Supporting Information

Yacoby et al. 10.1073/pnas.1103659108

SI Methods.

Plasmid construction. All of our gene fusions were optimized for *Escherichia coli* expression and synthesized by GeneArt (Germany) and inserted into the pMA vector at the NdeI and AvrII restriction sites (1, 2). These restriction sites were used to subclone the fusion gene into pHydEA at the original site of the algal [FeFe]-hydrogenases gene. A series of ferredoxin (Fd)-HydA fusions were created that incorporated different sized linkers. The restriction sites AgeI and NheI were engineered into the 5′ and 3′ ends, respectively. These sites enabled extension of the linker lengths from 15aa (amino acids) to 30aa (see Table S2), by digestion of pHydEFd-HydA with the AgeI and NheI, and insertion of a new linker. Linkers were generated from pairs of complementary DNA oligos (IDT) that encoded linkers listed in the Table S2. The linker pairs contained noncomplementary overhanging ends for ligation into AgeI and NheI sites. Each of the HydA, Fd fusion proteins contained the C-terminal extension 5′-ctcgagattgaaggccgtcaattgggctggagccatccgcagtttgaaaaataacctag-g-3′ that encoded a Factor Xa site upstream of the StrepTagII site.

Protein expression. Expressions were performed in Novagen strain Rosetta-2 (DE3). A 10-mL volume of LB containing 200 $\mu g\,mL^{-1}$ Ampicillin and 50 $\mu g\,mL^{-1}$ Streptomycin was cultured overnight at 37 °C. The cells were harvested and washed twice with fresh media and resuspended in 10 mL of LB. One milliliter of washed cells were diluted into 100 mL of Terrific-Broth solution (TB) with the same concentration of antibiotics and cultured at 37 °C with shaking at 250 rpm until absorbance value at A_{600} reached 0.4. Then 45 mL of this preculture was added into 1 L of fresh TB media and cultured until the A_{600} reached a value of 0.4. For induction, fresh ammonium ferric citrate (1 M) was added to a final concentration of 2.5 mM, and IPTG was added to a final concentration of 1.5 mM. Induction was carried out aerobically for 1 h at 30 °C and 150 rpm for the Fd-HydA fusions, and at 20 °C for native hydrogenase. After 1 h the media was transferred to 1 L bottles (Kimax-25) with V-shape rubber cap and sparged under argon for 1 h at 20 °C. Then the cells were transferred to an anaerobic glove box (Coy Laboratories) under 4% H_2/96% N_2 atmosphere and incubated over night at 20 °C.

Protein purification. Because the oxygen sensitivity of HydA is a known obstacle, during the entire purification process, all solutions contained 10 mM of sodium dithionite. Cells were collected by centrifugation at 7,000 × *g* for 7 min 4 °C, and resuspended in a 30-mL volume of buffer A (Table S3). Cell disruption was carried out by French press, and the lysate centrifuged at 144,000 × *g* in a 60Ti rotor (Beckman) for 30 min at 4 °C. Cleared lysates (usually 30–40 mL) were loaded on 3 × 5 mL DEAE fast flow columns (GE-Healthcare) preequilibrated in buffer B at a flow rate of 5 mL min^{-1}. Then the column was washed in two steps: first with 50 mL of buffer B, then with 50 mL of buffer C each at 5 mL min^{-1}. Elution was carried out with buffer D at a flow rate of 2.5 mL min^{-1} and the eluate collected. Brown fractions (usually 7–10 mL) were combined and the NaCl concentration was adjusted to 1 M. The enzyme pool was loaded onto 4 × 5 mL Strep-tactin columns (GE Healthcare) connected in series at a flow rate of 1 mL min^{-1}. The columns were preequilibrated with buffer E. The columns were washed with 50 mL of buffer E. Enzyme was eluted at the same speed with buffer F, and 1-mL fractions collected. Protein concentrations were quantified by Bradford assay (Bio-Rad) according to manufacturer instructions.

Protein gel electrophoresis. Protein gel electrophoresis was performed with Invitrogen NuPAGE® Novex® Bis-Tris Gel Systems according to manufacturer instructions.

Isolation of *Chlamydomonas reinhardtii* Thylakoids. A 2.1-L culture of *C. reinhardtii* grown photoheterotrophically in Tris-acetate-phosphate media was pelleted by centrifugation at 3,500 × *g* for 5 min. Cells were washed with a 1/5 vol of wash buffer (0.35 M sorbitol; 20 mM Hepes pH 7.5; 2 mM $MgCl_2$; 5 mM sodium ascorbate, SAc). Washed cells were resuspended in wash buffer to a final chlorophyll concentration of 1 $mg\,mL^{-1}$. Cells were broken by French press at 3,300 $lb\,in^{-2}$. Thylakoids were pelleted by centrifugation at 40,000 × *g* for 20 min at 4 °C.

