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Rational redesign of the ferredoxin-NADP$^+$-oxido-reductase/ferredoxin-interaction for photosynthesis-dependent H_2-production

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ABSTRACT

Utilization of electrons from the photosynthetic water splitting reaction for the generation of biofuels, commodities as well as application in biotransformations requires a partial rerouting of the photosynthetic electron transport chain. Due to its rather negative redox potential and its bifurcational function, ferredoxin at the acceptor side of Photosystem 1 is one of the focal points for such an engineering. With hydrogen production as model system, we show here the impact and potential of redox partner design involving ferredoxin (Fd), ferredoxin-oxido-reductase (FNR) and [FeFe]-hydrogenase HydA1 on electron transport in a future cyanobacterial design cell of *Synechocystis* PCC 6803. X-ray-structure-based rational design and the allocation of specific interaction residues by NMR-analysis led to the construction of Fd- and FNR-mutants, which in appropriate combination enabled an about 18-fold enhanced electron flow from Fd to HydA1 (in competition with equimolar amounts of FNR) in *in vitro* assays. The negative impact of these mutations on the Fd-FNR electron transport which indirectly facilitates H_2 production (with a contribution of $\leq$42% by FNR variants and $\leq$23% by Fd-variants) and the direct positive impact on the Fd-HydA1 electron transport ($\leq$23% by Fd-mutants) provide an excellent basis for the construction of a hydrogen-producing design cell and the study of photosynthetic efficiency-optimization with cyanobacteria.

1. Introduction

Cyanobacteria harvest light energy with particular efficiency and transform it into chemical energy - a process which goes along with CO_2-fixation and oxygen release. Due to the many steps involved in this process, the final efficiency of photosynthesis - based on the output of sugar or related compounds - is very low, routinely below 1% [1]. It is therefore tempting to modify the natural cyanobacterial metabolism to shorten the process of energy transformation as much as possible in order to increase the efficiency and to capture compounds suitable for long-term energy storage directly from photosynthesis. Hydrogen (H_2) fulfills these requirements and is one of the possible photosynthetic products of both, cyanobacteria [2] and green algae [3,4]. Besides its high energy content and unmatched environmental sustainability, H_2 is easily harvested due to its release from the cells into the medium. The S-deprivation metabolism of green algae like *Chlamydomonas reinhardtii* provides an impressive natural example for an enduring and productive photohydrogen evolution activity based on the [FeFe]-hydrogenase HydA1; remarkably, this enzyme is directly coupled to the photosynthetic electron transport *via* the plant-type ferredoxin PetF [5,6]. Several trials to implement and optimize an [FeFe]-hydrogenase-based photohydrogen metabolism in cyanobacteria emulating the example of *C. reinhardtii* have been made [7,8]. Among them, the heterologous expression of a clostridial [FeFe]-hydrogenase in *Synechococcus elongatus* sp. 7942 and its coupling to Photosystem 1 (PS1) *via* Fd of the host was most successful [9]. While photohydrogen evolution clearly exceeded the H_2-production activity of the native [NiFe]-hydrogenase, it did not reach the level of anaerobic *C. reinhardtii* cultures. This is

Abbreviations: C. reinhardtii, Chlamydomonas reinhardtii; Fd, PetF, ferredoxin; FNR, ferredoxin-NADP$^+$-reductase; HydA1, [FeFe]-hydrogenase of *Chlamydomonas reinhardtii*; *S.* 6803, *Synechocystis* PCC 6803; PS1, Photosystem 1

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possibly due to insufficient enzyme maturation and the rather low compatibility of the heterologous clostridial HYDA to the electron mediator PetF (Fd) of the host cell. A major reason is the complex structure of clostridial [FeFe]-hydrogenase that requires the synthesis of four additional [FeS]-clusters besides the active center cofactor (H-cluster) and that also has not been evolved to interact with plant-type ferredoxins. Both problems could be solved by using an algal-type [FeFe]-hydrogenase such as HydA1 of *C. reinhardtii* that requires only H-cluster maturation and is directly connected to the photosynthetic electron transfer (PET) through PetF [10]. Photohydrogen production is also limited by redox enzymes competing with [FeFe]-hydrogenase for electrons from Fd – especially ferredoxin-NADP-oxidoreductase (FNR). In *C. reinhardtii* a knock-down of FNR expression caused a 2.5-fold increase of photohydrogen production under sulphur-deprivation [11] which is explained by the K_M of Fd for FNR being approximately by a factor of 10 lower than for HydA1 [5,6].

