Enzymes Nanoconfined and Electrified for Fundamental Discovery: Antibiotic Resistance

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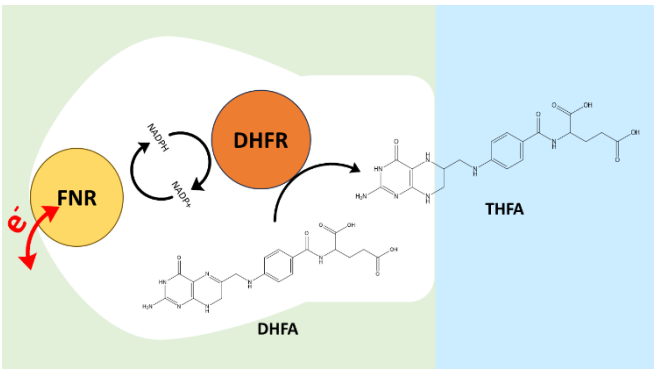
Introduction: The electrochemical leaf (e-Leaf) is a new electrochemical platform to drive and control enzymes using electricity in a nanoconfined space that is more like their natural environment. This is due to two key factors, the use of a photosynthetic enzyme, ferredoxin $NADP^+$ reductase (FNR) and the use of a highly porous metal oxide electrode (Scheme 1). In photosynthesis, FNR receives light-excited electrons (e^-) from ferredoxin and uses them to reduce $NADP^+$ to NADPH. The NADPH is then used by the Calvin cycle, the biosynthetic enzyme cascade that makes glucose in plants. In the e-Leaf, FNR is loaded into a porous electrode and e^- are provided directly to FNR's active site flavin cofactor from the electrode, by applying a potential (voltage); FNR then uses the electrons to catalyse the interconversion of $NADP^+$/NADPH. Extended enzyme cascades are also trapped in the pores which, like the Calvin cycle use the NADPH. The cascade must contain one enzyme that needs NADP(H) to allow it to couple to FNR. In this project, first, a reductive aminase (RedAM) enzyme was coupled to FNR in the e-Leaf as an exemplar system.[1] Then, the bacterial enzyme, dihydrofolate reductase (DHFR), from the folate pathway was monitored in the e-Leaf. DHFR catalyses the reduction of dihydrofolic acid (DHFA) to tetrahydrofolate (THFA) using NADPH (Scheme 1).



Scheme 1. DHFR coupled to FNR in the e-Leaf: pore (white) surrounding bulk solution (blue).

Aims of the project: The aim of this project is to monitor DHFR in the e-Leaf. Preliminary work by Dr Megarity showed that DHFR behaved differently when confined in the e-Leaf electrode compared to dilute solution. My objective then was to find out why that was happening. This will help achieve the overall aim of studying the bacterial folate pathway in the e-Leaf for antibiotic resistance research.

Methods

Expression and purification of FNR: FNR was overexpressed in *Escherichia coli* and purified by nickel-affinity chromatography as described.[2] Bacterial DHFR had been prepared previously and its concentration was measured using a nanodrop spectrophotometer (280 nm).

Electrode Fabrication by Electrophoretic Deposition: Conductive supports were used (indium tin oxide glass, titanium, and graphite disc electrodes), onto which particles of indium tin oxide (ITO) were deposited by electrophoretic deposition[3] to form the porous layer of the working electrodes.

Enzyme Loading: FNR and DHFR were loaded into the electrode pores by either drop casting a concentrated droplet of enzymes onto the surface and incubating for 30 minutes at 4°C or by sinking an electrode into a continuously stirred solution containing the enzymes for 30 minutes at 4°C. In both cases, before use in an experiment, the excess enzyme molecules were rinsed off so that only those in the pores remained.

Cyclic Voltammetry: A three-electrode system (working (ITO), counter (Pt) and reference electrode (Ag/AgCl)) was used. In cyclic voltammetry, the potential applied to the working electrode is swept linearly between two limits and the enzyme rate is directly measured as electrical current as a function of the potential, which is the driving force.

Results and outcomes

Expression and Purification of FNR

Figure 1 shows the purification gel for FNR. Lane 1 shows the overexpressed enzyme in cells and purified elutions are indicated. The enzyme was bright yellow due to its flavin cofactor, and its concentration was 0.47 mM after desalting and concentration.

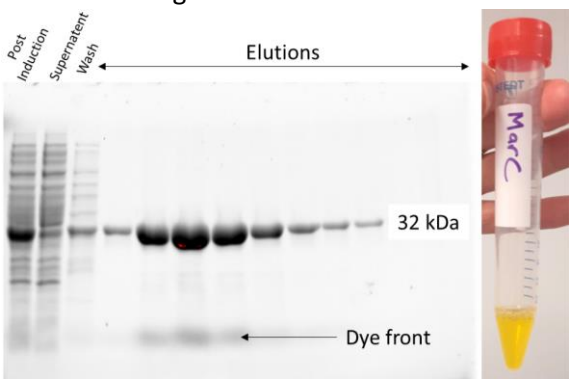


Figure 1. Expression and purification of FNR monitored by SDS gel electrophoresis.

