this model appeared in print, the subject unexpectedly leapt forward. In August 2010 a PSII crystal structure with true atomic resolution was announced. Importantly, this structure had very little beam damage and many resolved waters. The structure became available in spring 2011 and the spectroscopy and theory groups for Solar H_2 were extremely well placed to take advantage of the new playing field. The crystal structure had however undergone reduction by the beam and two of the teams in SolarH_2 optimised or “repaired” the cluster *in silico* (Ames et al. 2011, Grundmeier and Dau, 2012).



The final geometry fitted quite well with the preferred geometry from the earlier spectroscopy studies the team was able to move forward rapidly with further theoretical and spectroscopic studies to produce further insights and producing an attractive model for the electronic structure of S2 state. This in itself has repercussions for mechanistic models but to electronic structures for other S states will be needed in order to get a clear picture of the chemistry that occurs upon water oxidation. Throughout the project transfer of biological insights to the WP1 was continuous through excellent contacts within and between groups. Some team addressed the overlap directly in their publications (e.g. Dau et al. 2010). With the rapid changes in the field, increasingly relevant information was produced in the last phase of the

program. Insights from WP1 were also useful to the efforts to understand the molecular enzymology.

Task 2.2 Molecular enzymology of hydrogenases: understanding the structure and catalytic mechanism and transfer of knowledge to WP1 and WP3

One great success of the program was the over-expression of the algal FeFe Hydrogenase (see for example Camp et al 2008). This was the key to the subsequent biophysical studies on the enzyme which have gone on successfully through the course of this project. This enzyme and other bacterial FeFe Hydrogenases have been studied and compared and several advances were made in terms of studying assembly, function and inhibition (Czech et al 2011). Good progress has been made on obtaining the electron structure. Site-directed mutagenesis has been done to understand structure-function relationships and to engineer in changes for their use in artificial systems for light driven H_2 production (Lubner et al., 2010; Winkler et al., 2011; Lubner et al., 2011). The structure function studies show that the conserved amino acids surrounding the active site have a considerable influence on the catalytic cycle (Knörzer et al., 2012). Good progress has been made understanding the individual roles of specific amino acids. This is expected to be useful for the development of artificial systems.

The events occurring upon inhibition and destruction of the active site upon exposure to O_2 were studied in detail using x-ray absorption (Lambertz et al.2011). The model obtained is quite detailed, binding and reduction of O_2 at the FeFe site followed by dissociation of the $O_2^{\bullet-}$ or peroxide and its oxidative attack on the neighbouring FeS cluster. This could give insights on potential protective strategies.

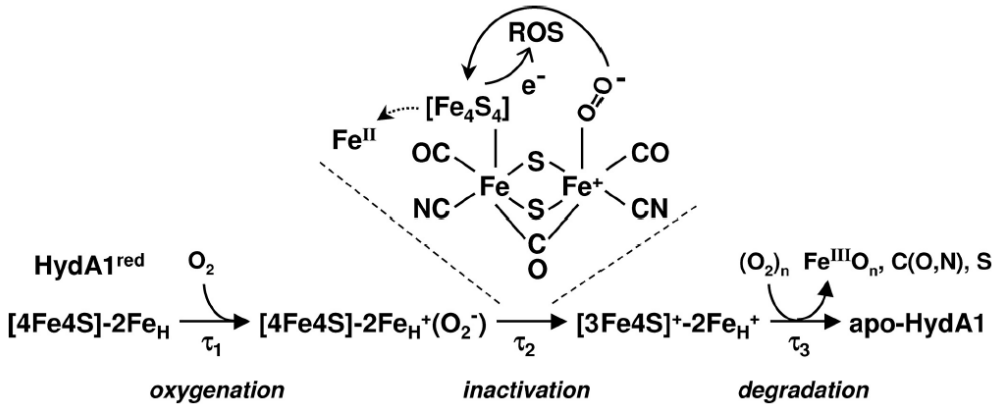


Fig.5 *Three kinetic phases of O_2-induced modifications at the H-cluster of HydA1. (Figure from Lambertz et al., J. Biol. Chem. 2011)*

The work on NiFe hydrogenases has powered ahead. With crystal structures providing some surprises (Ogata et al. 2010) and state-of-the-art spectroscopy providing detailed electronic structure for the *D.v.* Miyazaki F hydrogenase (Pandelia et al. 2010). A large body of work on these NiFe hydrogenases enzymes was accumulated providing insights on mechanistic issues.

Probably the most exciting results have been on the O_2 insensitive enzyme. A combination redox potentiometry, electrochemistry, spectroscopy all indicated exciting and unusual structure and mechanism in this enzyme (Goris et al 2011, Pandelia et al. 2011).

