Graduate Research Plan Statement

Introduction

Many metabolites are reactive and unstable, making them prone to undesired chemical modification outside of the intended pathways[1]. **While metabolism as a whole is well-studied, the biochemical mechanisms of managing such reactive metabolites are not.** Proteins which are closely metabolically involved with each other can frequently be found in protein-protein interactions which are essential for nearly all cellular function. It is currently believed that most, if not all, proteins participate in protein-protein interaction networks[2]. Such a network suggests the possibility of metabolic substrate channeling, in which a metabolite travels from one enzymatic active site to another without freely diffusing into the surrounding medium. Evidence for substrate channeling has been observed in enzymes of many major biochemical pathways and is being increasingly recognized as foundational to metabolic regulation[3]. Through substrate channeling, an intermediate metabolite may be retained for use in a specific pathway, protected from degradation, or prevented from causing damage to the cell[4].

The goal of my proposed research is to discover mechanisms by which unstable metabolites are managed in biochemical systems. This research will provide insight into cellular control over reactive metabolites, protein-protein interactions, and substrate channeling. To achieve this goal, I will uncover mechanisms of substrate channeling for the reactive metabolite Δ^1-pyrroline-5-carboxylate (P5C). P5C is an unstable intermediate at the intersection of proline, glutamate, and ornithine metabolic pathways (Figure 1). P5C has been shown to react with other metabolites and inhibit enzymes and is linked with human disease including hyperprolinemia type II[5].

Under physiological conditions, P5C exists in dynamic equilibrium with glutamic semialdehyde (GSA) via spontaneous nonenzymatic hydrolysis and condensation reactions. In the proline biosynthetic pathway, P5C serves as an intermediate for production of proline from glutamate. Glutamate is reduced to GSA by P5C synthetase (P5CS), then P5C is reduced to proline by P5C reductase (P5CR). Similarly, the glutamate biosynthetic pathway uses P5C as an intermediate in the production of glutamate from proline. Proline is oxidized to P5C by proline dehydrogenase (PRODH), then GSA is oxidized to glutamate by P5C dehydrogenase (P5CDH). In an alternative pathway, P5C can be produced or consumed by ornithine aminotransferase (OAT), which uses ornithine as a reactant or product for P5C production or consumption. Under normal physiological conditions, OAT functions in the "forward" direction from ornithine to P5C, however, under extreme dysregulation, OAT may catalyze the opposite "reverse" reaction from P5C to ornithine[6].

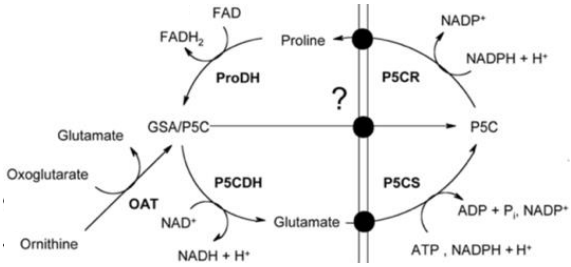

Figure 1. Involvement of P5C with proline, glutamate, and ornithine metabolism. Adapted from Stránská et al[6].

Aim 1: Protein-protein interactions among enzymes of the proline/glutamate/ornithine pathways.

Existing research already supports protein-protein interactions between PRODH and P5CDH[7]. However, the involvement of other enzymes in the proline, glutamate, and ornithine pathways is unknown. This aim will focus on potential protein-protein interactions between P5CS–P5CR, OAT–PRODH, and OAT–P5CDH. Because of the current discord surrounding the cellular locations of P5CS and P5CR (e.g., mitochondrial vs. cytosolic), OAT–P5CS and OAT–P5CR complexes will also be considered. In fact, the expression in some organisms of ornithine cyclodeaminase, which directly catalyzes ornithine to proline, supports potential protein-protein interactions between OAT and P5CR[8].

