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Metabolic engineering of cyanobacteria for the production of hydrogen from water

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Abstract

Requirements concerning the construction of a minimal photosynthetic design cell with direct coupling of water-splitting photosynthesis and H_2 production are discussed in the present paper. Starting from a cyanobacterial model cell, *Synechocystis* PCC 6803, potentials and possible limitations are outlined and realization strategies are presented. In extension, the limits of efficiency of all major biological components can be approached in a semi-artificial system consisting of two electrochemically coupled half-cells without the physiological constraints of a living cell.

Introduction

Life on Earth is mainly dependent on the process of photosynthesis that transforms light energy with high efficiency into chemical energy and also produces the oxygen that we breathe. However, although the primary reactions of this process occur with high efficiency (quantum efficiency of both photosystems under optimum conditions, nearly 100%; photosynthetic light transformation efficiency, ~40%), the solar conversion efficiencies for crop plants in terms of biomass that can be harvested typically do not exceed 1% [1], with a maximum of 5–7% for microalga in bubbled bioreactors having been estimated [2]. The major reason for this is the tremendous loss of energy between the primary 'light' reactions and the much slower 'dark' reactions, with differences in their kinetics of several orders of magnitude, combined with major losses as heat. In consequence, exploitation of photosynthesis as energetic basis for renewable energy production is only conceivable if such processes are as closely connected to the primary photosynthesis reactions as possible, i.e. by avoiding the dark reactions that produce biomass with major energy loss [3,4]. For a photosynthetic organism that has been optimized for using PS2 (Photosystem II) and PS1 (Photosystem I) to drive H_2 production, a theoretical solar-energy conversion efficiency of 10.5% has been determined, assuming, however, that all electrons and protons derived from water oxidation are used for H_2 formation [5].

The production of H_2 via photosynthesis is attractive for various reasons: the product can be produced by the direct coupling to PS1 via Fd (ferredoxin), i.e. with minimal intermediate steps, especially if hydrogenases of the Fe–Fe type are used. Also, H_2 as product is released from natural cells without requiring additional energy, it can be (technically) stored and its consumption does not involve CO_2. It should be considered, too, that, in contrast with, for instance, photovoltaics, a biofuel is produced directly by this process.

Problems which have to be overcome in order to produce biohydrogen with reasonable yields are the general O_2-sensitivity especially of [Fe–Fe]-hydrogenases on the one hand and their non-existence in cyanobacteria, which are the ideal 'design organisms' for this purpose owing to their simple organization and easy transformability, on the other hand.

Besides this potential practical application in the future, the combination of photosynthesis with biological H_2 production is also attractive from a basic research perspective as it challenges two principal questions, as follows.

(i) What is the maximum efficiency that can be practically achieved if photosynthetic primary processes are directly coupled to H_2 production? Combined with this, can model systems be used to estimate the maximum capacity of key components of the photosynthetic electron transfer chain even beyond the limitations of the physiological environment?

(ii) Up to what percentage can primary energy be used for alternative, i.e. 'engineered', processes without endangering the basic 'housekeeping' reactions of a photosynthetic cell, or, in other words, what is the basic requirement for survival of a photosynthetic cyanobacterial cell, if we regard the cell as a 'living enzyme' for a coupled photosynthesis-powered process?

'Biobattery' as model system for studying limitations of both photosystems and their energetic coupling to hydrogenases in a semi-artificial environment (i.e. free of physiological constraints)

Minimal systems are ideal for characterizing the central components of energy transduction and their limitations.

Key words: biofuel, biohydrogen, metabolic design, photobioreactor, photosynthetic electron transport, water splitting.

Abbreviations used: Fd, ferredoxin; FNR, ferredoxin:$NADP^+$-oxidoreductase; MV, Methyl Viologen; PQH_2, plastoquinol; PS, Photosystem; WT, wild-type.