The thylakoid pellet was resuspended in wash buffer, homogenized by vortexing, and recentrifuged at 1,200 × *g* for 30 s to pellet unbroken cells. The supernatant was centrifuged again at 11,000 × *g* for 12 min to remove soluble proteins. The pellet, which contained pure washed thylakoids, was resuspended in wash buffer to a chlorophyll concentration of 2.5 $mg\,mL^{-1}$.

Purification of Plastocyanin. Plastocyanin (PC) was purified from pea leaves during the process of photosystem I (PSI) isolation[2]. After the first addition of the detergent DDM and centrifugation, the supernatant was collected, and both NaCl (200 mM) and PEG2000 (7%) were added. This solution was centrifuged at 15,000 × *g* for 10 min at 4 °C, and an additional 9% PEG2000 was added to the supernatant. This solution was centrifuged at 15,000 × *g* for 10 min at 4 °C. The supernatant (about 500 mL) was diluted 1:4 in MES buffer (0.1 M pH 6.5) and loaded onto a DEAE column by gravity flow. The blue PC was eluted with a linear gradient of 0–500 mM NaCl in MES buffer, pH 6.5. The eluate containing the blue PC was concentrated and purified with gel filtration superdex 75.

Hydrogen production by solubilized *E. coli* whole cells expressing recombinant hydrogenase. Electron-donating buffer was prepared in the anaerobic glove box in a serum vial to a volume of 30 mL. Methyl viologen (MV) (Sigma) and sodium dithionite were added to 25 mL of buffer G (Table S3) to obtain a stock solution of 10 mM of MV and 20 mM of sodium dithionite. The solution was shaken and sparged with argon for 20 min to remove residual hydrogen from the glove box. Reaction solutions of 1 mL were prepared in buffer G in a 13-mL serum vial and 10-μL of sample protein solution was added. Sample solutions were sparged with argon for 5 min. After that, 1 mL of the electron-donating buffer was injected to the sample solution using a gas-tight syringe, mixed, and placed in a water bath at 37 °C for 10–20 min. The gas-phase level of hydrogen was measured by injection into a gas chromatograph (GC) Hewlett-Packard 5890 Series II.

Enzyme activity U ($\mu mol\,H_2\,mL^{-1}\ min^{-1}$) was calculated using the formula

$$\text{rate} = H_{2\,\text{nmol}}/1{,}000_{\text{nmol}/\mu\text{mol}} \times 26(V_{\text{vial}}/V_{\text{syringe}}) \times (1/V_{\text{prot}})/T_{\text{min}},$$

where $H_{2\,\text{nmol}}$ is the GC measured amount of hydrogen in 0.5-mL sample, V_{vial} is the volume of the vial (13 mL), V_{syringe} is the volume of the gas phase that was injected to the GC (0.5 mL), V_{prot} is the volume of the initial protein sample in mL, and T_{min} is the reaction time after electron-donating buffer was injected into the sample and the headspace hydrogen level measured by GC. When enzyme activities or concentrations were high, enzymes

were diluted up to 1,000-fold in buffer B. Specific activity of the protein was calculated using the formula

$$U = \text{rate}/C_{\text{prot}},$$

where C_{prot} is the protein concentration (mg mL^{-1}) of the sample solution.

Hydrogen photoproduction by thylakoids. The reaction mixtures were prepared in gloveboxes and sparged with argon for 5 min to remove hydrogen. Aliquots of HydA or Fd-15aa-HydA were added using a gas-tight syringe, the mixture sparged for 20 min to consume residual sodium dithionite. When testing for competition with ferredoxin:NADP$^+$-oxidoreductase (FNR)-mediated NADPH production, a 50-μL volume of NADP$^+$ was added with gas-tight syringe, and the reaction measured as described. Equilibrated reactions were illuminated using the halogen lamp of a Kodak slide projector equipped with 300W EXR halogen lamp as a light source. The amount of hydrogen was measured at 15 min intervals by GC. The activity of enzymatic hydrogen was calculated using the formula

$$\text{rate} = \text{H}_{2\,\text{nmol}}/1{,}000_{\text{nmol}/\mu\text{mol}} \times 26 V_{\text{vial}}/V_{\text{syringe}} \times (1/\text{Chl}_{\text{mg mL}^{-1}}/T_{\text{min}} \times 60_{\text{min h}^{-1}}),$$

where $\text{H}_{2\text{nmol}}$ is the measured amount of hydrogen in the gas-phase sample injected into the GC, V_{vial} is the volume of the vial (13 mL), V_{syringe} is the volume of the gas phase injected into the GC (0.5 mL), T_{min} is the time of the illumination in min, and $\text{Chl}_{\text{mg mL}^{-1}}$ is the concentration of chlorophyll in mg mL^{-1}. Hydrogen production rates were calculated in μmol H_2 mg Chl^{-1} h^{-1}. For each experiment, at least three independent measurements were carried out, including four readings. The final data points were calculated as a median of all of the readings, after manual exclusion of the most outlined values. Error bars represent standard deviation of a value.

