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Rapid Report

Wiring an [FeFe]-Hydrogenase with Photosystem I for Light-Induced Hydrogen Production[†]

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ABSTRACT: A molecular wire is used to connect two proteins through their physiologically relevant redox cofactors to facilitate direct electron transfer. Photosystem I (PS I) and an [FeFe]-hydrogenase (H_2ase) serve as the test bed for this new technology. By tethering a photosensitizer with a hydrogen-evolving catalyst, attached by Fe−S coordination bonds between the F_B iron−sulfur cluster of PS I and the distal iron−sulfur cluster of H_2ase, we assayed electron transfer between the two components via light-induced hydrogen generation. These hydrogen-producing nanoconstructs self-assemble when the PS I variant, the H_2ase variant, and the molecular wire are combined.

The exploitation of solar energy for the direct production of liquid and gaseous fuels represents a promising and clean approach to supplementing the world's energy requirements. Solar energy has always been involved in the synthesis of fuels, namely fossil fuels formed from decaying organic matter, by providing the energy necessary for photosynthetic organisms, and those that feed on them, to grow. In addition to fossil fuels, the biofuel ethanol can be produced from the degradation of the naturally occurring starch and cellulosic components in plants and algae. These processes require significant amounts of time and space before a useable fuel is generated, millions of years in the case of fossil fuels, and large amounts of land for the growth of starch and cellulose-producing crops. An alternative approach is to employ solar energy directly to generate a liquid or gaseous fuel, which can be captured and used in real time. Recently, this has been accomplished by using either whole organisms, such as cyanobacteria and algae, or purified photosynthetic components (*1*, *2*). When cyanobacteria or algae are used, the production of a fuel, generally either ethanol or hydrogen, is inextricably linked to complicated biochemical pathways within the cell. While much information about these processes has been gained, large increases in the efficiency of fuel production are still required. Because of this challenge, purified photosynthetic components are being considered for the direct generation of solar fuels (*3*−*6*).

The photosynthetic reaction centers present in oxygen-producing plants and cyanobacteria represent highly evolved systems for the conversion of solar energy to stored chemical energy. Oxygenic photosynthesis extracts electrons from water and transfers them to $NADP^+$, thereby carrying out the $2H_2O + 2NADP^+ + 4h\nu \rightarrow O_2 + 2H^+ + 2NADPH$ reaction. This otherwise thermodynamically unfavorable reaction is performed in two photochemical half-cells driven to completion by the energy of visible photons (*7*). Photosystem II (PS II) catalyzes the oxidation of water, while Photosystem I (PS I) and ferredoxin:$NADP^+$ oxidoreductase catalyze the reduction of $NADP^+$ through the intermediary carrier, ferredoxin. The $NADP^+$/NADPH half-cell couple has a standard biochemical midpoint potential of −324 mV, whereas the H^+/H_2 half-cell couple has a standard biochemical midpoint potential of −410 mV. Because the terminal Fe−S cluster in PS I, F_B, has a pH-independent standard midpoint potential of −580 mV, the reduction of $NADP^+$ to NADPH and the reduction of H^+ to H_2 by PS I are both thermodynamically favorable. Thus, it should be possible to modify PS I to generate H_2 from sunlight provided a suitable catalyst is employed.

The two approaches reported for direct solar H_2 production using PS I as the photosensitizer include the reductive deposition of noble metals (Pt and Au) on the PS I reaction center and the covalent attachment of Pt nanoparticles to PS I (*3*−*6*). These constructs, however, require relatively large amounts of rare and expensive noble metals. Only one system, in which the membrane-bound [NiFe]-H_2ase (MBH) from *Ralstonia eutropha* is fused to the PsaE subunit of PS I, currently utilizes a biological catalyst to generate light-induced H_2, albeit at a relatively modest rate of 0.58 μmol of H_2 (mg of Chl)$^{-1}$ h^{-1} (*8*). When the PS I−MBH fusion protein is bound to an electrode, the rate of light-induced H_2 production becomes orders of magnitude higher (*9*); however, the activity declines rapidly as a function of time.