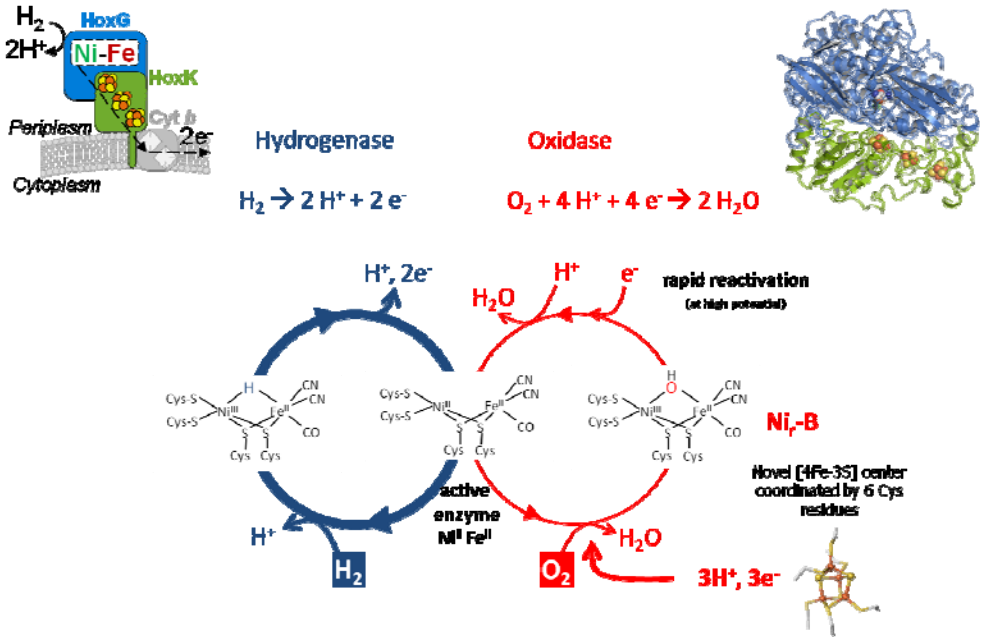


Fig. 7: A functional model of hydrogenase and oxidase activity in oxygen tolerant [NiFe]-hydrogenase showing competing catalytic cycles in an O_2-tolerant [NiFe]-hydrogenase.

Usual features were revealed by the crystal structures from the SolarH$_2$ team (Fritsch et al 2011) and simultaneously from Shomura et al (2011). This provided good chemical explanations for the insensitivity to O_2 a mechanism, fitting well with the mechanistic model derived from electrochemical studies. A structural change occurs upon oxidation by O_2, allowing it to provide 4 electrons to fully reduce the O_2 all the way to water without forming damaging reactive oxygen intermediates. This remarkable mechanism provides direct insights for designing O_2 insensitivity into hydrogenases and their models.

The new understanding of hydrogenases, their reactivity, sensitive and mechanisms for insensitivity (when present) provide useful insights for catalyst design. Particularly in the later phases of the project, the transfer of good chemical insights to WP1 has been evident.

Task 2.3 Regulation of ET associated with H_2 production in cyanos and algae

A good deal of work has been done in this general area. Some examples include: the effects of high light and UV light have been studied (Wu et al. 2011), the PSII repair cycle has received a good deal of attention (Cormann et al 2009ab. Grasse et al. 2011), the presence of PSII in heterocysts (Cardona et al 2009), alternative electron donors and acceptors have been proposed under different conditions and these too could be important to growth under stress conditions (e.g.Zhang et al., 2009). Electron transfer through the electron acceptors has received a good deal of study since this is often the rate limiting step and the acceptors are central to important regulatory mechanisms (Chernev, P et al 2011, Sedoud et al 2011).

One interesting aspect of research in this area that captured the attention of at least 5 of the partners in Solar H2 was the high light variants of the reaction centre protein D1 in cyanobacteria (e.g. Sugiura et al 2010a Boussac et al. 2011, Sander et al. 2010). The properties of the high light variant are likely to be advantageous for bioreactor growth.

References (non-SolarH2)

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Shomura Y; Yoon K-S; Nishihara H; et al. (2011) Nature 479, 7372 2011 Structural basis for a [4Fe-3S] cluster in the oxygen-tolerant membrane-bound [NiFe]-hydrogenase
Umena, Y., Kawakami, K., Shen, J.-R., Kamiya, N. (2011) Crystal structure of oxygen-evolving Photosystem II at a resolution of 1.9Å. **Nature** 473: 55-60

Work Package 3 – The genetic and metabolic foundation for "improvement" of microorganisms for photobiological hydrogen production (Report by Thomas Happe, Ruhr Universität Bochum, Partner 7, Germany)

The main objective of WP3 was to analyse, understand and engineer proteins and pathways of hydrogen metabolism in their natural context to generate efficient in vivo systems for applied hydrogen production. In detail, the goal was to gather detailed knowledge about the physiology of H_2 production in order to be able to engineer photosynthetic organisms with improved solar powered H_2 production capacity. The obtained knowledge could be used as connection between WP2 and WP4. Therefore the following individual goals were aspired for WP3:

1. Development and optimization of new overexpression systems to purify large amounts of hydrogenases, electron carriers (ferredoxins and flavodoxins) and photosystem I and II. This was necessary for the biophysical analysis in WP2.
2. Discovery of new insights into hydrogenase gene regulation and metabolic pathways involved or resulting in photobiological H_2 formation in known and newly discovered species of cyanobacteria and green algae. Selection of new organisms for efficient hydrogen production by screening natural and mutant strain collections and libraries.
3. Genetic engineering of already identified high hydrogen production strains to further improve sunlight to hydrogen conversion efficiencies. Most promising organisms were tested and employed in bioreactors in WP4.

