

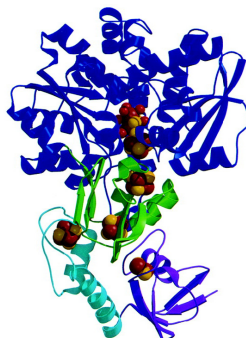
Extending Cell-free Protein Synthesis to Complex Targets: Expression of Active [FeFe] Hydrogenases



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Abstract

The study of many complex proteins has been impeded by the lack of an easily manipulated expression/maturation system. *In vitro* systems allow enhanced control over the translation and folding environment but have not been effective for proteins with complex activation requirements. Important examples include the [FeFe] hydrogenases, oxygen-sensitive metalloenzymes which are integral in microbial ecosystems and have potential for producing renewable hydrogen fuels. We have developed an effective combined transcription and translation cell-free system that activates both bacterial and algal [FeFe] hydrogenases in an *E. coli* cell extract containing heterologous helper proteins. We demonstrate that maturation is assisted by S-adenosyl methionine. This new system enables rapid protein production and activation and is particularly useful for screening populations of mutants. We expect that this same approach will be effective for a broad assortment of proteins, particularly metalloproteins, oxygen-sensitive proteins, and proteins requiring maturation enzymes.



[FeFe] hydrogenase I from *Clostridium pasteurianum*¹

Motivations for [FeFe] hydrogenase study:

- Rapid H₂ production rates
- Applications in biological production of hydrogen fuel
- Unique metal active sites

Difficulties include:

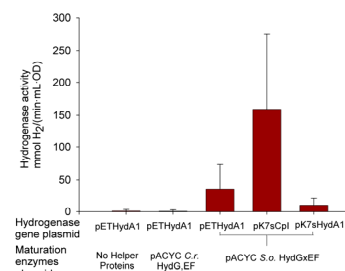
- Extreme oxygen sensitivity
- Complex metal clusters and uncommon ligands
- Activation requires unique maturation enzymes

Cell-free production system advantages:

- Access to and control over production and folding environment
- Small scale for testing variables and screening mutants
- Easy labeling of target protein

Introduction

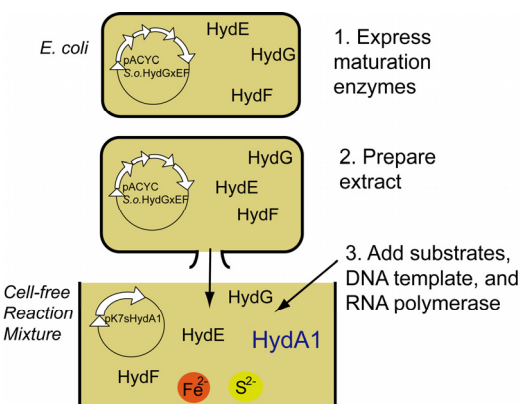
Traditional *in vivo* Production of [FeFe] Hydrogenases



[FeFe] hydrogenase genes were co-expressed in *E. coli* with maturation enzymes from either *Chlamydomonas reinhardtii* or *Shewanella oneidensis*. Enzymes from *S. o.* were able to mature both algal and bacterial hydrogenases and were more effective than enzymes from *C. r.* Traditional *in vivo* expression of [FeFe] hydrogenases resulted in low yields and high variability. A cell-free production system was developed to empower study.

Results

Cell-free Production of [FeFe] Hydrogenases



1. Express maturation enzymes

2. Prepare extract

3. Add substrates, DNA template, and RNA polymerase

Two major modifications to the cell-free system were required for production of [FeFe] hydrogenases:

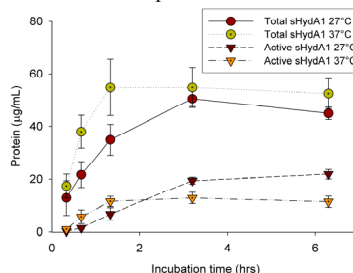
1. Heterologous maturation enzymes were expressed during cell extract growth
2. Extracts were prepared anaerobically

This system contains functional elements from three different organisms: *E. coli*, the eukaryotic alga *Chlamydomonas reinhardtii*, and the gram-negative bacteria *Shewanella oneidensis*.

Modified extracts were used for cell-free protein production within an anaerobic chamber. Both algal and bacterial hydrogenases were expressed and matured.

Production of Algal and Bacterial [FeFe] hydrogenases	HydA1	Cpl
Total Protein (mg/mL cell-free reaction)	45 ± 3	12 ± 3
Hydrogenase Activity (nmol H ₂ /(min·mL cell-free reaction))	20 ± 2	3 ± 0.2
Hydrogenase Specific Activity (mmol H ₂ /(min·nmol total protein))	21 ± 2	16 ± 4

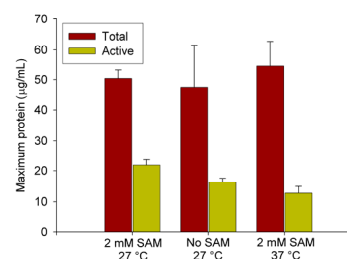
Kinetic profiles of hydrogenase production



Modified anaerobic extracts containing HydG, HydE, and HydF maturation enzymes can produce ~50 µg/mL total HydA1 protein and ~20 µg/mL active protein.

Kinetic profiles indicate that active protein accumulation is limited by something other than protein production.

Variables affecting expression and maturation of hydrogenase



Cell-free reactions can be conducted at the 15 µL scale in parallel to test the effects of multiple reaction variables. The following variables positively affected active protein accumulation:

57% increase when reactions were incubated at chamber temperature (27 °C) versus 37 °C.

35% increase when 2 mM S-adenosyl methionine was supplemented in reactions

Conclusions

- Maturation enzymes from *Shewanella oneidensis* are capable of activating both algal and bacterial hydrogenases and work well in *E. coli*
- Cell-free extracts can be modified by the expression of heterologous maturation enzymes to assist target protein folding
- Cell extracts can be prepared anaerobically for use with oxygen-sensitive targets.
- 20 µg/mL of active HydA1 produced under optimal conditions

Acknowledgments

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