In this work, we employ an entirely novel approach to the issue of transferring electrons between Photosystem I and hydrogenase. A molecular wire is utilized to tether the redox cofactors in both PS I and H_2ase, thereby connecting directly the electron transfer chains of both proteins. We show here that PS I and the [FeFe]-H_2ase from *Clostridium acetobutylicum*, which is biased toward the reduction of protons (in contrast with the

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[NiFe]-H_2ases) (*10*, *11*), can be connected through their physiologically active redox cofactors using an alkane dithiol molecular wire, thereby eliminating diffusional constraints that would otherwise limit electron transfer were soluble shuttle molecules to be used (Figure 1). PS I and [FeFe]-H_2ases contain low-potential [4Fe-4S] clusters. The protocol for tethering two proteins at their physiologically relevant redox centers takes advantage of the highly conserved iron−sulfur (Fe−S) cluster binding motifs in which cysteine residues ligate the iron atoms. By changing surface-located Cys residue to a Gly, either an open or a modifiable coordination site can be introduced into the protein. We have previously reported that Cys 13 on PsaC, which ligates the terminal F_B cluster, can be modified to a Gly (C13G) (*12*, *13*). When the C13G PsaC apoprotein is expressed in *Escherichia coli* and its Fe−S clusters are inserted *in vitro* using $FeCl_3$, Na_2S, and 2-mercaptoethanol (*14*) by a ligand-exchange reaction, a functional protein is assembled, with retention of a 2-mercaptoethanol at the Gly 13 site (*15*). The 2-mercaptoethanol can be exchanged with other thiol-containing compounds, including 1,6-hexanedithiol (*15*). This generalized method provides the basis for tethering any two Fe−S proteins, provided they each contain a surface-located cysteine residue. Here, we report a similarly engineered Cys to Gly change at the distal [4Fe-4S] cluster of the [FeFe]-H_2ase from *C. acetobutylicum*, and we describe the construction of a tethered nanoconstruct comprised of PS I and an [FeFe]-H_2ase.

PS I was reconstituted from P_{700}−F_X cores (*16*), the C13G variant of PsaC, which harbors a 2-mercaptoethanol molecule on the F_B cluster, and recombinantly expressed PsaD. As shown in Figure S1 of the Supporting Information, the presence of the external rescue ligand at Gly 13 does not alter the charge recombination kinetics from the terminal Fe−S clusters. Furthermore, the PS I variant was able to transfer electrons at a rate similar to that of the wild type, as shown by the light-induced steady-state kinetics of flavodoxin (Fld) reduction [1053 μmol of Fld (mg of Chl)$^{-1}$ h^{-1} in wild-type PS I complexes vs 1049 μmol of Fld (mg of Chl)$^{-1}$ h^{-1} for the PS I variant]. The external ligand, 2-mercaptoethanol, was subsequently exchanged for 1,6-hexanedithiol to provide a short (dithiol sulfur−sulfur distance of ~11.5 Å) covalent connection between the variant PS I and H_2ase enzyme.

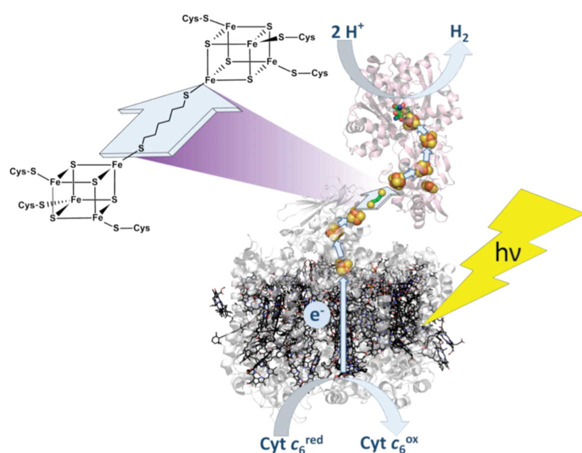

FIGURE 1: Schematic of the PS I−molecular wire−[FeFe]-H_2ase nanoconstruct. Assembled photosynthetic nanoconstructs in which the connecting wire is 1,6-hexanedithiol. Soluble electron donors include cytochrome c_6, ascorbate, and PMS. Arrows indicate electron transfer through the system, including reduction of protons to H_2.

The HydA [FeFe]-H_2ase enzyme in *C. acetobutylicum* contains a [2Fe-2S] cluster as well as a chain of three [4Fe-4S] clusters that constitute the electron transfer pathway between the H-cluster at the active site and the surface of the enzyme (*17*). The C97G variant of HydA from *C. acetobutylicum* was expressed in both *E. coli* (see the Supporting Information) (*18*) and *C. acetobutylicum* (*11*). The wild-type and variant proteins showed a broad absorption around 430 nm, consistent with S → Fe charge-transfer bands from the Fe−S cluster(s). Electrochemical analysis of the wild-type and C97G variant enzymes on a graphite rotating disk electrode showed similar voltammetric wave shapes, indicating that the transfer of electrons to and from the active site had not been significantly altered (not shown). When the variant expressed in *C. acetobutylicum* was tested for H_2-evolving activity, the resulting activity was 138.7 mol of H_2 (mol of H_2ase)$^{-1}$ s^{-1} compared to 556.8 mol of H_2 (mol of H_2ase)$^{-1}$ s^{-1} for wild-type HydA (Table 1).