All of the deliverables and milestones of WP3 had been fulfilled very successfully during the funding period (see periodic reports). Moreover, the collaborations between the different SolarH2 researches within or with other work packages were extremely positive and fruitful. The big advantage of this consortium was the fact that it has brought together researcher from different scientific areas like mathematics, chemists, biologists and engineers. Because of the intensive collaborations, the people from different European countries from Finland in the North to Spain in the South became friends over the years. The most important academic outputs of WP3 are reflected by the 115! articles (only 40 papers were promised in the original proposal) that were published in highly ranked scientific journals. Most of the publications were written together by two or more researchers of the consortium. The following short review which discusses the promised milestones of the application can only cover some of the experimental highlights (for more details see the periodic reports).

Milestone 3.1 *Novel hydrogenase expression systems for characterisation of different H_2-producing enzymes*

In order to elucidate the catalytic mechanisms of hydrogenases and photosystems as well as the reaction of active sites with inhibitors or destructive agents like oxygen in case of [FeFe]-

hydrogenases, large amounts of pure and fully active enzymes had to be provided to be analysed by biophysical techniques (**WP2**). In addition, the generation of protein variants with single or multiple amino acid exchanges allowed the characterisation of structure-function relationships. The establishment of powerful overexpression systems to produce both wild type and mutant enzyme forms was the first purpose of **Milestone 3.1**.
Heterologous systems for the overproduction of hydrogenase enzymes have been established. The system employing the bacterial hosts *Clostridium acetobutylicum* was optimised regarding yields. 3 mg hydrogenase with a specific activity close to the natively isolated enzyme could be purified from one litre of host cell culture. Since then, this system has been used to generate significant amounts of wild type HydA1 to be analysed by various biophysical techniques in order to gain insights into the active-site structure, the catalytic mechanism and the influence of gaseous inhibitors on the algal [FeFe]-hydrogenase. For example, the active site cluster (H-cluster) and the function of conserved amino acid side chains were analysed by applying various spectroscopic techniques (Knörzer et al., 2012). Additionally, the H-cluster destroying reactions triggered by molecular oxygen could be elucidated (Lambertz et al., 2011) and specifically altered hydrogenase variants could be employed in efficient artificial systems for light driven H_2 production (Lubner et al., 2011).

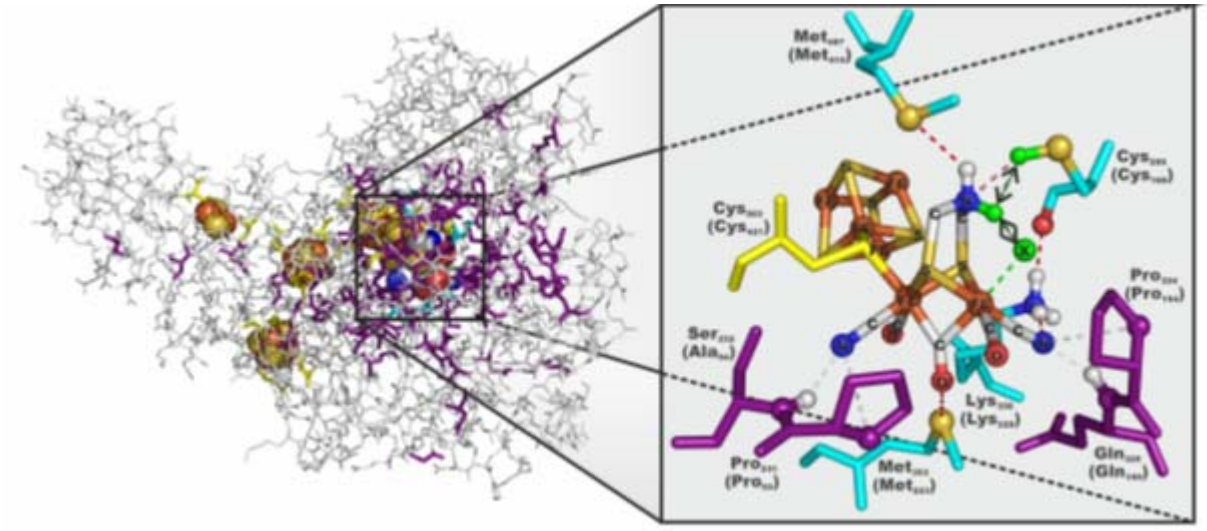

Figure 1: Interaction between the [2Fe] subcluster of [FeFe]-hydrogenases and surrounding amino acids in the [FeFe]-hydrogenase CpI of *Clostridium pasteurianium*.

