



Advances in Cell-Free Expression and Maturation of Active [FeFe] Hydrogenase

Jon M. Kuchenreuther¹, Marcus E. Boyer¹, James A. Stapleton¹, James R. Swartz^{1,2}
Stanford University ¹Department of Chemical Engineering, ²Department of Bioengineering



The Biohydrogen Project

The overall objective of the project is to construct a system for sustained H₂ production. The sun's energy will be captured by a photosynthetic microorganism. An [FeFe] hydrogenase will subsequently produce H₂ *in vivo*.

Major Challenges of the Biohydrogen Project

- Engineering of an O₂-tolerant mutant [FeFe] hydrogenase.
- Engineering *Synechocystis* sp. PCC 6803 to express and mature the mutant hydrogenase as well as additional enzymes required for maturation of the hydrogenase active site.
- Metabolic engineering of *Synechocystis* for *in vivo* production of H₂ via the mutant hydrogenase.
- Bioreactor design for sustained growth of *Synechocystis* and accumulation of H₂.

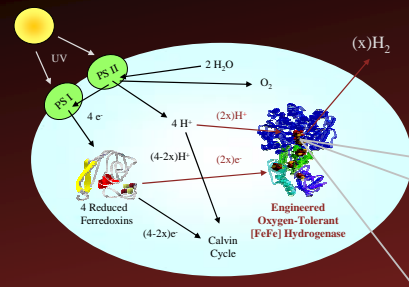
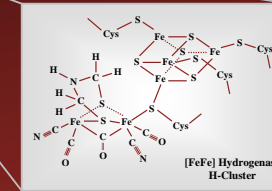


Figure 1: The cyanobacterium *Synechocystis* sp. PCC 6803 engineered for heterologous expression and maturation of an O₂-tolerant [FeFe] hydrogenase. Photosystems I and II capture the sun's energy, producing protons and electrons. The cyanobacteria will be engineered to direct some protons and electrons to the hydrogenase for H₂ production; other protons and electrons will be utilized for production of biomass. Not shown in the diagram are the maturation enzymes (HydE, HydF, and HydG from *Shewanella oneidensis*) that are involved in the maturation of active hydrogenase.

[FeFe] Hydrogenase and the H-Cluster

The hydrogenase active site, known as the H-cluster (depicted below), contains a [4Fe-4S] cluster connected to a [2Fe-2S] cluster that is decorated with several ligands. The radical SAM enzymes HydE and HydG as well as the GTPase HydF function in [FeFe] hydrogenase maturation, although the mechanism has not been elucidated. Like the hydrogenase, the maturation enzymes also contain O₂-sensitive [4Fe-4S] clusters.



Evolution of an O₂-Tolerant [FeFe] Hydrogenase

We are evolving the HydA1 hydrogenase from *Chlamydomonas reinhardtii*. HydA1 does not contain N-terminus Fe/S clusters associated with electron transport to the H-cluster, making HydA1 less complex than the Cpl hydrogenase from *Clostridium pasteurianum* (depicted in Figure 1). Active HydA1 has also been easier to express in an anaerobic cell-free system compared to Cpl.

Directed evolution techniques employed by our lab to create libraries of mutant [FeFe] hydrogenases include error-prone PCR and site-directed mutagenesis. Library expression is carried out in an anaerobic cell-free protein synthesis system.

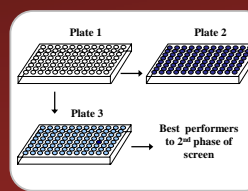


Figure 2: Mutant hydrogenase genes are diluted to single molecules (sm) and then amplified with smPCR. Amplified genes are expressed in anaerobic cell-free reactions (Plate 1). Activity of each mutant hydrogenase is measured with a methyl viologen reduction assay (Plate 2) using a portion of the cell-free reaction. Remaining cell-free hydrogenase is exposed to O₂ with O₂-saturated buffer, and activity of each mutant is again measured (Plate 3).

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

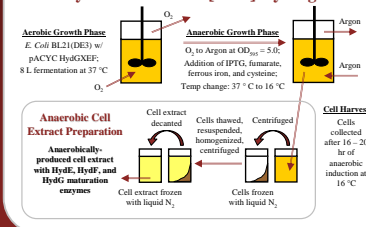


Figure 3: *E. coli* cell extract for production of active cell-free hydrogenase contains heterologous HydE, HydF, and HydG from *S. oneidensis*. *In vivo* expression of the *S. o.* HydG:KDF operon is induced with 500 μM IPTG 30 min after switching to anaerobic growth. Fermentation temperature is reduced to 16°C after 60 min of induction, which then proceeds for an additional 16–20 hr. Cells are harvested maintaining strict anaerobic conditions. The cell extract is made in an anaerobic glove box by lysing cells with high-pressure homogenization, and then removing both insoluble material as well as genomic DNA via centrifugation.

