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Research Plan

a. RATIONALE:

Oxidoreductases are enzymes used in a massive range of applications. Some include synthesis of chiral compounds, such as chiral alcohols, aldehydes and acids; preparation and modification of polymers, especially biodegradable or biocompatible polymers; biosensors for a variety of analytical and clinical applications; and degradation of organic pollutants. Capture of CO2 by employing oxidoreductases can be a sustainable method to combat excess CO2 overwhelming our environment today. The direct capture of carbon dioxide using enzymes such as formate dehydrogenase could be the ultimate carbon neutral process.

However, if these oxidoreductase enzymes can be used as biocatalysts you need an easy, inexpensive way to separate out its cofactor from its product. My research will be the stepping-stone to more efficient use of oxidoreductases as biocatalysts and a maintainable process to limit excess CO2 in the atmosphere.

b. RESEARCH QUESTION(S), HYPOTHESIS(ES), ENGINEERING GOAL(S), EXPECTED OUTCOMES:

Can 3’NADP be used as a novel model for NAD-capped RNA? If mutations are made to CboFDH then, 3’NADP will show activity with it. In this study, formate dehydrogenase from Candida boidinii (CboFDH) will be mutated through site-saturation mutagenesis, changing its cofactor specificity to demonstrate activity with 3’NADP, providing sufficient evidence for activity with NAD-capped RNA. A phosphate is attached in the same location in 3'-NADP as the RNA attaches in the NAD-capped RNA therefore, 3’NADP should be a successful model.

c. PROCEDURES/METHODS/DATA ANALYSIS

Organisms:

Formate Dehydrogenases from methylotrophic yeasts are easy to manipulate and are highly conservative. These yeasts are NAD dependent and have been used in developing NAD+ regeneration systems in organic synthesis to produce added-value products. The FDH will be extracted from live cultures of *Candida boidinii.*

Resuspending CboFDH Gene Fragment:

A dried CboFDh gene (Sigma-Aldrich) will be used and centrifuged for 5 seconds at 300xg. 100ul of ddH20 will be added to reach a concentration of 10ng/ul. The solution will be incubated in 50^{o}C water for 20 minutes then, vortexed.

TOPO Cloning Reaction:

Using the TOPO cloning kit (ThermoFisher Scientific), pipette 2 ul of the resuspended CboFDH gene, 1 ul of TOPO salt solution, 2 ul of ddH20, and 1 ul of TOPO vector into a PCR tube. Mix the reaction gently at room temperature, then store the reaction overnight at -20^{o} C.

Transforming TOPO reaction into DH5alpha:

DH5alpha E.coli cells are specifically engineered to have the greatest transformation efficiency. Before transformation, all materials including ligation products, cuvette, SOC media, and tips are to be pre-cooled for 10 min. Begin by thawing a vial of (C2987H) NEB alpha competent E.coli cells and thaw on ice for 10 minutes. Near a flame, add 2 ul of TOPO reaction product to a vial of competent cells and flick the tube 5 times to mix, then incubate on ice for exactly 30 minutes. Place the tube in a 42oC water bath for 30 seconds. Place on ice for 5 minutes then, pipette 950 ul of room temperature SOC into the mixture and pipette mixture into a falcon tube. Place the falcon tube at 37^{o}C in a shaking incubator for 1 hour. The contents of the tube are to be spread onto LB-Kanamycin plates using glass beads and incubated overnight at 37^{o}C.

Analyzing TOPO-CboFDH clones:

Isolated colonies are going to be chosen from the LB-Kanamycin plates in the TOPO reaction, to create bacterial overnights. These colonies should be scraped into a falcon tube with 3ml TB along with 3 ul Kanamycin stock for the overnights. The following day 500 ul of each overnight are to be mixed individually with each 500 ul in a 2ml cryotube and placed in an -80^{o}C. The remainder bacteria should be miniprepped. The overnights should be spun down at 5000 rpm for 5-10 minutes and the supernatant dumped. 250 ul of buffer P1 is going to be added to each pellet and vortexed till the mixture is resuspended. Then, 250 ul of buffer P2 is added, capped the Eppendorf, and inverted 4-6 times to mix. Buffer N3 is added and mixed into solution by inverting 4-6 times. The solution is centrifuged for 10 minutes at 13,000 rpm and the supernatant is pipetted into a spin column. The column should then be centrifuged for 1 minute and flow through discarded. 750 ul of PE is added to the column, centrifuged and flow through discarded, this step should be done twice. The empty column is to be centrifuged for 4 minutes, and the tip from the spin column taken and placed in a new Eppendorf tube. 30 ul of warmed ddH20 should be pipetted into the center of the filter, left standing for 1 minute, and centrifuged for 4 minutes at 13,000rpm. The concentration of the miniprepped samples will be checked using nanodrop quantification and sent for DNA sequencing with the forward and reverse primers to confirm the gene was correct.