Enzymes used in these studies will be expressed as His-tagged proteins, as done previously with PRODH and P5CDH[7]. Stable protein-protein interactions will be examined through coimmunoprecipitation with anti-His-tag antibodies and supplemented with pull-down assays via Ni-NTA chromatography. In each experiment, the identification of specific proteins will be accomplished through Western blot.

Transient protein-protein interactions will be observed through surface plasmon resonance (SPR) at the BIAcore 3000 instrument maintained by the Nanomaterials Characterization Core Facility at the University of Nebraska Medical Center. For SPR, an enzyme's His-tag will be used to anchor the protein to a Ni-NTA sensor chip. SPR will allow the characterization of protein-protein interaction association and dissociation constants. For cellular characterization of protein-protein interactions, fluorescence resonance energy transfer and yeast two-hybrid system experiments will be carried out[2,7].

Aim 2: Kinetics of P5C channeling among enzymes of the proline/glutamate/ornithine pathways.

Even in the case of weak, transient protein-protein interactions, substrate channeling may occur. Substrate channeling of P5C has been identified in the glutamate biosynthetic pathway but has yet to be characterized among other protein pairs of the proline, glutamate, and ornithine pathways[7].

Previous studies with PRODH and P5CDH have used a free diffusion two-enzyme model to simulate reaction progress curves. In these models, the presence of substrate channeling can be inferred by comparing theoretical non-channeling versus experimental differences in transient time[7]. In addition, P5C trapping studies will be carried out to support results from the simulated progress curves. In these experiments, *ortho*-aminobenzaldehyde (*o*AB) is conveniently used to "trap" P5C in a spectrophotometrically-monitored *o*AB–P5C complex. In the presence of P5C channeling, less P5C will freely diffuse to be complexed with *o*AB as compared to a negative non-channeling control. In addition, I will use stopped-flow kinetics to measure the transient time of P5C channeling between the enzymes.

Intellectual Merit

My proposed graduate research plan to examine unstable metabolite management is a natural continuation of research I have been involved with in the Becker lab at the University of Nebraska-Lincoln. Previously, I conducted research investigating P5C channeling in the glutamate biosynthetic pathway, so I am well-prepared to expand into protein-protein interactions and broader aspects of substrate channeling. I have experience with fundamental methods used for protein and metabolic research, including protein overexpression, purification, UV-visible spectroscopy, stopped-flow, and steady state enzyme kinetics.

This research will be well-supported in the Becker lab at the University of Nebraska-Lincoln, home of the Center for Biological Chemistry and the Redox Biology Center. My projects will be pursued in collaboration with existing structural biology partners in the Tanner lab at the University of Missouri.

Broader Impacts

It is estimated that over 80% of proteins rely on protein-protein interactions[2], and substrate channeling has been identified in many major metabolic pathways[3]. My research will provide specific knowledge in unstudied interactions and channeling between proteins of the proline, glutamate, and ornithine pathways, offering insight into the biochemical methods of unstable and reactive metabolite management. Additionally, this research will serve as a case study to lay the foundation for metabolite management experiments in other major pathways. Research surrounding P5C has implications for biological mechanisms of metabolism relevant to all life.

Outside of strict academics, I will leverage this graduate research in a way that benefits the scientific community and the general public. I will present results at regional and national conferences and publish using accessible language in open-access journals. Through this research, I will take advantage of important mentorship opportunities. Mentoring undergraduate or beginning graduate students in the lab will allow me to help develop the next generation of scientists as I provide a rigorous yet supportive environment to foster academic, professional, and personal growth.

References

[1]Lerma-Ortiz et al. *Biochem. Soc. Trans.* 2016. [2]Berggård et al. *Proteomics.* 2007. [3]Sweetlove et al. *Nat. Commun.* 2018. [4]Liu et al. *Arch. of Biochem. & Biophys.* 2017. [5]Farrant et al. *J. Biol. Chem.* 2001. [6]Stránská et al. *Plant Sig. & Behav.* 2008. [7]Sanyal et al. *J. Biol. Chem.* 2015. [8]Goodman et al. *Biochem.* 2004.