Producing Fuels the Old-Fashioned Way: Using Biology

Direct Solar BioHydrogen: Part II

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H₂
Energy History: Planet Earth

Solar Energy

Microorganisms, Plants

H₂O CO₂

O₂ Biomass

Biological Ecosystems

Energy (Fossil fuels)

Consumption

CO₂
Sustainable Energy Future: Planet Earth

Solar Energy

Our Overall Proposal

Engineered Photosynthetic Microorganisms

Energy (H₂ Economy)

Fuel Cells, Power Plants, Automobiles

H₂O → H₂ → Fuel Cells, Power Plants, Automobiles → O₂ → H₂O

Solar Energy → Engineered Photosynthetic Microorganisms → Energy (H₂ Economy) → Fuel Cells, Power Plants, Automobiles → O₂ → H₂O
The solar resources for generating power from concentrating solar power systems is plentiful. For instance, enough electric power for the entire country could be generated by covering about 9 percent of Nevada – a plot of land 100 miles on a side* – with parabolic trough systems.

* Equivalent to 1.7% of Current Cropland

http://www.energylan.sandia.gov/sunlab/overview.htm#solres
The Direct Conversion Concept

- Incident Sunlight
- Large Surface Area Collector/Reactor
- Engineered Organisms
- Rejuvenate Culture
- One Candidate Collector/Reactor Cross Section
- Transparent Cover
- Transparent Gas Permeable Membrane
- Vacuum
- Organism Suspension
- Cooling Fluid

- Low Pressure
- H₂ & O₂ Harvest
- Temp. Control Fluid In
- Temp. Control Fluid Out

- Low Pressure
- Engineered Organisms
- Rejuvenate Culture
- Large Surface Area Collector/Reactor
- Incident Sunlight

Direct PhotoBiological Hydrogen Production: Building a New Electron Pathway

Aerobic Process

\[ 2 \text{H}_2\text{O} \xrightarrow{h\nu} \overset{\text{Sunlight}}{\text{PS II}} \overset{h\nu}{\text{PS I}} \overset{\text{Reduced}}{\text{Ferredoxin}} \overset{\text{Growth}}{\text{Synechocystis Catabolism}} \]

Anaerobic Process

\[ \text{Glucose} \rightarrow \overset{\text{Pyruvate}}{\text{Reduced Ferredoxin}} \rightarrow \overset{\text{Hydrogenase}}{4 \text{H}^+} \rightarrow 2\text{H}_2 \]

New Pathway

\[ \text{Clostridium pasteurianum Catabolism} \]

4 H+
Direct PhotoBiological Hydrogen Production: Building a New Electron Pathway

Goal: Engineered *Synechocystis* Bacterium
Direct PhotoBiological Hydrogen Production: Building a New Electron Pathway

Goal: Engineered *Synechocystis* Bacterium
Comparison of Different Fe-Fe Hydrogenases

Clostridium pasteurianum

Desulfovibrio desulfuricans

Scenedesmus obliquus

Chlamydomonas reinhardtii
The 3-D Structure is Known for the Hydrogenase from *Clostridium pasteurianum*

\[
2H^+ + 2\text{Fd}^{\text{red.}} \rightleftharpoons \text{H}_2 + 2\text{Fd}^{\text{oxid.}}
\]
The Oxygen-Sensitive Active Site is Very Complex
A Recent Molecular Dynamic Model Suggests Two Oxygen Channels

Hydrogenase CpI from *Clostridium pasteurianum*¹

\[ 2H^+ + 2Fd^{\text{red}} \leftrightarrow H_2 + 2Fd^{\text{oxid}} \]

Proposed model for O₂ diffusion in CpI²


We Will Use Directed Evolution to Produce Oxygen Tolerant Hydrogenases

Process of Directed Evolution

Create Collection of Hydrogenase Genes with Genetic Diversity

Mutate Genes of Oxygen Tolerant Hydrogenases

Express Genes in Cell-Free System

Identify Candidates With Increased Oxygen Tolerance

Evaluate In Synechocystis

2H^+ + 2Fd^{red} \rightleftharpoons H_2 + 2Fd^{oxid}
Cell-Free Protein Synthesis (CFPS) – Can Easily Conduct Multiple Parallel Reactions

Combined Transcription/Translation: *E. coli*

1. Grow and Lyse *E. coli*
2. Prepare Extract
3. Add Substrates, Salts, and Folding Aids
4. Add Template
5. Incubate

Provides Direct Access and Control and Rapid Analysis
Screening Strategy

Generate Diversity

- PCR
  - Error-Prone PCR, Family Shuffling, Rational Design

Isolate Mutants by Dilution

- Mutant Library

Amplify Mutants

- PCR

Cell-Free Protein Synthesis:

- Gene Library → Protein Library

Oxidized Methyl Viologen Hydrogen Consumption Assay

\[ 2 \text{MV}_{\text{ox}} + H_2 \leftrightarrow 2 \text{MV}_{\text{red}} + 2H^+ \]

Anaerobic Chamber, 3% H₂

Exposure to Oxygen by Addition of Air-Saturated Buffer
Single Molecule PCR (smPCR) Allows Clonal Separation

- Dilution to single molecule level is crucial
- Poisson distribution describes single molecule statistics
- For Leu66 (10⁷ dilution of library):

\[ P(k) = \frac{m^k e^{-m}}{k!} \]

P is the probability of getting k DNA molecules in a specified well, when \( m \) = average # DNA molecules per well