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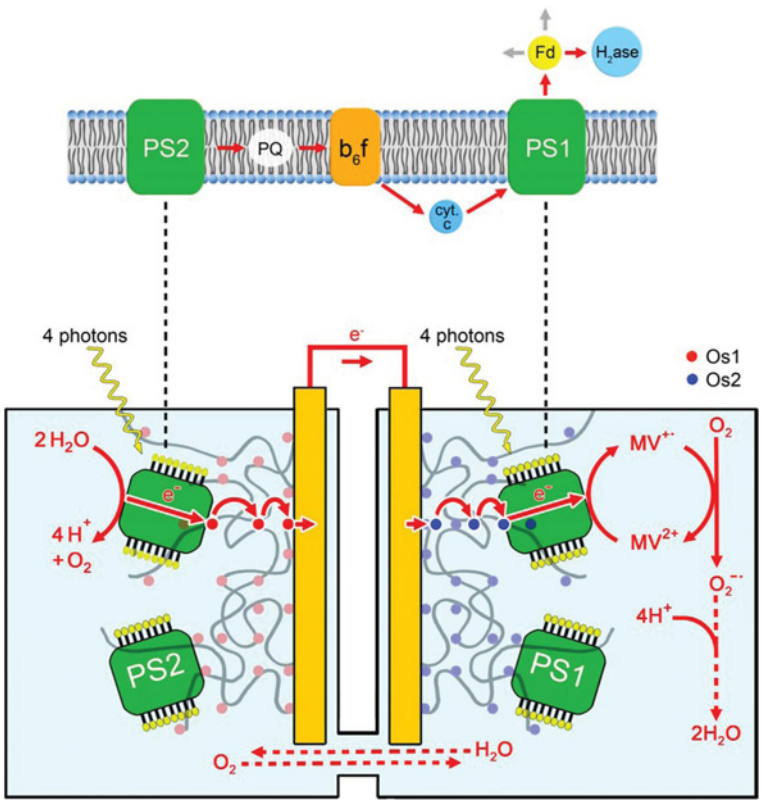
Figure 1 | Photosynthetic electron transport in the natural system, thylakoid membrane (upper part), and in a semi-artificial system (lower part) involving osmium-based redox polymers

The rate-limiting step of the natural system, reoxidation of PQH_2 at the cytochrome b_6f-complex ($t_{1/2} = 20$ ms), is bypassed in the semi-artificial system by two osmium polymers with appropriate redox potentials at the acceptor side of PS2 (Os1) and at the donor side of PS1 (Os2) respectively. Owing to this 'wiring' of the photosystems to the electrodes via the osmium polymers, these electron transfer reactions are no longer diffusion-limited. In the semi-artificial system realized to date, oxygen is the final electron acceptor from MV and will be replaced by hydrogenase in the final version. Thermostable PS1 and PS2 have been isolated from *Thermosynechococcus elongatus* as described in [25,26] respectively. See [6] for more details.



Concerning the two photosystems, the following limitations have to be overcome (Figure 1).

In natural cells, electron transport through both photosystems is limited by both linear and cyclic electron transport through a membrane which separates two reaction compartments with different biochemical environment. Down-regulation of this natural system (Figure 1, upper half) is dominated by the reoxidation of plastoquinol (PQH_2) at the cytochrome b_6f complex and other restrictions directly or indirectly combined with it (i.e. build-up of a pH gradient across the thylakoid membrane). Also, the capacity is limited by the rather slow CO_2-fixation reactions. Combined with this is the limitation of electron transport due to diffusion-controlled processes at the acceptor side of PS2, i.e. PQH_2 to cytochrome b_6f, and at both donor and acceptor sides of PS1, i.e. cytochrome *c* and Fd respectively.

The semi-artificial approach allows us to overcome these limitations by using redox hydrogels with appropriate redox potentials instead of diffusible electron carriers; in parallel, these hydrogels also function as stabilizing matrices for the immobilization of both isolated photosystems on gold electrodes [6]. By optimizing both photo-triggered reactions in separate half-cells, the photocurrent of immobilized PS2 dimers (i.e. the anodic side of the biobattery) could be increased up to 45 μA [7], whereas most recent studies with isolated PS1 trimers achieved more than 300 μA (T. Kothe, personal communication), i.e. one order of magnitude higher than recently published results [8]. The fact that the lifetime of the especially light-sensitive PS2 complexes could be increased by a factor of ~14 in comparison with PS2 immobilized as monolayer on gold electrodes [9] indicates that efficient electron removal at the acceptor side contributes considerably to PS2 stability and offers new possibilities to study the impact of oxygen-induced damage on the lifetime of this photosystem [7]. In comparison, osmium polymer usage at the PS1 half-cell (i.e. the cathodic side of the biobattery) leads to unrestricted electron transfer at the donor side and acceptor-side limitation due to accumulation of reduced MV (Methyl Viologen), which requires oxygen as final acceptor. Although this reaction has already been optimized, the future