Hydrogen Photoproduction by PSI. All reactions were performed in sealed 13-mL serum vials. Reaction mixtures consisted of a 900-μL volume of buffer A (Table S4), 0.2 μL of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (stock of 70 mg mL^{-1}), 0.5 μL of dichloroindophenol (DCIP) (stock of 2.9 mg mL^{-1}) and 10 μL of SAsc (stock of 1 M). A volume of 10–40 μL of PC from a 3 mg mL^{-1} stock solution was added, after mixing both Fd and FNR were added at variable concentrations. Manipulations of PSI were performed in the dark. The amount of PSI in the reaction solution was adjusted to a final chlorophyll concentration of 10 μg mL^{-1}. This mixture was sparged under argon for 5 min, and the desired amount of HydA or Fd-15aa-HydA was added by a gas-tight syringe; the solution was resparged for 20 min, followed by addition of 50 μL NADP$^+$. The reaction mixtures were illuminated by a halogen lamp, as described above. The hydrogen concentrations were measured at 15-min intervals by GC, and hydrogen production rates calculated using the equation above.

SI Results

Study of Hydrogen and NADPH Photoproduction with *C. reinhardtii* Thylakoids. Unlike plants, thylakoids prepared from the unicellular green algae *C. reinhardtii* lose the lumenal-localized PC during purification. Thus, PC has to be added to in vitro reaction mixtures to ensure transfer electrons from the *cytb6/f complex* to PSI. We observed that there is a high degree of structural and functional conservation between higher plant and algal thylakoids with respect to photosynthetic electron transport. To ascertain whether this conservation also exists with respect to in vitro hydrogen production and NADP$^+$ reduction, we have performed these assays with both plant and algal thylakoids. We found that, similar to plant thylakoids, FNR activity is retained in the membranes of washed algal thylakoids. The presence of FNR results in more than 75% inhibition of hydrogen production by added algal hydrogenase in the presence of NADP$^+$. In addition, we observed that washed algal thylakoids photoreduce NADP$^+$ exactly as plant thylakoids. And, most importantly, the Fd-15aa-HydA fusion was able to divert at least 60% of the photosynthetically generated electrons from FNR and NADP$^+$ reduction toward hydrogen production.

Table S1 shows the hydrogen photoproduction measurements ($U = \mu$mol H_2 mg Chl^{-1} h^{-1}) by thylakoids in the presence of either HydA or the Fd-15aa-HydA fusion, and the allocation of photosynthetically generated electrons between H_2 production and NADPH generation. The addition of NADP$^+$ (2.5 mM) and Fd (10 μM) to isolated thylakoids (which contained membrane-bound FNR) resulted in competition between H_2 production and NADP$^+$ reduction. The noncompetitive mode was achieved by excluding NADP$^+$ from the reaction. The right column for each case (HydA and Fd-15aa-HydA fusion) shows the percentage of electrons that are diverted to hydrogen production vs. NADPH production.

NADPH Production by *C. reinhardtii* Thylakoids. The maximal measured rate was in the range of 12–18 μmol NADPH mg Chl^{-1} h^{-1}. The addition of an excess of external FNR increased the rate to 19 μmol NADP mg Chl^{-1} h^{-1}. In the absence of added PC (30 μL of 3 mg mL^{-1}) the observed rate was negligible (about 1 μmol NADPH mg Chl^{-1} h^{-1}), independent of whether external FNR was present or not.

1. King PW, Posewitz MC, Ghirardi ML, Seibert M (2006) Functional studies of [FeFe] hydrogenase maturation in an Escherichia coli biosynthetic system. *J Bacteriol* 188:2163–2172.