For \( K = 0 \)

\[ P(0) = \frac{m^0 e^{-m}}{0!} = e^{-m} \quad m = -\ln[P(0)] \]

The 10⁷ dilution contains 1.23 molecules/µL

Data from Phil Smith

Methodology developed by Jim Stapleton
We Began with Ferredoxin. It also Needs an Fe-S Center

Cell-Free Produced Ferredoxins are Fully Active

Synecocystis Ferredoxin

Marcus Boyer

Activity of Ferredoxins on per Iron basis

Activity (M Cyt / M Iron / min)

In-Vivo  KC1  pRKISC

Fd Sample

Cell-Free  Cell-Free
The Active Site of Fe-Fe Hydrogenases is Complicated: Stabilized by Cysteines, Carbon Monoxide, and Cyanide
Discovery of Two Novel Radical S-Adenosylmethionine Proteins Required for the Assembly of an Active [Fe] Hydrogenase*

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FIG. 5. Hydrogen production rates from purified HydA1 heterologously expressed in E. coli either alone or co-expressed with the indicated Hyd proteins.

Hydrogen production was measured using the methyl viologen-based assay. The data shown represent the average of four independent experiments; average deviations from the mean are shown.

*Genes Taken from Chlamydomonas reinhardii
In-vivo Co-expression of Maturation Enzymes and Hydrogenases

Hydrogenase Activity [mmo H2/(min*mL*OD)]

Marcus Boyer, Chia-Wei Wang, Jackie Ng

Hydrogenase Expression in E. coli Identified Much Better Helper Proteins
Producing Active Cell Extract Is a Complicated Procedure

**Anaerobic Production of Cell Extract for Cell-Free Synthesis of Active [FeFe] Hydrogenase**

**Aerobic Growth Phase**

*E. Coli* BL21(DE3) w/ pACYC HydGXEF; 8 L fermentation at 37 °C

**Anaerobic Growth Phase**

O₂ to Argon at OD₅₉₅ = 5.0; Addition of IPTG, fumarate, ferrous iron, and cysteine; Temp change: 37 °C to 16 °C

**Anaerobic Cell Extract Preparation**

Anaerobically-produced cell extract with HydE, HydF, and HydG maturation enzymes

- Cells thawed, resuspended, homogenized, centrifuged
- Centrifuged
- Cells frozen with liquid N₂
- Cell extract decanted
- Cell extract frozen with liquid N₂

**Cell Harvest**

Cells collected after 16 – 20 hr of anaerobic induction at 16 °C

Marcus Boyer, Jim Stapleton, Jonny Kuchenreuther, Chia Wei Wang
Activity of Cell Extracts Can be Significantly Increased by “Reconstitution”

The Helper Proteins HydE, HydG, and HydF are all 4Fe-4S Proteins.*

“Reconstitution” is Effected by Incubation of Cell Extract with:
1mM Fe(NH$_4$)$_2$(SO$_4$)$_2$, 1mM Na$_2$S, and 1 mM Dithiothreitol (DTT)

Jonny Kuchenreuther and Marcus Boyer

*Brazzolotto, …, Marc Fontecave, J. Biol. Chem. 281:769, 2006
Improved Translational Initiation Gives Higher Active Yields

Jonny Kuchenreuther
New Insights: The Devil is in the Details

We must better understand:
The Function and the Activation of the Helper Proteins
as well as any other Requirements for Hydrogenase Activation:

A) Required for Effective Screening of Hydrogenase Mutants

B) Required for Activation of Hydrogenase when it is Expressed in Photosynthetic Organism.

Fontecave’s work suggests requirements for:
Guanosine Tri-Phosphate (GTP)

and S-Adenosyl Methionine (SAM)

What do GTP and SAM do?
What is the Origin of the Active Site Atoms?
Post-Synthesis Activation (Maturation) of ApoHydrogenase Enables Detailed Studies of Maturation Requirements

A. Hydrogenase polypeptide is produced without helper proteins and under aerobic conditions. Small molecules are removed.

B. Cell Extract with Helper Proteins is Reconstituted under anaerobic conditions. Small molecules are removed.

C. Under anaerobic conditions, the preparations are mixed and candidate small molecules are added.

Marcus Boyer and Jonny Kuchenreuther
Screening Strategy

Generate Diversity

PCR
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Isolate Mutants by Dilution

Amplify Mutants

PCR

Cell-Free Protein Synthesis:
Gene Library → Protein Library

Oxidized Methyl Viologen Hydrogen Consumption Assay

Anaerobic Chamber, 3% H₂

Expose to Oxygen by Addition of Air-Saturated Buffer

2MV_{ox} + H₂ ⇌ 2MV_{red} + 2H^+

Jim Stapleton
Initial Screening Assays Were Highly Variable

Pre-exposure Reproducibility

For Unmutated Hydrogenase
We are Working to Make the Screen Performance More Reproducible

Jim Stapleton,
Sean Kendall,
Phil Smith
Conclusions

- Solar BioHydrogen Appears to be Technically and Economically Feasible
- BUT, We must first Evolve an Oxygen Tolerant Hydrogenase
- We can express Fe-Fe Hydrogenases With Cell-Free Technology Using Activated Cell Extracts
- We can accomplish Single-Molecule PCR for Clonal Separation
- GTP, SAM, and NAD are Required for H$_2$ase Activation
- We Have Gained Significant Knowledge To Help Us Express The Hydrogenases in Photosynthetic Organisms
- The Search for Oxygen Tolerant Hydrogenases is Underway
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