Milestone 3.2 *- Stepwise increase of photon to H2 conversion efficiency in green algae by single targeted engineering attempts and by genetic crossing of newly developed phenotypes*

A significant improvement of H_2-evolution by *Chlamydomonas* was the isolation of the *Chlamydomonas* mutant *pgrl1* (Tolleter et al., 2011) affected in cyclic electron flow (CEF). This mutant was discovered using a genetic approach within the framework of the Solar-H2 program which was developed to identify new regulatory mechanisms of photosynthesis and explore novel strategies to improve H_2 production. The screen of 15,000 mutants led to the isolation of a dozen of mutants differentially affected in chlorophyll fluorescence transients. One of these mutants harbored a unique insertion of the paromomycin resistance cassette in the *PGRL1* gene. The *pgrl1* mutant was found to produce about 3 - 5 times more H_2 than the

wild type strain either during short-term or long-term experiments realised in conditions of sulfur deficiency. Based on extensive physiological characterisations which were carried out on the *pgrl1* mutant using a wide range of techniques (chlorophyll fluorescence, flash-induced absorption changes, mass spectrometry…), it was concluded that the *pgrl1* mutant is affected in the activity of CEF around PSI. Using an uncoupling agent, it was demonstrated that the proton gradient generated by CEF provokes a strong inhibition of electron supply to the hydrogenase in the wild-type strain which is released in the *pgrl1* mutant. The control of the trans-thylakoidal proton gradient by PGRL1 expression or activity opens promising perspectives in the reprogramming of the bioenergetic metabolism of microalgae towards improved hydrogen photo-production.

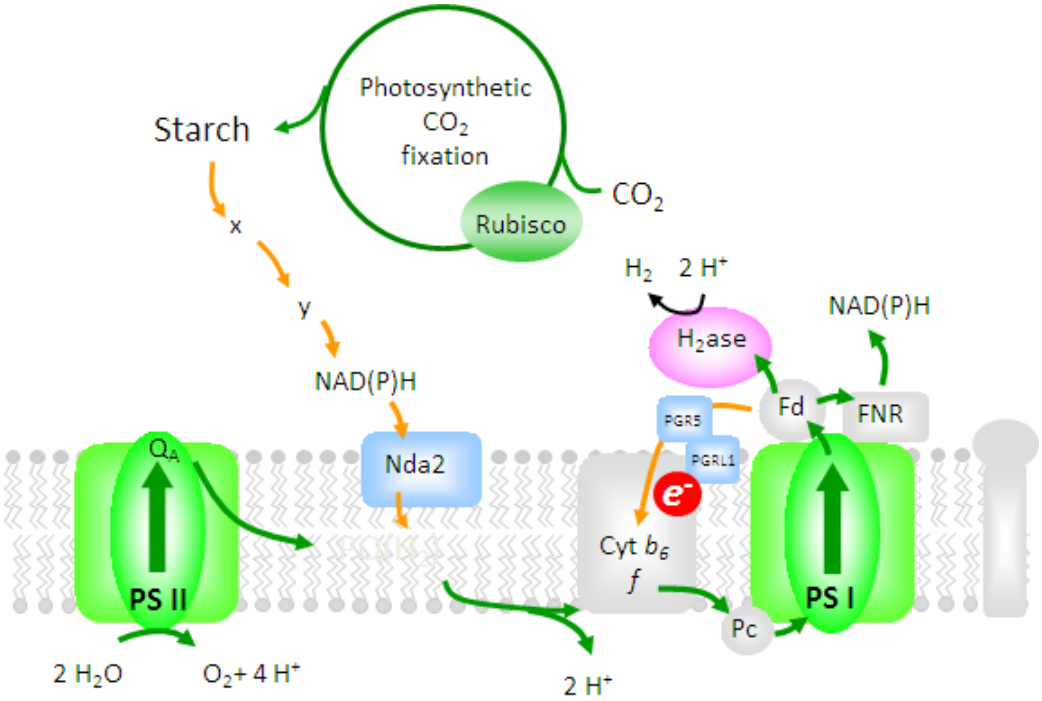

Figure 2: Model of photosynthetic and auxiliary routes of electron transfer reactions in chloroplasts of eukaryotic algae. The site of PGRL1 (and PGR5) action during cyclic electron transport is indicated by the red circle.

Milestone 3.3 *Understanding of the transcriptional regulation of native cyanobacterial hydrogenases.*

In close connection to the cyanobacterial bidirectional hydrogenase, the main H_2-evolving enzyme in non-nitrogen fixing strains, two novel transcription factors have received increasing attention over the past five years: a LexA-related protein and the AbrB-like family members. Recent work on these regulators has produced new insights and advances towards the understanding (and possible interconnection) of several regulatory networks in cyanobacteria, namely nitrogen metabolism, redox response, toxin production, CO_2 concentrating mechanisms and H_2 metabolism. The fact that a LexA-related protein and AbrB-like family members have been co-purified in independent laboratories studying different sets of cyanobacterial genes suggests a possible common and/or complementary function of these regulators.

Detailed studies were performed on the regulation of hydrogenase gene expression in cyanobacteria and have demonstrated that the DNA binding transcription factor AbrB (=CalA) may regulate H_2 production. The filamentous, heterocystous, nitrogen-fixing cyanobacterium *Nostoc* sp. strain PCC 7120 may contain, depending on growth conditions, up to two hydrogenases directly involved in H_2 metabolism.
In the framework of WP3, one partner is engaged in analysing the regulation and maturation of the oxygen tolerant [NiFe]-hydrogenases of *Ralstonia eutropha* as well as in constructing and characterising genetically engineered operons that encode O_2-tolerant [NiFe]-hydrogenases. Functional constructs could be introduced into cyanobacteria in order to connect oxygenic photosynthesis directly to H_2 production. Understanding the mechanism of O_2-tolerance of the *Ralstonia* [NiFe]-hydrogenases was of special interest for the final goals of SOLAR-H2, since this knowledge could also be used to engineer O_2-stable hydrogenases with a higher specific activity.