PCR amplification of CboFDH from TOPO-CboFDH:

Forward and reverse primers for TOPO-CboFDH are going to be diluted with ddH2O to create 10 mM solutions: adding 382 ul to the forward primer and 313 ul to the reverse primer. While PCR (polymerase chain reaction) is completing, an agarose gel is to be made to demonstrate the

success of the PCR, using 10 ul of 1.0kB DNA ladder as DNA standard. Purple loading dye from BioLabs will be used in each sample to stain the DNA for visibility. DNA, restriction digests and PCR products will be visualized on a 1% (w/v) agarose gel in 1x TAE buffer (40 mM Tris acetate, pH 8.2, 1 mM EDTA). Then, the samples that showed successful cloning into DH5 alpha E. coli cells will be transformed, using the same transformation method as previously discussed.

PCR linearization of pET-28a-TADH:

The linear sequence of FDH calls for the complement of a linear pET-28a-TADH vector. After running general PCR protocol, the sample will be subjected to Dpnl treatment. Since Dpnl cleaves only when a recognition site is methylated, restriction enzyme digestion will be able to be performed with reliable restriction endonucleases.

Transformation into BL21(DE3) Competent Cells:

After proof of pET-28a linearization, the linear CboFDH will be combined with the pET28a to create a circular plasmid (pET28a-CboFDH). This will be transformed into BL21, for its key genetic markers and inducibility of protein expression. The BL21(DE3) competent cells are from New England Biolabs. The bacteria will then be grown on LB-kanamycin plates. The following day the bacteria will be miniprepped. An agarose gel will be made to demonstrate the success of the PCR using 10 ul of 1.0kB DNA ladder as DNA standard. Purple loading dye from New England BioLabs (Ipswich, MA) will used in each sample to stain the DNA for visibility.

Site-saturation mutagenesis of CboFDH:

Six rounds of PCR are going to be used to mutate six CboFDH mutants: D195A, D195N, D195Q, D195S, Y196H, and D195S-Y196H. They will then be transformed into BL21 (DE3) competent cells. Hi-Fi DNA assembly Master Mix, Q5 site-directed mutagenesis kit, and E. coli BL21(DE3) expression cells will be from New England Biolabs.

Mutation Analysis:

Samples of each of the six mutations will be miniprepped and each mutation concentration will be checked using nanodrop quantification. Samples of each mutation were then sent for DNA sequencing. Using Benchling, the sequence sent back of the mutated CboFDH will be compared with the expected mutated sequences.

Expression of pET28a-CboFDH using IPTG:

The vectors containing the genes of interest will be transformed into E. coli BL21(DE3) cells and plated on Luria-Bertani (LB) agar plates containing kanamycin. For the FDH seed culture, LB (50 ml) supplemented with antibiotic (100 μg/μl Amp for FDH-N or 50 μg/μl Kan for FDH-C) will be inoculated with a single colony and incubated overnight at 37°C with shaking at 230 r.p.m. The seed culture (10 ml) will then be diluted into LB (1 l) supplemented with antibiotic and the culture will be grown at 37°C. When the culture reaches an OD600 of ~0.6, isopropyl β-D-1-thiogalactopyranoside (IPTG) will be added.

Purification of pET28a-CboFDH protein:

After the CboFDH is in frozen pelleted form, 40mM imidazole buffer will be added to aid in the resuspension of the pellet. Once thawed, the pellet will be vortexed gently then sonicated. The sample should be centrifuged for one hour at 7000xg. The supernatant from the sample after sonication will be separated and kept for the column purification. 3ml of Ni-NTA resin (Clontech) will be poured into a column and the manufacturers protocol will be followed. The supernatant is applied onto the Ni-NTA column and washed with 2 column volumes (CV) of working buffer. Column will be washed with 30 mM imidazole in working buffer to remove impurities. A supernatant, 0%, 5%,15%, 20%, 40% and 100% fractions will be collected for each mutation and unmutated CboFDH from purification through the column. The fractions of the unmutated CboFDH are going to be tested in a protein gel. A Novex Hi-density TBE sample buffer will be used as a protein stain and Novex Sharp Pre-stained Protein Standard will be used as a ladder.

Enzyme Activity Assays:

A multi-well plate will be filled testing each of the CboFDH mutations. Using a SpectraMax M2 Spectrophotometer (Molecular devices) absorbance is monitored at 340 nm with a background absorbance at 800 nm. Enzyme activity will be characterized in a NuPAGE MES SDS running buffer. Kinetic measurements are to be recorded every 20 seconds and change in absorbance at 340 nm will be fit to a linear model.

d. BIBLIOGRAPHY:

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Post Research Summary:

There were no changes were made to the research plan.