2. Ben-Shem A, Frolow F, Nelson N (2003) Crystal structure of plant photosystem I. *Nature* 426:630–635.

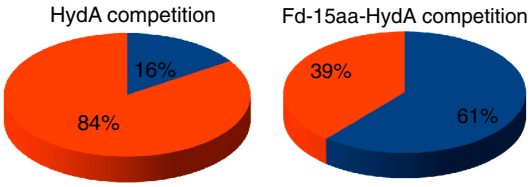

Fig. S1. *Summary of the results from Table S1*. The pie charts show the percentages of electrons used in NADP$^+$ reduction (orange) vs. hydrogen production (blue) with *C. reinhardtii* thylakoids. (*Left*) The pie chart shows the electron partitioning in the presence of HydA. (*Right*) The pie chart shows the electron partitioning upon addition of Fd-15aa-HydA. As was observed with plant thylakoids, Fd-15aa-HydA was able to divert electrons from FNR, increasing the basal level of H_2 production by approximately 4-fold.

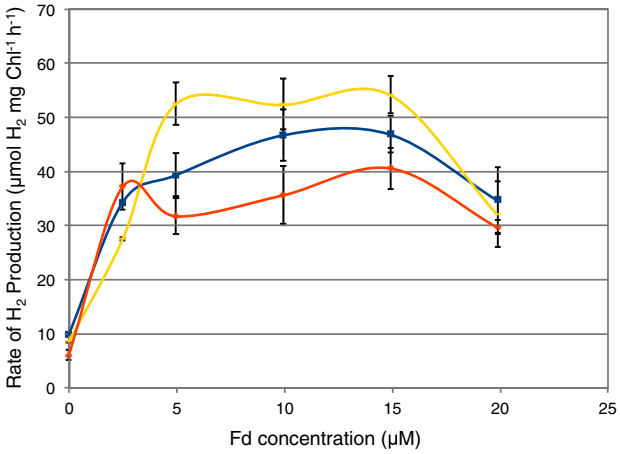

Fig. S2. *Hydrogen photoproduction by Fd-15aa-HydA with purified PSI and Fd*. In order to evaluate the NADPH production and H_2 production kinetics in a purified system, PSI from plants was used in lieu of thylakoids. The figure shows that the optimum working concentration for Fd was 10 μM. The Fd-15aa-HydA fusion bypasses inhibition by FNR (orange line), as observed in reactions containing thylakoids from plant (Fig. 4, main text) or algae (Table S1). The experiments were performed under three reaction conditions: (*i*) noncompetitive, without addition of FNR (blue line); (*ii*) noncompetitive, with the addition of heat inactivated FNR (yellow line); and (*iii*) competitive, with the addition of active FNR (red line). Each reaction mixture contained PSI (10 μg), PC (8 μM), and Fd-15aa-HydA fusion (1 μM). Noncompetitive, heat inactivated FNR (100 nM) and $NADP^+$ (2.5 mM). Competitive, active FNR (100 nM) and $NADP^+$ (2.5 mM).

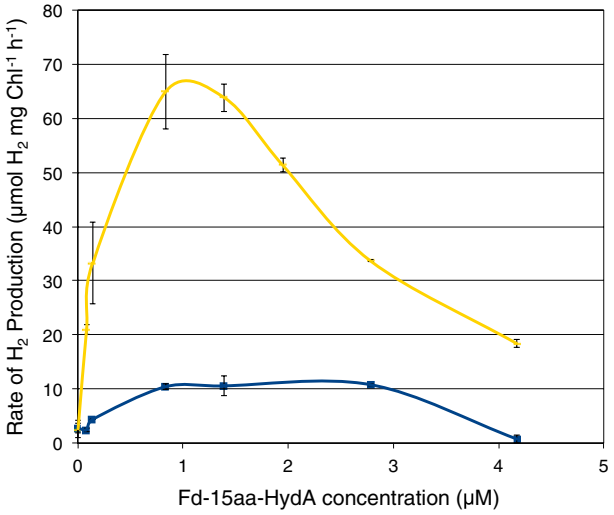

Fig. S3. *Dependence of hydrogen photoproduction by PSI on Fd-15aa-HydA concentration*. This experiment was done to validate the optimum concentration of the 15aa fusion. The experiment was performed with purified plant PSI under two conditions: (*i*) addition of 10 μM Fd (yellow line) and (*ii*) without Fd (blue line). Both reaction conditions contained PSI (10 μg), PC (8 μM), and Fd-15aa-HydA fusion at a final concentration of 0–4 μM. Under both reaction conditions, the optimum concentration range for Fd-15aa-HydA was 1 μM.