Milestone 3.4 *Novel cyanobacteria with increased level of hydrogenase gene expression, containing a gene encoding a foreign hydrogenase*

In order to design a cyanobacterium which produces high amounts of hydrogen using the electron transport pathways of oxygenic photosynthesis to deliver electrons to an oxygen-stable, highly efficient hydrogenase, it is necessary to **a)** understand and optimize electron transfer to the hydrogenase enzyme and **b)** to yield high and stable production of a heterologous hydrogenase.
Within the SOLAR-H2 framework, the possibility of higher hydrogen production has been explored by increased electron flow to the hydrogenase protein. The cyanobacterial nitrate assimilation pathway is a potential competing pathway which may reduce the electron flow to the hydrogenase and thereby limit hydrogen production. To improve H_2 production, the nitrate assimilation pathway in *Synechocystis* PCC 6803 was disrupted. Engineered strains disrupted in either nitrate reductase (Δ*narB*) or nitrite reductase (Δ*nirA*) or both nitrate reductase and nitrite reductase (Δ*narB:*Δ*nirA*) were constructed and tested for their ability to produce hydrogen (Baebprasert et al., 2012). H_2 production and Hox-hydrogenase activities in all mutant strains were higher than those in wild-type. The results suggested that the high rate of H_2 production observed in the Δ*narB:*Δ*nirA* strain of *Synechocystis* PCC 6803 is the result of redirecting the electron supply from the nitrate assimilation pathway, through genetic engineering, towards the bidirectional Hox-hydrogenase.
A mutant of *Synechocystis* PCC 6803 was analysed having a C-terminal truncation of the Epsilon-subunit of its ATPase. This mutant strain showed a partial uncoupling of proton and electron transport. The lowered efficiency of the ATPase is compensated by increased PS2 activity leading to a two-fold higher linear electron transport rate. The growth of this organism does not differ significantly from the growth of the wild type under normal light/dark conditions. Therefore, this strain is a promising candidate for achieving a considerably higher hydrogen production rate by transferring electrons from water-oxidation at PS2 to an oxygen-tolerant hydrogenase.

For stable hydrogen production rates continuous cultivation conditions using chemostatic or turbidostatic process control are superior to batch conditions in respect to both space-time-yield and cost efficiency. Based on a 5 L flat-panel photobioreactor a turbidostat process was realised using a computer-controlled feedback loop between media pumps and online measurements of cell density. Stable growth and production conditions enabled continuous cultivation of the *Synechocystis* mutants over periods of more than 9 months without significant drops in culture quality.

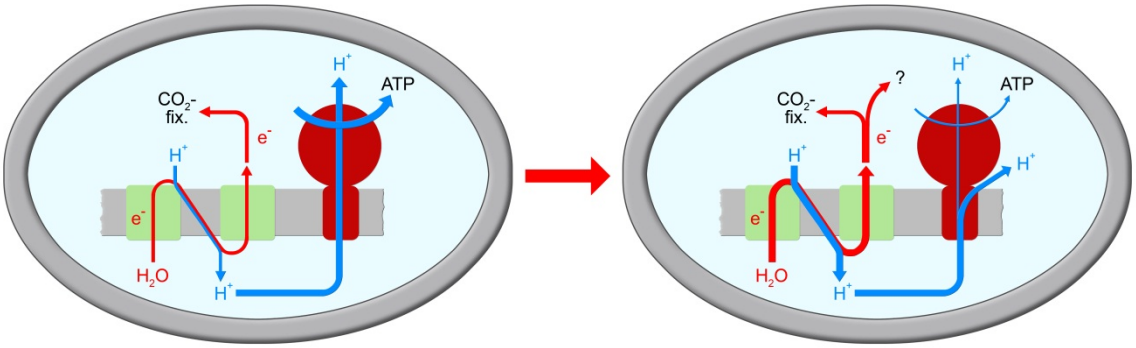

Figure 3: Scheme of linear photosynthetic electron transport in the *Synechocystis* PCC 6803 wild type (left) and in the mutant having a C-terminal truncation of the Epsilon-subunit of the ATPase resulting in partial uncoupling (right).

Conclusion

The findings of WP3 and the whole SolarH2 consortium have contributed significantly to a better understanding of H_2-production in algae and cyanobacteria and the enzymes which are necessary for this metabolism (hydrogenases, photosystems, electron carriers). Many of the findings are promising to contribute to targeted engineering of organisms and enzymes in order to achieve higher and industrially relevant H_2-yields either from living cells or isolated biocatalysts. In some cases, a significant enhancement of H_2-evolution capacities was already achieved. These milestones for a future H_2-economy could only be achieved in the network which was made possible by funding the SolarH2 consortium. The scientific findings and also the cooperations which were born during the SolarH and SolarH2 period have contributed significantly to the field of biological H_2-production and will continue doing so.

