RURALS: Review of Undergraduate Research in Agricultural and Life Sciences

Volume 1 | Issue 1 Article 3

2006

Development of a System for Directed Evolution of *Arabidopsis* Formate Dehydrogenase to Utilize NADP as a Cofactor

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Cover Page Footnote

The authors are grateful for UCARE grants from the University of Nebraska to BLP and JRW. JRW is from Columbus, NE and graduated from the University of Nebraska Biochemistry program in 2005; he is now a M.S. Student in the Department of Agronomy and Horticulture. BLP is from Grand Island, NE and graduated from the U.N. Biochemistry program in 2006; she is now a graduate student in the Biosciences Program at the University of Iowa. The faculty sponsor for this project is Dr. Patricia L. Herman, Research Assistant Professor, School of Biological Sciences, University of Nebraska-Lincoln, Lincoln, NE 68588-0118; E-mail address: pherman1@unl.edu; Phone: 402-472-6084. Review coordinated by Professor Kenneth Nickerson, School of Biological Sciences, University of Nebraska-Lincoln.

1. Introduction

Formate dehydrogenase (FDH; EC 1.2.1.2) is a NAD-dependent enzyme distributed among methylotrophic bacteria and yeast as well as in plants. This enzyme catalyzes the reversible oxiion of formate to CO_2. Full or partial cDNA sequences encoding FDH have been reported for a number of plant species including *Arabidopsis*, potato, rice, pine, maize, barley and oak. The enzyme is localized in the mitochondrion (Oliver, 1981; Colas des Francs-Small et al., 1993) and can comprise as much as 9% of the total mitochondrial protein in nonphotosynthetic tissues (Colas des Francs-Small et al., 1992).

The best-studied plant FDH at the biochemical level is that in *Arabidopsis thaliana*. The enzyme was originally purified from leaf mitochondria (Li et al., 2000). It was subsequently noticed that the *Arabidopsis* FDH is synthesized with a signal sequence that is predicted to result in import by both mitochondria and chloroplasts. A chloroplastic location was confirmed by the presence of the activity in Percoll-purified chloroplasts and by immunogold localization and electron microscopy (Herman et al., 2002). Our research group confirmed that the *Arabidopsis* FDH is specific for NAD as a cofactor (Olson et al., 2000).

The ability to manipulate the sequence of an enzyme and potentially change the substrate specificity, pH optimum, enantiomeric selectivity or stability has been an area of basic research with applied implications. One approach to modify the properties of enzymes is 'directed evolution' (Farinas et al., 2001; Schmidt-Dannert, 2001). This involves a series of *in vitro* manipulations that mimic the random process of natural mutation, but done in a punctuated fashion, coupled with a selective screening to uncover the genetic changes producing desirable enzymatic properties. Generally, the process involves a library of genetic variants produced by error-prone PCR replication. Because most mutations are neutral or deleterious with respect to the desired catalytic property, the process generally employs low mutation frequencies and multiple rounds of selection. Directed evolution has been successful in obtaining new enzymes with desirable properties such as modified substrate specificity (Meyer et al., 2002; Dixon et al., 2003).

We are interested in whether it is possible to use FDH to alter the metabolism of the plant leaf. It has been demonstrated *in vitro* that FDH can efficiently reduce CO_2 with NADH if the formate generated is removed by other enzymatic reactions (Obert and Dave, 1999). The plant cell mitochondrion is different from other cellular compartments in that it provides high NADPH/$NADP^+$ ratios that could facilitate reduction reactions. Transformation of plants with a NADP-utilizing FDH targeted to mitochondria may make it possible to provide a sec-

ond site for CO_2 fixation. Formate in plant cells is rapidly assimilated by 10-formyl-tetrahydrofolate (THF) synthetase (Prabhu et al., 1996). Increased flux of formate to 10-formyl-THF increases the availability of formyl- and methylene-THF, the latter metabolite being used by the serine hydroxymethyltransferase to convert glycine to serine. For every mitochondrial methylene-THF formed from formate, there would be one fewer glycine molecule processed by the glycine decarboxylase during photorespiration. Thus, the ability to produce and assimilate a low level of formate within the plant cell has the potential to improve photosynthetic efficiency and use the currently wasteful photorespiratory cycle as a second cycle for production of reduced carbon (Ramberg et al., 2002). The goal of our research is to develop a directed evolution system to generate an altered FDH with a high affinity for NADP as a cofactor and use it to transform plant cells, testing the hypothesis that such an activity may have an impact on photosynthetic efficiency.