For this reason, manipulation of the electron flow at the acceptor side of PS1 that funnels about 90% of the reducing equivalents *via* Fd and FNR into CO_2 fixation is a decisive step for light driven hydrogen production in a cyanobacterial "design cell" [12]. It requires a more detailed understanding about the interactions between reduced ferredoxin and both competing redox partners, *i.e.* FNR and HydA1, on the molecular level, followed by their modifications: Specific weakening of the affinity between Fd and FNR will minimize the activity of the Calvin-Benson cycle and redirect electrons to HydA1. As the extend, up to which percentage electrons can be re-routed without serious effects on the whole cell survival, is unknown, various FNR- and Fd-mutants are necessary in order to be able to fine-tune this manipulation and achieve maximal rates of hydrogen production from a stable phototrophic culture. Although a similar strategy has already been applied for the respective homologs of *C. reinhardtii* [13], cyanobacterial systems are attractive as they are much easier to manipulate genetically and have a much simpler metabolism.

Generally, redox interactions between separate proteins are realized by transient, non-covalently bound complexes [14]. Batie and Kamin [15,16] reported the first FNR mediated electron transfer from Fd to $NADP^+$/NADPH, with Fd interacting in a 1:1 complex with FNR [17]. In this complex electron tunneling is ensured by the close proximity of their two prosthetic groups [18,19]. For the Fd/FNR complex of *Z. mays*, a distance of 6 Å has been determined between the [2Fe-2S] cluster of Fd and FAD of FNR [19]. Also, structural analysis reveals predominantly basic areas on the FNR- and acidic areas on the Fd-surface within their corresponding binding sites. This indicates that the transient interaction between Fd and FNR is mainly driven and guided by electrostatic forces [20]. The same principle applies for the complex formation between ferredoxin and HydA1, resulting in a large overlap of the Fd interaction surface areas for HydA1 and FNR [21]. This was also determined in a nuclear magnetic resonance (NMR) titration experiment with *C. reinhardtii* proteins comparing the effects of HydA1 and FNR titration on the ^1H-^{15}N-HSQC spectrum of ^{15}N-labeled PetF [13].

Synechocystis ferredoxin (Fed1, PetF) is an 11 kDa soluble, acidic protein with strongly negative redox potential (E_m: −412 mV; [22]) and contains a [2Fe-2S] cluster switching between oxidized Fe^{3+}-Fe^{3+} and reduced Fe^{3+}-Fe^{2+} state [23,24]. It is involved in both, cyclic and linear photosynthetic electron flow.

The current study evaluates the impact of single exchange variants of Fd and FNR from *Synechocystis* on their protein-protein interaction and on the partition of reduced Fd for FNR and HydA1, respectively. Positions for FNR-mutants have been selected on basis of the Fd/FNR complex structure from *Z. mays* [19] and analysis of their Fd-dependent enzyme kinetics. Also, five Fd variants have been generated based on a thorough NMR analysis of the Fd/FNR/HydA1 interaction profiles. All mutants have been evaluated in an *in vitro* competition assay between FNR and HydA1 which yields valuable information for design principles of protein-protein interaction and for the construction of a future hydrogen producing cyanobacterial design cell.