WP4: Characterization, modelling and optimization of photobioreactor system for biohydrogen gas production by photosynthetic microorganisms . (Report by Prof Jack Legrand, CNRS Nantes, France)

Significant scientific breakthroughs are required in several domains to envisage in the long term the use of photosynthetic microorganisms to produce H_2 efficiently. Those breakthroughs range from the physiological aspects (especially in the understanding of the complex bioenergetic and metabolic pathways involved during the H_2 release), to the effects of the culture conditions (like the light supply, anoxia conditions…). The tight coupling between all influencing conditions and the resulting dynamic behavior of the culture makes essential to conduct investigations in a fully-controlled environment, adapted for dynamic supervision of the process. The strategy of the project was stressed upon the synergy between (i) the fundamental research on the hydrogen metabolisms and their regulations in green microalgae *Chlamydomonas reinhardtii*, (ii) the development of a production system with an original and fully characterized geometry especially adapted for photosynthetic microorganisms cultivation, and (iii) the merger of advanced control algorithms, based on knowledge-based modeling of the photosynthetic microbiological cultures in photobioreactors. WP4 was organized in 4 tasks.

Task 4.1 Development of a lab-scale photobioreactor system (6 months) and experimental investigations of H_2 production under fully controlled conditions (48 months) (Partner 12, scientists involved in WP3)

An experimental set-up dedicated to investigate H_2 production under fully controlled and monitored conditions has been developed. This set-up combines a photobioreactor (1.5 l, illuminated surface to volume ratio is equal to 25 m^{-1}) based on a torus geometry that allows to obtain a high control of culture conditions (including light and mixing) with an online analysis of gas produced or injected in the reactor using mass flow rate controllers. The radiation-field inside the cultivation can be easily controlled, that is essential especially if a modelling approach has to be conducted for further analysis (as done in Task 4.2 and 4.3).

The combined effect of light and acetate (a usual compound of H2-producing protocols) on the set-up of anoxic conditions was investigated. A carbon mass balance confirmed the important CO_2 release when using acetate in the medium (Degrenne *et al*., 2010). An alternate protocol was then developed to produce hydrogen in autotrophic conditions and without affecting photosynthetic capacities (as with sulphur deprivation). This was obtained from the statement of the important role of the illuminated and dark zones on PBR running. This was here investigated in case of hydrogen production for setting anoxic conditions in combination or not with acetate consumption.

Photobioreactor biomass productivity for eukaryotic cells as *C.reinhardtii* revealed highly linked to the illuminated fraction (named γ) that represents the repartition in PBR volume between light and dark zones. Partner 12 demonstrated that even if all the light should be absorbed in the culture volume to obtain a full conversion of incoming light, requesting thus a sufficiently high biomass concentration, a dark zone in the PBR revealed to have a negative effect on PBR productivity due to mitochondrial respiration (Takache *et al.*, 2010). Biomass and oxygen productions being directly linked in photosynthetic growth, this conclusion was extended to the case of anoxia establishment in illuminated culture and strictly autotrophic conditions. The aim was to achieve anoxic conditions under light by only changing the photons flux density, as a simple operating parameter directly modifying light radiative transfer conditions in the culture volume. As a result, photosynthetic capacity of the cells is hoped to be not be affected like that is the case for example of sulphur deprivation. This new protocol revealed efficient, with a sustainable hydrogen production during several days, here in fully-autotrophic conditions and without any mineral limitation. This protocol of high interest for photobioreactor application will be further investigated after Solar-H2 program (Degrenne *et al.*, 2011a).

Whatever the protocol of hydrogen production with algae, a strong coupling is emphasized between environmental conditions (PFD, sulfur concentrations) and biological responses (photosynthetic activity, oxygen and hydrogen production). Modeling interactions between physical and biological phenomena appears thus interesting to set-up and optimize hydrogen producing strategies at PBR level. With this regards, a kinetic modeling on the induction of anaerobic conditions has been proposed by Fouchard *et al*., 2009. The resulting model described the kinetics of extra- and intracellular sulfur, total biomass and intracellular starch concentrations as a function of environmental conditions. It was shown the model to be able to describe different phenomena induced by sulfur-deprivation and leading to H_2 producing conditions. The modeling approach has allowed determining operating conditions allowing producing hydrogen gas in a PBR with green microalgae by using the sulfur deprivation protocol. The mechanistic model proposed (Fouchard *et al*., 2009) was validated with autotrophic production. Despite the systematic optimization applied, hydrogen production when expressed per gram of biomass (specific hydrogen production) was found closely constant, whatever the conditions tested (Degrenne *et al.*, 2011b). It indicates certainly a biological limitation in the H_2 producing metabolism. It encourages thus to pursue the

research effort on the optimization of strains and bioenergetics pathways. Productivities and yield are indeed still low. It was shown for example that only 0.2% of the total energy used (PFD and biomass degradation) was recovered in hydrogen production phase. Assuming that one gram of dry biomass contains a calorific capacity of about 30 KJ per gram, only 9% of the energy contained in the biomass is thus transformed in hydrogen.

Task 4.2 Development of models H_2 production (48 months) (Partner 12)

The objective was to design a multivariable control law based on the feedback linearizing theory using a continuous-time multi-state model. The biomass concentration and the hydrogen ions concentration – hence the pH – are the online measurable output variables which were selected to be controlled. The biomass concentration was controlled hydraulically through the dilution rate D whereas the pH was regulated chemically by means of the CO_2 gas whose dissolution modifies rapidly the ratio between the dissolved CO_2 and the bicarbonate ions which is a function of pH. The proposed control algorithm was proved to globally stabilize the photosynthetic growth process of *Chlamydomonas reinhardtii* in the torus photobioreactor developed within the Task 4.1.