The Gly 97 variant of HydA was combined with the PS I−molecular wire construct in an equimolar ratio. The PS I−molecular wire−[FeFe]-H_2ase nanoconstruct self-assembles due to facile exchange with an external thiolate at the iron−sulfur coordination bond. Because [FeFe]-H_2ases are rapidly inactivated by oxygen, the nanoconstructs were assembled and characterized under strictly anoxic conditions. The operation of the PS I−molecular wire−[FeFe]-H_2ase half-cell requires the use of a sacrificial donor. Among the electron donors tested, a combination of cytochrome (Cyt) c_6, ascorbate, and phenazine methosulfate (PMS) was found to provide the highest rates of light-induced H_2 production. The electron donors were added to dark-adapted PS I−molecular wire−[FeFe]-H_2ase nanoconstructs at pH 8.3, and the solution was illuminated as a 5 mm thick solution with broad-spectrum white light at an intensity of 996 μmol photons m^{-2} s^{-1}. Measured from the headspace as a function of time, the rate of light-induced H_2 production was 30.3 μmol of H_2 (mg of Chl)$^{-1}$ h^{-1} for the C97G HydA variant expressed in *C. acetobutylicum*.

Illumination was necessary for H_2 production. No H_2 was generated when the C97G HydA variant, the C13G variant of PS I, or the 1,6-hexanedithiol was omitted from the solution. Similarly, no H_2 was generated when the C13G variant of PS I was replaced with either wild-type PS I or PS I cores lacking the PsaC subunit, or when the C97G HydA variant was replaced with wild-type H_2ase. When fresh ascorbate was added to test the activity, the PS I−molecular wire−[FeFe]-H_2ase construct showed little or no loss of light-induced H_2 production when stored at room

Table 1: Comparative Rates of Hydrogen Uptake and Hydrogen Evolution in Wild-Type (WT) and Variant H_2ases[a]

H_2ase source	[FeFe] H_2ase	mol of H_2 (mol of H_2ase)$^{-1}$ s^{-1}	μmol of H_2 (mg of H_2ase)$^{-1}$ min^{-1}	μmol of H_2 (mg of Chl)$^{-1}$ h^{-1}
C. acetobutylicum	WT HydA	556.8 ± 39.9	522.1 ± 42.6	0
C. acetobutylicum	C97G HydA	138.7 ± 5.4	130.0 ± 5.8	30.3 ± 0.8

[a]Hydrogen evolution was measured in purified enzymes with reduced methyl viologen as the electron donor, and light-induced hydrogen evolution was measured in PS I−molecular wire−[FeFe]-hydrogenase nanoconstructs with Cyt c_6, ascorbate, and PMS as electron donors.

temperature under room illumination and assayed over a period of 64 days. Because a fraction of non-hydrogen-producing constructs may be present in the solution because of PS I−PS I dimers or H_2ase−H_2ase dimers, the rate of light-induced hydrogen production is likely to be underestimated.

The relatively high rate of light-induced H_2 evolution is achieved because of the direct connection between the F_B cluster of PS I and the distal Fe−S cluster of the [FeFe]-H_2ase enzyme. The molecular wire tethers the two redox centers at a short distance so that electrons can quantum mechanically tunnel from PS I to the [FeFe]-H_2ase at a rate faster than the rate of inherent charge recombination between P_{700}^+ and F_B^-. The rate of electron tunneling is affected by the nature of the intervening medium (*19*), and in this nanoconstruct, the covalent pathway of σ bonds between the two Fe−S clusters affords a more favorable route than pathways through solvent. This direct coupling strategy affords a significant increase in the rate of light-induced H_2 generation over current photobiological constructs under similar conditions (*3*, *4*, *6*, *8*, *20*). Moreover, this work represents the first instance of an *in vitro* wiring of two enzymes through their physiologically relevant redox centers and may be applied to other Fe−S cluster-containing metalloproteins. Not only does this technique allow for the production of a variety of alternative fuels, but also if wiring could be performed *in vivo*, the possibility exists for a fully self-assembled and self-repairing mechanism for the generation of commercially valuable products.

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SUPPORTING INFORMATION AVAILABLE

Detailed materials and methods, discussion of H_2ase enzymes expressed in *E. coli*, and voltammograms of H_2ase enzymes. This material is available free of charge via the Internet at http://pubs.acs.org.

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