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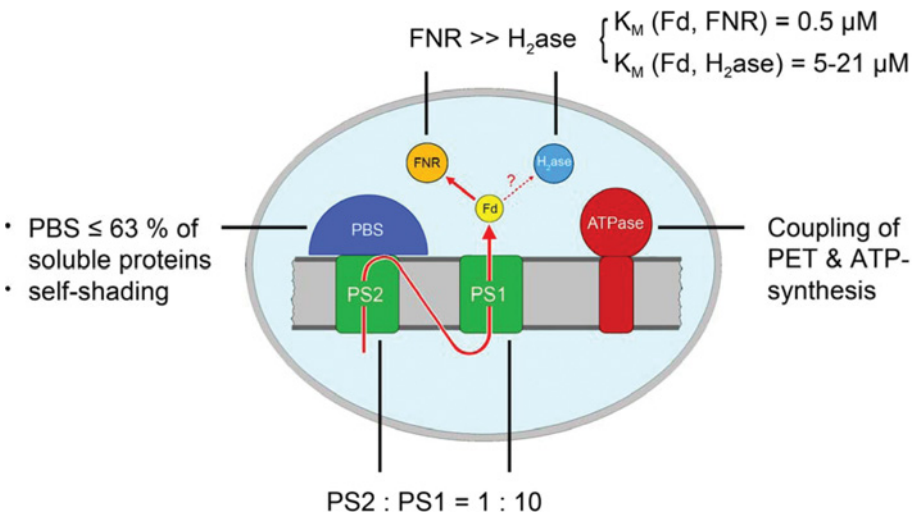
Figure 2 | Decisive parameters with impact on the construction of the future design cell for photosynthesis-based H_2 production
Data for PBS (phycobilisome) content [13] and the PS2/PS1 ratio (F. Mamedov, personal communication) have been determined for *Synechocystis* PCC 6803; K_m values of Fd for FNR (M. Winkler, personal communication) and for hydrogenase [27,28] was determined for *C. reinhardtii* (as a future source of hydrogenase). PET, photosynthetic electron transport.



coupling with the PS2 half-cell as electron donor for PS1 and an [Fe–Fe]-hydrogenase via MV as 'sink' at the PS1 acceptor side will show the efficiency of this approach.

Owing to the separation of anodic and cathodic half-cell reaction, available oxygen-sensitive [Fe–Fe]-hydrogenases can now be included into this set-up on the cathodic side, which can be kept anaerobic. This should enable the energetic balancing of the whole reaction from water-splitting up to H_2 production, including optimization of protein–protein interactions between PS1 and hydrogenase by testing various Fds. In summary, this 'biobattery' produces oxygen and H_2 similarly to the technical process of water electrolysis, but with biological catalysts that are powered by solar energy. This model system allows the *in vitro* optimization of engineered protein–protein interactions and also the evaluation of the maximum yield of such a system, which could be a benchmark for the construction of the living 'design cell' (see below).

Cyanobacterial 'design cell' as a model system for maximally exploiting photosynthetic energy (and minimal biomass production)

Owing to the limited lifetime of the semi-artificial system and its high costs, the construction of a living cell that directly couples photosynthetic water oxidation to a hydrogenase is mandatory for an efficient up-scaling of such a system. Such a cell should finally be regarded as a self-reproducing enzymatic system that can be kept under constant conditions for an unlimited time using continuous cultivation set-ups. The self-reproduction and self-repair competence is especially required for the PS2 complex, in which the D1-subunit has to be replaced every 20–30 min under full-light conditions. Cyanobacteria are good candidates for the construction of such a design cell as they have a short generation time, are easy to transform and are suitable for mass cultivation in fresh or sea water. Also, strains adapted to extreme environmental conditions ranging from psychrophilic to thermophilic are available. As *Synechocystis* PCC 6803 is one of the best characterized cyanobacteria with many mutants being available, we have chosen this strain as a starting organism for the construction of the design cell, i.e. as proof-of-principle. Figure 2 shows the most limiting parameters of this WT (wild-type) strain regarding an efficient future photosynthesis-based H_2 production.