A model enzyme in the e-Leaf: Reductive aminase

Before starting the experiments with DHFR, we evaluated a reductive aminase (RedAm) enzyme as our model system since it is active in the e-Leaf and is well characterised. Figure 2 shows catalysis by RedAm coupled to FNR measured by CV. RedAm and FNR were coloaded in the pores with only the cyclopropylamine and $NADP^+$ present in the solution from the start. The black trace corresponds to the interconversion of $NADP^+$/NADPH by FNR. The red trace shows the coupled activity of RedAm upon addition of cyclohexanone (reaction scheme shown above CV).

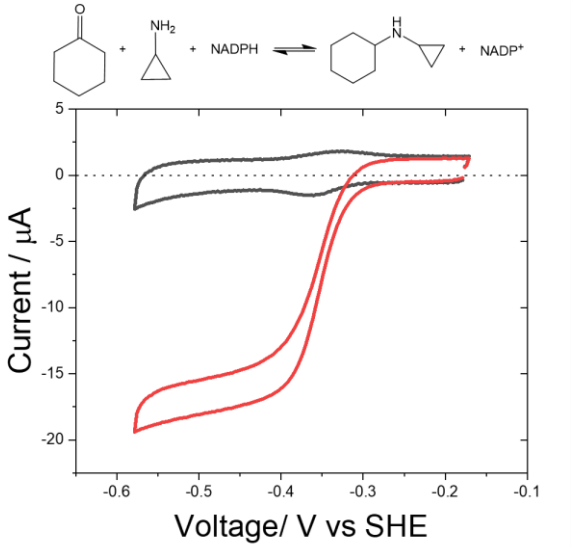


Figure 2. Reductive amination catalysed by RedAm coupled to FNR in the e-Leaf (red trace). 20 µM $NADP^+$, 20 mM cyclohexanone and 50 mM propylamine, scan rate 1 mVs^{-1}

DHFR: UV-VIS and the e-Leaf

The activity of DHFR was first tested by UV-VIS spectroscopy by measuring the decrease in absorbance at 340 nm due to the depletion of NADPH. The enzyme was active, and the reaction went to completion. Different conditions were tested for DHFR in the e-Leaf including pH, enzyme loading and substrate concentration. The best activity of DHFR in the e-Leaf is shown in Figure 3 (red trace). As it can be observed, the catalytic current due to DHFR-FNR coupling is significantly lower than that for RedAm. Our current hypothesis to explain this is that DHFR's product release, which is the rate limiting step, is even more limiting in the pore environment because, once released, it stays in the pore and can bind again with DHFR.

Future directions: The next step will be to include another enzyme that uses the product of DHFR as substrate to prevent it from binding again to DHFR. Ultimately, the whole bacterial folate pathway will need to be studied.

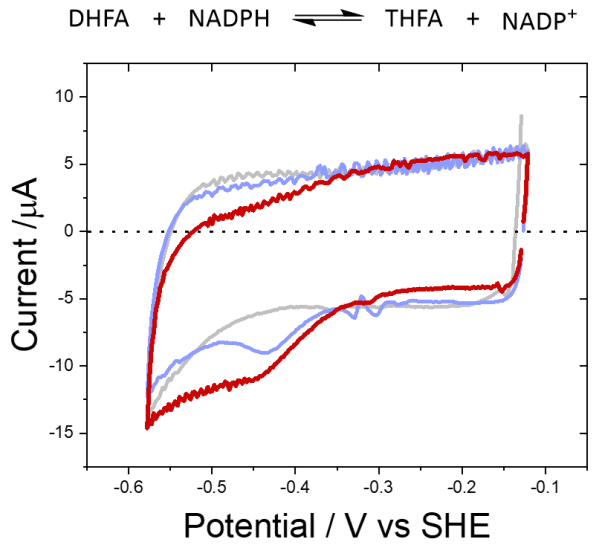


Figure 3. Reduction of DHFA catalysed by DHFR coupled to FNR in the e-Leaf (red trace). 100 µM $NADP^+$, 0.2 mM DHFA.

Departures from original project plan: We did not complete the dilute solution kinetic analysis for DHFR beyond initial activity tests because we focused our work in the e-Leaf to achieve a promising result. We added the work on RedAm as a parallel study.

Value of the studentship to the student and the research group: In Dr Megarity's group, I have developed many lab skills, such as, cell culture, electrochemistry, and anaerobic glovebox work. I have also enriched many transferable skills, such as, time management, an essential skill in the lab due to the timed experiments I conducted. I also learnt how to keep detailed lab books which is essential as a scientist. As time passed, I felt more comfortable and secure in the lab. I also improved my scientific communication skills as we analysed our experiments. I also met a lot of other PI and Ph.D. students, which gave me an insight into their experience. This reassured me of my decision to end up in academic research. All the data I collected from my experiments is of significant help to Dr Megarity's group. I will help them to understand a little bit the odd behaviour of DHFR in the e-Leaf.

References:

1. Megarity *et al.*, Angewandte Chemie International Edition, 2019, 58, 4948-4952.

2. Megarity *et al.*, ChemCatChem, 2019, 11, 5662-5670.

3. Siritanaratkul *et al.*, Chemical Science, 2017, 8, 4579-4586.