The technical literature lacks of advanced control algorithms implemented on luminostat systems, but the necessity of such formulations is obvious (Takache *et al.*, 2010). An efficient method to control the incident light is to use a light uptake ratio (L_u) which represents the amount of light per biomass unit: $L_u=q_0/X$. Unlike the turbidostat, this type of control is suitable for batch cultures, but it can be also integrated in multivariable controllers. The multivariable nonlinear algorithm designed for the photosynthetic growth was found to give an excellent response stabilizing the closed loop. The expression of the pH control variable showed clearly that a proper growth model is fundamental in the control of photobioreactors. The present algorithm can be applied to other types of reactors and photosynthetic organisms by adapting the light transfer model to their geometry and by re-identifying the parameters which depend strictly on the shape of the reactor, optical properties and specific growth rate of the organism under study.

Task 4.3 Development of models for light regime optimization in photobioreactors (48 months) (Partners 11 & 12)

4.3.1 Modelling of the dynamic light regime and the biological response in a photobioreactor (Partner 11)

The specific growth rate and biomass yield on light energy of *Chlamydomonas reinhardtii* has been evaluated for L/D dark cycles of various frequencies (high frequencies in Partner 11 and low frequencies in Partner 12). The goal of these measurements was to evaluate the influence of rapidly fluctuating light conditions in photobioreactors. Such light fluctuations are caused by rapid mixing of microalgae cultures in combination with full light attenuation along the (short) optical path. In the ideal scenario microalgae would 'integrate' the light during such a cycle. In case full light integration is reached, the algae will use light flashes of over-saturating nature with the same high efficiency as low light continuous illumination. From the experiments we could conclude that a short flash time (≈1 ms) and high flash frequency (100 Hz or more) are necessary to achieve full light integration. Longer flash times and lower frequencies resulted in a lower degree of light integration and at frequencies approaching 1 Hz the microalgae appeared to respond to the instantaneous light intensities during the flash resulting in a low photosynthetic efficiency in case of over-saturating flashes.

The ultimate goal of this study is to develop a model which can predict algal growth (and biomass yield on light energy) in fluctuating light regimes. In order to establish the model, partner 11 developed a biological oxygen monitor (BOM) in which the effect of

different L/D ratios and light intensities on photosynthetic oxygen evolution was studied in a short time frame. Different datasets have been obtained for *Chlamydomonas*. A simple model is being developed based on these experimental data. The model is based on a flexible storage pool of energy generated during photosynthesis. Such a capacitor effect should allow photosynthesis to run at higher rates during light flashes in a dynamic light regime until the storage pool is completely filled. In addition, we are including a dissipation term for the light absorbed which depends on the size and degree of reduction of this hypothetical storage pool.

4.3.2 Hydrodynamic and 'radiative' modeling of torus photobioreactor (48 months) (Partner 12)

Optimization of light use by modification of hydrodynamic conditions requires the formulation of two key-problems: knowledge of light history experienced by flowing cells, and the prediction of photosynthetic response to this history. If both are known, a modeling approach can be conducted to propose an innovative tool for hydrodynamic optimization of light use in PBRs. Modeling in a predictive way the photosynthetic response in dynamic light regime seems today unrealistic, the global response being the result of numerous possible interacting intracellular reactions, with various timescales, some of them being certainly unknown. On the contrary, the coupling between light transfer and cells displacement is predictable, each one being fully determined physically by radiative transfer theory and fluid dynamics.

One limitation of assimilating a biological response to a simple chemical reaction is in its dependence on the physiological state, which is often the result of the cell life. In that case, the biological phase can be considered as composed of single elements, each having its own history. This is obtained using a Lagrangian approach. Trajectories of cells being known, their history with respect to the nutrient concentration can then be determined. Obviously, because the abiotic phase is not history dependent, an Eulerian approach can still be applied to the nutrient transport modeling. It was demonstrated (Pruvost *et al*., 2008) that such a simple coupling cannot be made. Considering radiative transfer to be independent of mixing conditions will generate a methodic error in the Lagrangian formulation which introduces a non-existing influence of flow on the use of light. Calculation method of the radiative transfer will have to be modified to consider the effect of a non-ideal mixing. To avoid simulating a great amount of cells to represent their effects on light attenuation in the culture, an original method was presented, in accordance with the objective of a limited computation time to keep a formulation tractable for PBR modeling. Flow effect was introduced in the radiative two-flux model, which proved to be efficient for PBR application, using a parameter representative of the heterogeneous residence time spent by flowing cells along the depth of culture. The proposed formulation was demonstrated to be coherent with an energetic analysis of the PBR on both the photonic and material phases. The validated Lagrangian formulation was next applied for a theoretical investigation of a possible effect of flow on the use of light in PBRs. Lagrangian formulation was associated to a simple kinetics model of photosynthetic growth of *Chlamydomonas reinhardtii*. This allows simulating batch culture under non-ideal mixing conditions.