2. Materials and Methods

2.1. *Plasmid constructs*.

A full length FDH cDNA previously isolated from an *Arabidopsis* λPRL2 library and characterized (Olson et al., 2000) was cloned as a *Sal*I/*Not*I fragment into the pET28a expression vector (Novagen) to generate a construct designated as FDH-pET28a (Herman et al., 2002). The 87 bp sequence that codes for a transit peptide was subsequently deleted from the 5' end of the FDH cDNA in this construct. This modified plasmid was used as the template in a PCR reaction with primers that were designed to introduce an *Afe*I restriction site followed by a C nucleotide (i.e., AGCGCTC) at the 5' end of the FDH coding sequence and an *Eco*RI restriction site at the 3' end. These two restriction sites are not present elsewhere in the *Arabidopsis* FDH cDNA. The *Afe*I restriction site was positioned so that digestion of the PCR product with *Afe*I produced a blunt 5' end (GCTC) that was in frame with the coding sequence for the mature FDH polypeptide. The amplified DNA was sequenced to verify its accuracy and then ligated as an *Afe*I/*Eco*RI fragment into the *Eco*RV and *Eco*RI sites of the prokaryotic expression vector pETBlue-1 (Novagen). The resulting construct, designated pFDH-0, has an ATG initiation codon that is optimally positioned relative to the ribosome-binding site of the vector to ensure strong expression of the FDH protein in appropriate *Escherichia coli* strains.

2.2. *Mutagenesis*

Using pFDH-0 as a template, mutations were induced using the Diversify PCR Random Mutagensis Kit (BD Bioscience) and primers designed to maintain the *Afe*I and *Eco*RI sites (5'-GAG ATA TAA GCG CTC GCA TCT TC -3' and 5'-CCC GGG CAG GAA TTC CTC AAA AGC -3'). Mutagenesis was carried out with 0.16 mM $MnSO_4$ in a 30 cycle program using a 30 s denaturation at 94° and 75 s combined annealing and extension step at 64°C. Following PCR, the products were purified using a Qiaquick PCR purification kit (Qiagen). The putative mutant sequences were digested with *Afe*I and *Eco*RI and ligated into pETBlue-1 cut with *Eco*RI and *Eco*RV (http://www.emdbiosciences.com/docs/docs/PROT/TB055.pdf). The ligation reaction mixture was transformed into the *E. coli* expression strain Tuner (Novagen) and cells were grown at 37°C in LB medium containing 50 μg mL^{-1} carbenicillin.

2.3. *Assays*

Putative mutant colonies were selected and cells were placed in individual wells of a 96-well microplate containing 200 μL LB with 50 μg mL^{-1} carbenicillin. The microplates were shaken at 150 rpm for 18 hours at 37°C. A replica plate was made of each original microplate and stored at 4°C. Prior to assay, the microplate was centrifuged at 2000 x *g* for 20 min and the supernatant fraction discarded. Cells were lysed by addition of 100 μL BugBuster (Novagen). The microplate was shaken for 15 min at 200 rpm and room temperature and then centrifuged at 2000 x *g* for 40 min. The supernatant fractions were transferred to a new 96-well microplate to be assayed.

The NAD^+ assay for FDH was in a 200 μL volume using the conditions previously described (Baack et al., 2003), except that 0.1 M MOPS (pH 7.5) buffer was used rather than phosphate buffer. NAD(P)H formation was measured with a Bio-Tek PowerWave XS plate reader at 340 nm and 37°C. The assay for NADP-linked FDH was performed similarly, but with 4 mM $NADP^+$ rather than 1 mM NAD^+.