Table S1. Competition between HydA or Fd-15aa-HydA and FNR in *C. reinhardtii* thylakoids

Assay conditions	HydA		Fd-15aa-HydA	
	H_2 production activity, U*	Ratio of H_2/NADPH, %/%	H_2 production activity, U*	Ratio of H_2/NADPH, %/%
Noncompetitive†	5.05	100/0	5.88	116/0
Competitive†	0.8	16/84	3.09	61/39

The table shows the hydrogen photoproduction measurements ($U = \mu$mol H_2 mg Chl^{-1} h^{-1}) by thylakoids in the presence of either HydA or the Fd-15aa-HydA fusion, and the allocation of photosynthetically generated electrons between H_2 production and NADPH generation. The addition of $NADP^+$ (2.5 mM) and Fd (10 μM) to isolated thylakoids (which contained membrane-bound FNR) resulted in competition between H_2 production and $NADP^+$ reduction. The noncompetitive mode was achieved by excluding $NADP^+$ from the reaction. The right column for each case (HydA and Fd-15aa-HydA fusion) shows the percentage of electrons that are diverted to hydrogen production vs. NADPH production.

*$U = \mu$mol H_2 mg Chl^{-1} h^{-1}.

†Noncompetitive, in the absence of $NADP^+$; competitive, in the presence of $NADP^+$.

Table S2. Linker oligonucleotides used to create Fd-HydA fusions

Linker		Oligonucleotides
15aa	S	5′-ccggtggaggatccggaggtggaggatccggcggcggcg-3′
	A	5′-ctagcgccgccgccggatcctccacctccggatcctcca-3′
20aa	S	5′-ccggtggaggatccggaggtggaggatccggaggtggaggatccggcggcggcg-3′
	A	5′-ctagcgccgccgccggatcctccacctccggatcctccacctccggatcctcca-3′
25aa	S	5′-ccggtggaggatccggaggtggaggatccggaggtggaggatccggaggtggaggat-ccggcggcggcg-3′
	A	5′-ctagcgccgccgccggatcctccacctccggatcctccacctccggatcctccacctccggat-cctcca-3′
30aa	S	5′-ccggtggaggatccggaggtggaggatccggaggtggaggatccggaggtggaggat-ccggaggtggaggatccggcggcggcg-3′
	A	5′-ctagcgccgccgccggatcctccacctccggatcctccacctccggatcctccacctccggat-cctccacctccggatcctcca-3′

Abbreviations: S—sense strand; A—antisense strand.

Table S3. Buffers used for protein purification

Buffer	Purpose	Composition
A	Cells lysis	Tris-HCl 100 mM at pH 8; glycerol 5% vol; sodium dithionite 10 mM; protease inhibitor cocktail 1 mL for 4 L of cells solution; lysozyme 84 μg mL^{-1}; benzonase nuclease (Sigma) 20 μL for 4 L of cells solution
B	1st wash buffer for DEAE	Tris-HCl 100 mM at pH 8; glycerol 5% vol; sodium dithionite 10 mM
C	2nd wash buffer for DEAE	Tris-HCl 100 mM at pH 8; glycerol 5% vol; sodium dithionite 10 mM; NaCl 0.05 M
D	DEAE elution buffer	Tris-HCl 100 mM at pH 8; glycerol 5% vol; sodium dithionite 10 mM; NaCl 0.5 M
E	Strep-tactin wash buffer	Tris-HCl 100 mM at pH 8; glycerol 5% vol; sodium dithionite 10 mM; NaCl 1 M
F	Strep-tactin elution buffer	Tris-HCl 100 mM at pH 8; glycerol 5% vol; sodium dithionite 10 mM; desthiobiotin 12.5 mM
G	Buffer for enzyme activity measurement	Tris-HCl 100 mM at pH 8; glycerol 5% vol; KCl 0.25 M; triton X-100 0.2% vol.

Table S4. Buffers and solutions used to measure enzyme activity

Buffer	Purpose	Composition
A	Reaction buffer	Tris-HCl 50 mM at pH 7.4; bovine serum albumin 3.35 mg mL^{-1}; $MgCl_2$ 10 mM, sucrose 200 mg mL^{-1}
DCMU	Blocks PSII activity	3-(3,4-dichlorophenyl)-1,1-dimethylurea (Sigma), 0.3 mM in dimethyl sulfoxide
DCIP	Electron donor for plastocyanin	2.6-dichloroindophenol sodium salt hydrate (Sigma), 0.01 mM
SAsc	Electron donor for DCIP	Sodium ascorbate (Synthesized from ascorbic acid by titration with sodium hydroxide), 1 M
GOx	Consumes oxygen	Glucose oxidase (Sigma), 30 mg mL^{-1}
Glucose	GOx substrate	Glucose 5 M
Cat	Hydrogen peroxide removal	Catalase (Sigma), 10 mg mL^{-1}
$NADP^+$	FNR substrate	β-nicotinamide adenine dinucleotide phosphate sodium salt (Sigma), 0.04 mM