4.3.3 Validation of the predictive value of a combined hydrodynamic, 'radiative' and kinetic model (48 months) (Partners 11 & 12)

The studies concern the investigation of parameters governing photobioreactors productivities. Because of the need of answering various constraints (such as optimizing the light supply or mixing conditions), photobioreactors are indeed of very different geometries. Results obtained in different systems are thus often difficult to compare. In this context, partner 12 has investigated *Chlamydomonas reinhardtii* growth in two different PBRs having

the same specific illuminated area, but with different conception (geometry and volume), corresponding to the two types of PBR most usually encountered, namely cylindrical and flat panel PBR (the torus photobioreactor falling in this category). Same maximal volumetric biomass productivities were achieved for a given incident PFD, confirming thus the essential role of the specific illuminated area. Nevertheless, the obtained results for the microalgae *C. reinhardtii* also demonstrated that for eukaryotic microorganisms having short-time respiration in the dark, it was necessary to accurately control the field of radiation in the PBR in order to reach the maximum biomass productivity. This was perfectly represented using a dimensionless parameter, the illuminated working fraction γ, this parameter representing the photobioreactor partitioning in dark and illuminated zone due to rapid light attenuation in the photobioreactor. For value remaining in the range $\gamma = 1 \pm 15\%$, same maximal productivities were achieved in both photobioreactors although very different in concept (Takache *et al*., 2010). As a result, this contributes to identify the main engineering factors governing light-limited photobioreactors functioning, that consist mainly in three basic parameters which are the specific illuminated area, the illuminated working volume fraction in the photobioreactor γ, and the mean value of the incident hemispherical PFD.

Task 4.4 Metabolic Flux Analysis (48 months) (Partners 2, 7, 12)

A constraint-based modeling approach was developed to investigate the metabolic response of the eukaryotic microalgae *Chlamydomonas reinhardtii* under photoautotrophic conditions. The model explicitly includes thermodynamic and energetic constraints on the functioning metabolism. A mixed integer linear programming method was used to determine the optimal flux distributions with regard to this set of constraints (Cogne *et al.*, 2011). It enabled us in particular to highlight the existence of a light-driven respiration depending on the incident photon flux density in photobioreactors functioning in physical light limitation. Because of low reliability of the tools for predicting the subcellular localization of enzymes in *Chlamydomonas*, cell compartment was not taken into account in the reconstruction process except a thylakoid compartment for proton exchange along the photosynthetic electron transport chain. Composition of each of the biomass assembled building blocks (PPC, proteins, carbohydrates, lipids, nucleic acids) is assumed to be constant over the range of tested operating conditions, so that stoichiometry of the main assembly reactions is not affected except the biomass formation equation. The metabolic network was built up with 280 reactions, of which 10 are transport reactions, and 278 metabolites. Finally, the system was found to exhibit 12 degrees of freedom.

Interesting information can be collected through the observations of the energetic pathways. First, it is observed that respiratory flux is very low compared to the photosynthetic flux. The proposed method was shown to give useful information in the way that respiratory pathway and photosynthesis interact in microalgae with changes in the incident photon flux density under maximal growth rate conditions in light limitation. It was clearly shown that respiratory activity can be considered as part of the photosynthetic process, because it is needed to regulate the redox state of the cells during photosynthesis, and to maintain the ATP supply. Changes in the $P/2e^-$ ratio through photosynthetic electron transport pathways appear thereby to be one driving parameter underpinning adaptation of microalgae to modifications in their environment when changing light influx.

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Highlight clearly significant results

- The illuminated fraction (the repartition in culture volume between light and dark zones) plays an important role in the achievement of maximal biomass production in photobioreactors and also in the establishment of anoxia under light-limited conditions.
- A new hydrogen producing protocol was developed, with the achievement of anoxic conditions under light by only changing the PFD, as a simple operating parameter directly modifying light radiative transfer conditions in the culture volume. As a result, photosynthetic capacity of the cells is not affected like that is the case for example of sulphur deprivation. A sustainable hydrogen production was obtained during several days.
- Two controllers have been established for culture cultivation in turbidostat mode and in luminostat (all the photobioreactor is illuminated with a total light absorption) mode.
- Even at the lowest flash frequency tested (5 Hz), the biomass yield on light energy was increased in comparison to continuous illumination with over-saturating light (equal to flash intensity).
- Flow effect was introduced in the radiative two-flux model, which proved to be efficient for PBR application, using a parameter representative of the heterogeneous residence time spent by flowing cells along the depth of culture. The proposed formulation was demonstrated to be coherent with an energetic analysis of the PBR on both the photonic and material phases. The validated Lagrangian formulation was next applied for a theoretical investigation of a possible effect of flow on the use of light in PBRs. The combination of this model with a detailed modeling of the dynamic response of photosynthesis when submitted to flashing light remains to be conducted.
- The main engineering factors governing light-limited photobioreactors are the specific illuminated area, the illuminated working volume fraction in the photobioreactor, and the mean value of the incident hemispherical PFD.
- A constraint-based model for autotrophic growth of *C. reinhardtii* was developed, including thermodynamic and energetic constraints. The current model was shown to

capture the global interactions between photosynthesis, respiration at light and the central metabolism. However, hydrogenase activity still remains to be implemented.

Publications – Communications

Publications

- Cogne G., Rügen M, Bockmayr A, Titica M, Dussap C-G, Cornet J-F, Legrand J (2011). A model-based method for investigating bioenergetic processes in autotrophically growing eukaryotic microalgae: application to the green alga *Chlamydomonas reinhardtii*. Biotechnology Progress, 27:631-640.

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