Cell lines that appeared to possess significant NADP-FDH activity were grown in LB medium containing 50 μg mL^{-1} carbenicillin, lysed with BugBuster and assayed for activity in a Shimadzu UV1601 spectrophotometer. Protein concentrations were determined using the method of Bradford (Bradford, 1976).

3. Results and Discussion

One concern in developing our protocol for directed evolution of FDH was whether there would be a significant number of transformed cells that contained a plasmid without an insert sequence following the ligation and transformation of the products generated by random PCR. To test for this potential problem, we tested several hundred colonies from several different ligations in the NovaBlue strain using blue-white screening (Sambrook et al., 1989) to determine the frequency of cells containing plasmids without insert sequences. Over 90% of the cells selected with carbenicillin contained a recombinant insert in the plasmid as judged by formation of white colonies. Thus, we felt that we could routinely omit this step. Five of the white colonies were selected at random for sequencing and a mutation frequency of approximately 1.5 per thousand base pairs was found. This would produce a population of PCR products with approximately one mutation each for screening.

An additional concern about developing the screening for putative mutants developed in this project was whether the growth of cells and assay of FDH in the 96-well microplates would be sufficiently consistent to permit identification of lines with significantly different activities. The reproducibility of the assay was confirmed by creating two plates (174 wells) with identical FDH-0 enzyme extracts in each well, resulting in an average specific activity of 27.8 mU mg^{-1} ± 0.58 (SEM). Thus the growth of cells and expression of the enzymes appears to be sufficiently consistent that we should be able to expect to identify a mutant with an increase or decrease of 25% in activity.

Our initial screen for mutants involved the growth and assay of approximately 1300 lines derived from random PCR amplification of the pFDH-0 sequence. These assays identified approximately 100 putative mutants that had very small rates of activity with $NADP^+$. Of these cell lines, four were sufficiently consistent that they were grown in 5 mL of LB broth and extracts assayed in a 1 mL volume to confirm whether they would catalyze the oxidation of formate with $NADP^+$. One mutant line, pFDH-18, has been found to possess reproducible $NADP^+$-FDH activity. The kinetic constants of this mutant (from three separate determinations) are compared with those of the wild-type in Table 1. The NAD^+ K_m value of pFDH-18 is approximately three times greater than that of pFDH-0. This result is consistent with a previous report which demonstrated that an increase in the affinity of the *E. coli* isocitrate dehydrogenase for $NADP^+$ results in a decrease in affinity for NAD^+ (Hurley et al., 1996).

Table 1. Kinetic constants of the wild-type and pFDH-18 mutant *Arabidopsis* formate dehydrogenase.

Plasmid	K_m NAD^+	K_m $NADP^+$	K_m Formate (with NAD^+)	K_m Formate (with $NADP^+$)	v_{NADP+} / v_{NAD+}*
pFDH-0 (Wild type)	0.065 mM	----	10 mM	----	0
pFDH-18 (Mutant)	0.18 mM	4.2 mM	25 mM	118 mM	0.04

* Determined with 100 mM formate and 1 mM NAD^+ or 4 mM $NADP^+$.

Sequence analysis of pFDH-18 showed a single T→G mutation which changes the isoleucine (Ile) residue at position 188 in the wild-type enzyme to a methionine. This particular Ile residue is conserved in the known FDH sequences from higher plants and is located in the region of the enzyme that contains the binding domain for the NAD cofactor (from residues 156 to 338).

Thus, we have developed a system capable of screening large numbers of putative mutants in an effort to use directed evolution to modify the cofactor specificity of the plant formate dehydrogenase enzyme and validated that it is capable of identifying authentic mutants. We plan to use this system in an effort to examine whether such a mutant enzyme can impact photosynthetic efficiency in plants.

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