(i) The amount of water-splitting PS2 per cell governs the amount of electrons available for (future) H_2 production. Unfortunately, with a PS2/PS1 ratio of 1:10, this is very unfavourable in WT cells of *Synechocystis* PCC 6803. Reduction of the phycobilisome antenna size results in an up to 4-fold increase in linear electron transport in the olive mutant (deletion of phycocyanin-binding subunits) and a 6-fold increase in the PAL mutant [10] with completely deleted PBS (phycobilisome) genes [11]. Such truncated phycobilisome antennae have additional beneficial effects for our purpose: they reduce the oversaturation by high sunlight intensities and also the self-shading effect of cells, i.e. they allow a more uniform illumination of the culture resulting in higher cell densities, combined with more electrons per volume for fuel production [12]. On the other hand, the cellular metabolism of the phycobilisome mutant cells saves considerable energy, as phycobilisomes constitute to 63% of all soluble proteins in *Synechocystis* WT cells [13]. Similarly, positive effects of antenna size reduction on solar-energy conversion efficiencies have been reported for green algae [14].

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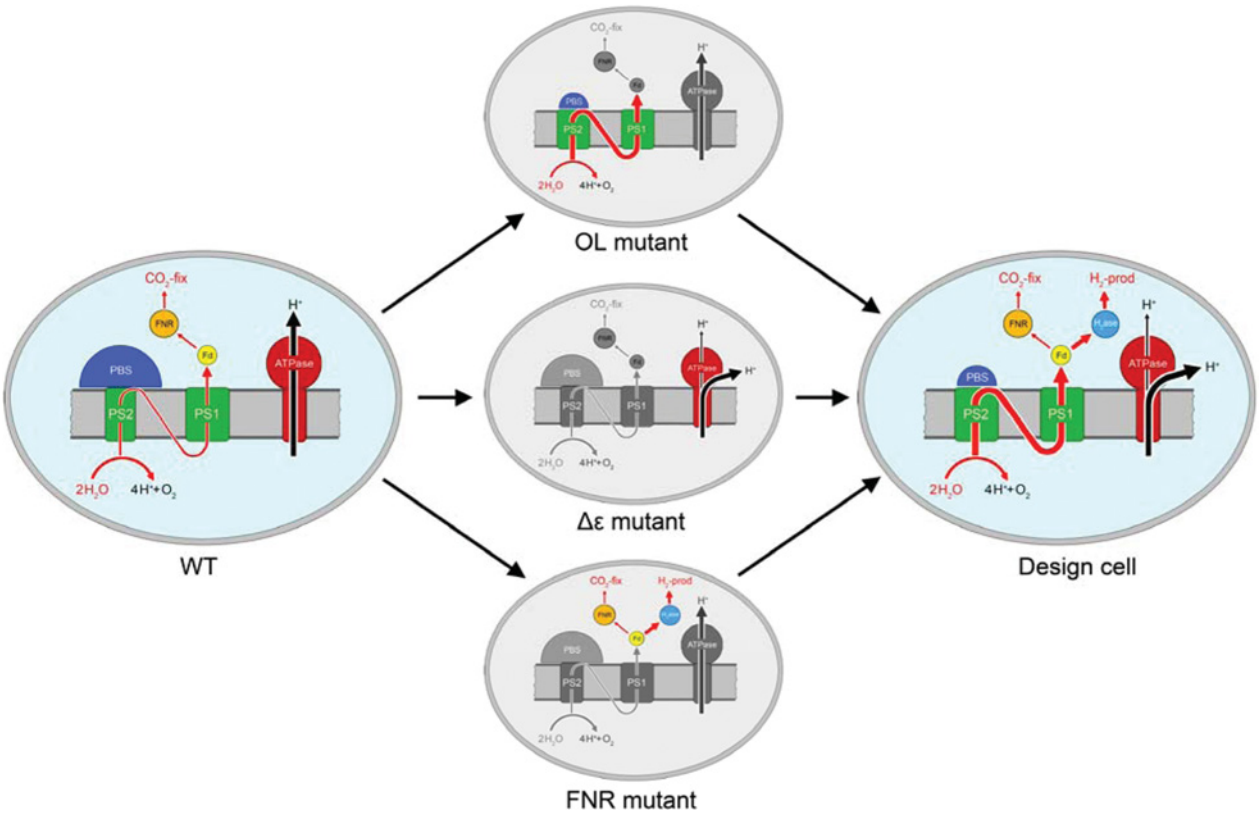
Figure 3 | **Strategy to construct a design cell based on mutants with engineered linear electron flow**

Starting from WT cells, the most appropriate directed mutant cells are selected and finally combined to the 'design cell'.