Reconstitution of Maturation Enzymes in an Anaerobic Cell Extract

Recent literature²³ discusses the aerobic expression of the individual maturation enzymes HydE, HydF, and HydG from *Thermotoga maritima*. Data from these reports indicate *in vitro* reconstitution of the Fe/S clusters along with subsequent enzymatic activity.

We hypothesized that the maturation enzymes present in the cell extract may not be fully active, and *in vitro* reconstitution could restore activity.

Our data illustrate *in vitro* reconstitution of the maturation enzymes prior to anaerobic cell-free synthesis increases active HydA1 yields approximately 4–5 fold (Figure 4), while total and soluble protein yields remained the same (data not shown).

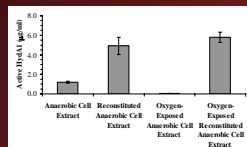


Figure 4: Anaerobic cell-free synthesis of active HydA1. *In vitro* reconstitution was done by incubating cell extract with 1 mM Fe(NH₄)₂(SO₄)₂, 1 mM Na₂S, and 1 mM DTP for 1–2 hr at 27°C in an anaerobic glove box. Oxygen-exposed cell extracts were exposed to atmospheric conditions for 60 min prior to *in vitro* reconstitution. 15 μl cell-free reactions using the PANOX-SP system were carried out at 27°C for 3–4 hrs. Active HydA1 was measured with a methyl viologen reduction assay.

Cell-Free Synthesis of Active HydA1 Using Aerobic Cell Extracts

Based on data in literature²³ as well as our reconstitution data (Figure 4), we hypothesized that anaerobic expression of the maturation enzymes along with maintaining anaerobic conditions during cell extract preparation may not be necessary to obtain a cell extract capable of producing active hydrogenase.

Aerobically-produced cell extract containing anaerobically induced HydE, HydF, and HydG is able to produce active HydA1 (Figure 5). Anaerobic cell-free yields of active HydA1 are comparable between the two types of cell extracts.

The ability to aerobically express the O₂-sensitive maturation enzymes and subsequently activate them may play an important role in expressing active hydrogenase *in vivo* in *Synechocystis*. The cyanobacteria do not grow under strict anaerobic conditions and can only survive anoxic conditions given occasional 5 min pulses of light⁴.

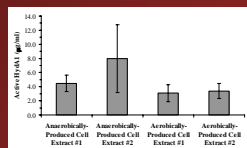


Figure 5: Anaerobic cell-free synthesis of active HydA1 using either an anaerobically-prepared cell extract (Figure 3) or an aerobically-prepared extract. Aerobically-prepared cell extracts were made from cells grown under aerobic conditions. Induction of HydE, HydF, and HydG was also done under aerobic conditions. All cell extracts were reconstituted prior to anaerobic cell-free synthesis of HydA1. Active hydrogenase was measured using a methyl viologen reduction assay.

In Vitro Maturation of Cell-Free Apohydrogenase

Given the O₂-sensitive maturation enzymes can be expressed in the presence of O₂ and subsequently activated, we postulated the [FeFe] hydrogenase may also be able to be translated in the apo-form and post-translationally matured using maturation enzymes present in the anaerobically-prepared cell extract.

Cell-free apo-HydA1 was first produced in an aerobic cell-free reaction in the absence of the maturation enzymes. In a separate *in vitro* reaction, HydA1 is capable of undergoing post-translational activation by incubating the apohydrogenase with supplemented cofactors as well as reconstituted anaerobically-prepared cell extract containing HydE, HydF, and HydG.

Utilizing the *in vitro* maturation system, we identified three molecules that significantly contribute to the process of [FeFe] hydrogenase maturation: S-adenosyl-methionine (SAM), GTP, and NAD (Figures 6a, 6b).