2. Material and methods

2.1. Design of expression constructs of ferredoxin

Plasmid constructs for the expression of *Synechocystis* ferredoxin-(*petF*)-variants in *Escherichia coli* were generated based on the vector pASK-IBA7 (IBA-GmbH in Göttingen, Germany). Primers AGTGTAGCT AGCTATACCGTTAAATTGATCACCCCCGATGG and ATGGTAGGTCTCA TATCAGTAAGGTCTTCTTCTTTGTGGG were used to amplify *petF* from genomic DNA isolated from the *Synechocystis* sp. PCC 6803 culture. The amplicon was cloned into pASK-IBA7 *via* restriction sites *Nhe*I and *Bsa*I thereby excising the DNA region which encodes the Strep-tag II peptide. The resulting construct pASK-petF was used as template for site-directed mutagenesis using Phusion High Fidelity DNA-Polymerase (Thermo Fisher Scientific) yielding *petF* variants D22A, D61A, D67A and E71A. Oligonucleotides used for each individual site directed mutagenesis are listed in Table S2.

2.2. Protein expression and purification

Due to the fact that no substantial catalytic differences were observed between the long and the short form of FNR – FNR_L and FNR_S, respectively (see Fig. S4), all experiments have been performed with FNR_S, which will be designated FNR hereafter (in case of FNR *wild type*: FNR-WT).

For the heterologous overexpression of FNR variants or FNR_L in *E. coli* strain BL21 (DE3) the His_6-tagged *petH* gene of *Synechocystis* sp. PCC 6803 (slr1643) was subcloned into the expression vector pASK-IBA37+ (IBA-Lifesciences, Goettingen, Germany) yielding plasmid IBA37-petH. The expression construct was also used as template for site directed mutagenesis using Phusion High Fidelity DNA-Polymerase (Thermo Fisher Scientific) yielding *petH* variants D71K, K75A/D and K78A/D. Primers for *petH* gene amplification and mutagenesis primers are listed in Table S2. The expression culture (8 × 500 ml) was inoculated with 10 ml of a 100 ml LB pre-culture grown over night from a single colony and cultivated under constant shaking (180 rpm) at 37 °C up to an OD_{578} of 0.6. FNR expression was induced during the following 6 h of continuous cultivation by the addition of Anhydrotetracycline (AHT) up to 0.2 µg/ml. Cells were harvested by centrifugation at 4 °C (20 min, 15,900 g), followed by snap freezing of the pellet with liquid nitrogen and storage at −20 °C until further use. *E. coli* cell disruption was achieved by passing the resuspended pellet at 4 °C 7-times through a French press (1000 psi) (SLM Amico French Pressure Cell Press). For protein purification an immobilized metal ion affinity chromatography (IMAC) with 5 ml HisTrap crude FF (GE Healthcare) using an ÄKTA purifier 100 (GE Healthcare) exploiting the presence of an N-terminal His_6-tag was combined with a hydrophobic interaction chromatography (HIC) (10 ml Toyopearl Butyl-650 M gravity flow column, Tosoh). FNR was obtained in 50 mM Tris/HCl pH 7.5, 100 mM NaCl buffer (for the activity assays) or in 50 mM potassium phosphate pH 6.8, 50 mM NaCl buffer (for NMR titration experiments) and stored at −80 °C.

E. coli BL21 (DE3) Δ*iscR* was used as host strain for the heterologous expression of both, [FeFe]-hydrogenase HydA1 and Fd variants.

Due to the deletion of the gene encoding *isc*-repressor ISCR this strain provides an elevated [FeS]-cluster biosynthesis backgound to ensure the cofactor coverage of overexpressed [FeS]-proteins [25]. For Fd-expression 2 liter-flasks containing 500 ml VB-medium [26] supplemented with 100 µg/ml ampicillin, 50 µg/ml kanamycin and 4% glucose were inoculated with 20 ml LB preculture (300 ml LB with 0.1% glucose including both antibiotics).

For the expression of ^{15}N-labeled Fd, $Na(NH_4)HPO_4$ of the VB-medium was substituted for Na_2HPO_4 and $^{15}NH_4Cl$ (1.787 g/l VB medium) was added after autoclaving. Also, the preculture was washed with ^{15}N-VB Medium (10 min, 3838 g, 12 °C) and further cultivated for

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