(ii) The supply of electrons from water splitting is further limited by the tight coupling of luminal proton efflux with ATP synthesis. By partial uncoupling, using an ATPase mutant with a truncated ε subunit, we could show that a distinctly lower ATP synthesis rate than WT during illumination impairs neither cell growth nor survival. The partial uncoupling resulted in a 2-fold higher linear electron transport and a drastically reduced ΔpH across the thylakoid membrane (<20% of WT), built up ~2.5-fold slower [15]. These results suggest survival of the cells under even lower ATP and NADPH levels, both of which are mainly used for CO_2 fixation via ribulose bisphosphate carboxylase. As cell growth and CO_2 fixation should be minimized anyway in our approach, the additional electrons from water splitting may also be used for biofuel production.

In conclusion, the energetic threshold of these cells may be oversized for standard conditions and could be used to a much larger extent for biofuel production at the expense of minimized biomass production.

(iii) The third decisive parameter is the partitioning of photosynthetic electrons between biomass and biohydrogen production (Figure 2). Although Fd is the switching point for many metabolic reactions at the acceptor side of PS1, in our scenario especially the electron channelling between FNR (ferredoxin:$NADP^+$-oxidoreductase) and (introduced) [Fe–Fe]-hydrogenase is decisive. Major problems are the much higher affinity of Fd for FNR than for the [Fe–Fe]-hydrogenase from *Chlamydomonas reinhardtii* (K_m differs by a factor of 10–40; Figure 2) and the unlikeliness of achieving cellular protein concentrations of this enzyme which are similar to FNR. Besides the construction of Fd–hydrogenase fusion proteins [16], one strategy to manipulate electron partitioning between both enzymes is to engineer the FNR-binding site for lower Fd affinity. Owing to detailed 3D structures being available for both Fd and various FNR mutants [17], a rational design is possible. Preliminary functional characterization of several mutants showed that K_d values could be shifted by a factor of more than 10 as determined with isolated FNR from WT and directed mutants (K. Wiegand and K. Cormann, personal communication). Consequences for electron routes under physiological conditions in the transformed design cells are now under investigation. The successful tuning of this metabolic switching point will be a decisive prerequisite for shifting the cell metabolism from biomass to biofuel production. It will also yield fundamental information on the unsolved question of to what extent 'housekeeping energy' can be minimized in a prokaryotic photosynthetic cell.

The individual design approaches via construction of directed mutants as shown above have been summarized in

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Figure 3. In the next steps, these individual mutants have to be combined for the future H_2-producing design cells. This will also yield valuable information on both the potential and limitations for the reorganization of cellular energy metabolism.

Outlook

The design cell approach has to be complemented by intensive efforts to construct and improve new low-cost photobioreactors as outlined in [18]. Of central importance is the realization of continuous culture conditions which is a prerequisite to optimize light and other environmental parameters for the engineered cells [19]. Such reproducible conditions have enabled the detailed characterization of the engineered design cells in comparison with WT under ambient and stress conditions by both proteomic and metabolomic approaches [12]. Similarly, the iterative modification of central metabolic steps has to be monitored with respect to energy distribution within the cell in order to identify new bottlenecks in the metabolic circuits. Last, but not least, this project may also contribute to the principal question which affects the light–dark cycle and the 'milking' of photosynthetic energy has on the biomass, and bioenergy balance of phototrophic cells, a new aspect that has partly been addressed in [20].

Problems to be solved which have not been addressed in detail in the present article concern engineering of [Fe–Fe]-hydrogenases for oxygen tolerance, their incorporation into cyanobacterial genomes and their maturation as eukaryotic enzymes in a prokaryotic cell [21,22]. Whereas the latter has already been shown to be possible, although still under anaerobic conditions [23], the engineering of oxygen tolerance is being addressed in various laboratories. Progress on this issue has a strong impact on the final success of this approach and on its feasibility as has been estimated by a life cycle analysis involving all present and future scenarios, especially the design cell and an upscaling of the developed flatbed photobioreactors [24].

In summary, these examples should illustrate that, from the point of view of photosynthetic biofuel production, many inefficient or limiting steps in the natural system are amenable to improvement by genetic engineering and certainly also by involving progressive techniques of synthetic biology and photobioreactor design.

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