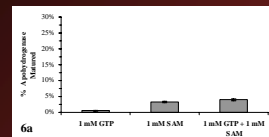
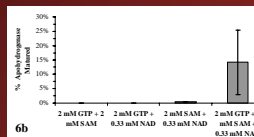


Figure 6: *In vitro* maturation of HydA1 [FeFe] hydrogenase. Apohydrogenase was first produced in an aerobic cell-free reaction using KC6 cell extract that does not contain the HydE, HydF, and HydG maturation enzymes. The apoprotein solution was buffer exchanged and concentrated into 100 mM Tris-HCl pH 8.0. Anaerobically-prepared cell extract was used to supply HydE, HydF, and HydG. Prior to its reconstitution, the anaerobically-prepared cell extract was either not dialyzed (Figure 6a) or dialyzed overnight versus S30 buffer (Figure 6b). Anaerobic maturation reactions (10–20 μl) included apo-HydA1 (20% v/v), reconstituted anaerobically-prepared cell extract (50–65% v/v), 0.1 mM tetracycline, and supplemented small molecules (GTP, SAM, and/or NAD) based on the particular reaction. Maturation reactions were carried out for 3–5 hrs in an anaerobic glove box at 27°C. Active HydA1 was measured using a methyl viologen reduction assay.



The capability to express apohydrogenase in the presence of O₂ and then mature the enzyme to its holo-form offers two advantages:

- This system provides a means to probe [FeFe] hydrogenase maturation and determine important factors associated with the assembly of the H-cluster. These factors will be vital to understand when engineering *Synechocystis* for *in vivo* expression and maturation to active hydrogenase.
- The *in vitro* maturation results reinforce the possibility of *in vitro* hydrogenase expression in *Synechocystis* with O₂ present, followed by maturation of the hydrogenase during a short phase of anaerobicity or microaerobicity.

N-Terminal Modification of the HydA1 Hydrogenase

Although we have developed an anaerobic cell-free system that is capable of producing active [FeFe] hydrogenase, both soluble and active HydA1 cell-free yields are very low (Figure 7b, HydA1). Moreover, the O₂-tolerant mutant hydrogenase screen utilizes linear DNA templates, which provide even lower yields than DNA plasmids (Figure 7b, HydA1 from Linear Templates). Poor expression of HydA1 from linear DNA templates has resulted in inconsistent screening data.

Recently published work^{5,6} has demonstrated that modification of the N-terminus to include the downstream box of chloramphenicol acetyl transferase (CAT) can lead to increased cell-free expression.

Addition of the first 9 amino acids from CAT to the N-terminus of HydA1 (CAT9-HydA1) increased cell-free expression of soluble hydrogenase 5–8 fold (Figure 7a, 7b). Furthermore, the amount of cell-free active hydrogenase also increases when expressing the gene from either a plasmid or a linear template (Figure 7c).

Secondary structure of mRNA near the N-terminus plays an important role in translation initiation, where GC-rich sequences like that of HydA1 may lead to slow initiation rates. Increased cell-free HydA1 yields when including the CAT downstream box on the N-terminus suggests that translation initiation of this protein may cause low cell-free yields. Also, successful expression of active CAT9-HydA1 from linear templates has led to improved screening for an O₂-tolerant [FeFe] hydrogenase.

Poor expression of HydA1 in *Synechocystis* may also be a problem. Future work will include further examination of N-terminus modification of the HydA1 hydrogenase.

Acknowledgements

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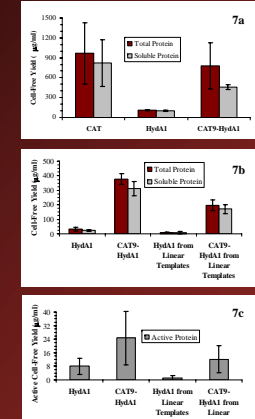


Figure 7: Cell-free synthesis of CAT, HydA1 hydrogenase, and CAT9-HydA1 hydrogenase. Total and soluble yields were measured after aerobic cell-free protein synthesis using KC6 cell extract and plasmids for each of the three proteins (Figure 7a). 15 μl cell-free reactions were carried out with the PANOX-SP system at 37°C for 4 hrs. Total, soluble, and active yields for HydA1 and CAT9-HydA1 were measured from plasmids as well as linear templates in 15 μl anaerobic cell-free reactions (Figures 7b, 7c). The PANOX-SP system along with reconstituted anaerobically-prepared cell extract containing HydE, HydF, and HydG were used for these reactions. Anaerobic cell-free reactions were 3–4 hrs long at 27°C. Total and soluble yields were determined via L-[U-¹⁴C]-leucine incorporation and a TCA precipitation assay. Active yields were measured with a methyl viologen reduction assay.