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Mutant Study of Sinorhizobium meliloti Proline Utilization A (PutA)

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Introduction

Proline Utilization A (PutA) is a flavoenzyme in bacteria that catalyzes the oxidation of proline to glutamate in two steps. The first step involves the proline dehydrogenase (PRODH) domain which coverts proline to 1-pyrroline-5-carboxylic acid (P5C) coupled to the reduction of FAD. The second step involves the P5C Dehydrogenase (P5CDH) domain which converts P5C to glutamate coupled to the reduction of NAD+.

One variety of PutA enzymes are trifunctional. Trifunctional PutA's such as *Escherichia coli's* have those two domains plus a DNA Binding Domain which represses transcription of the put regulon which contains the gene encoding PutA. Whether the enzyme is performing its membrane bound or cytosolic function is determined the redox state of the bound of flavin. When there is no proline present, the flavin is oxidized and the enzyme is bound to the DNA to carry out its regulatory function. In the presence of proline, the flavin is reduced. The ezyme undergoes a conformational change and binds to the membrane to carry out its enzymatic function.

The PutA in *Sinorhizobium meliloti* is bifunctional and lacks the DNA Binding Domain. In this work, we investigated SmPutA to see if it functionally switched, despite its lack of a known cytosolic activity. In addition we used site-directed mutagenesis to create two variants of SmPutA, D306N and E308A, that differed at key residues in the flavin binding site.

SmPutA Channeling Assays

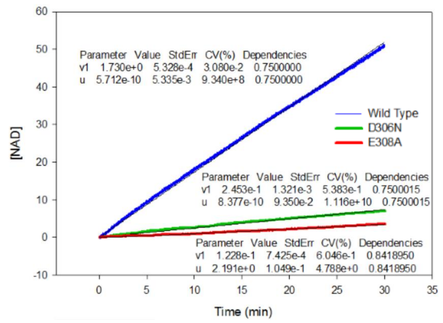
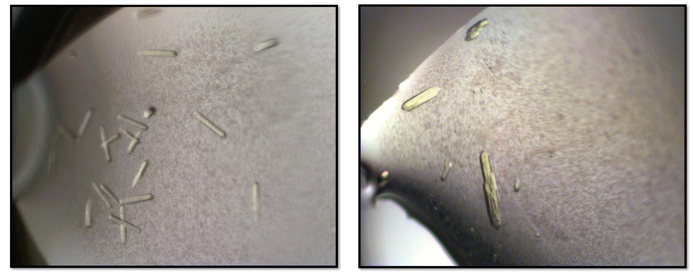


Figure 3. Mutant BjPutA Channeling Assays. *:* A channeling assay following the conversion of proline to glutamate via NADH formation for all SmPutA mutants. D779Y, D779W, and N943Y make very little NADH over the course of 10 minutes. *Right:* A schematic representing where mutations are located with respect to the channel.

Crystalization of SmPutA

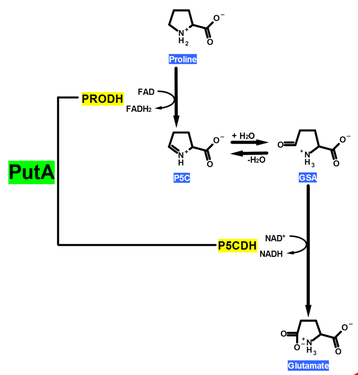


I-67 Opt.
10 mM THFA
pH 6.2
25% PEG 3350
0.1M Bis-Tris
0.2M $(NH_4)_2SO_4$

INSERT I90 Condition

Proline Oxidation

Figure 1. Reaction scheme for the oxidation of Proline. In bifunctional PutA's, proline undergoes a four electron oxidation. Two electrons are lost when cofactor FAD is reduced at the PRODH domain, forming pyrroline-5-carboxylate (P5C). P5C is then hydrolyzed to glutamic semialdehyde (GSA), which undergoes a second two electron oxidation at the NAD-dependent P5CDH domain, resulting in glutamate. Figure adapted from Arentson et al[2].



SmPutA FRET Assays

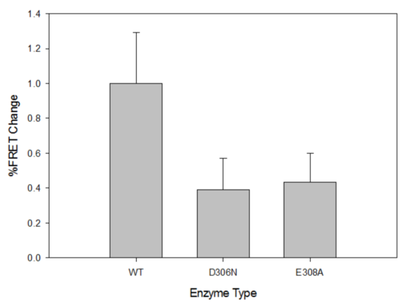


Figure 4. D779Y and D779W NADH formation. *Left:* Fluorescent channeling assays of 4 different concentrations of D779Y compared to wild-type. *Right:* Fluorescent channeling assays of 4 different concentrations of D779W compared to wild-type.

E308A Structure

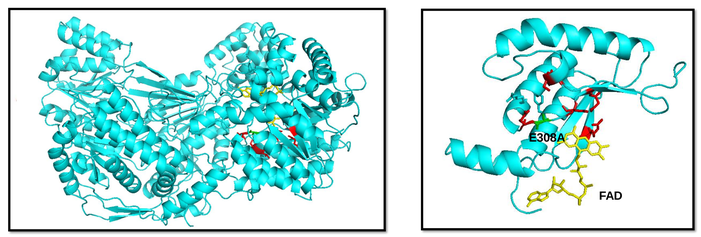


Figure 2. Crystal structures of SmPutA. *Left:* Overall butterfly structure of BjPutA showing two subunits: each monomer is composed of a red PRODH domain and an orange P5CDH domain. FAD is shown at the distal ends of the monomers within the ProDH domain, while NAD^+ is shown near the intersection of the two subunits within the P5CDH domains. A 41 Å channel is thought to exist connecting the two domains. *Right:* Crystal structure revealing spatial placement of mutated residues.

Proline Titrations

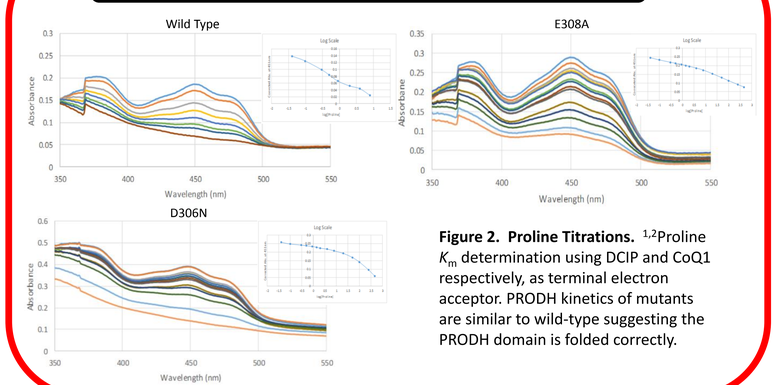


Figure 2. Proline Titrations. [1,2]Proline K_m determination using DCIP and CoQ1 respectively, as terminal electron acceptor. PRODH kinetics of mutants are similar to wild-type suggesting the PRODH domain is folded correctly.

Functional Memrbane Association Assays

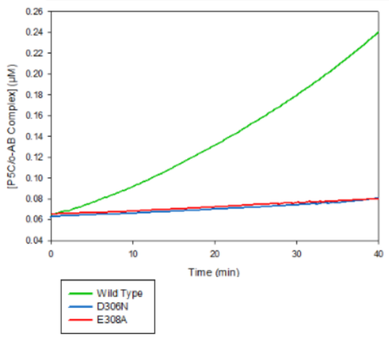


Figure 5. N943Y Lag phase. *Left Upper:* Channeling assays depicting wild-type BjPutA alongside a non-channeling control. The non-channeling control contains a lag phase that can be fit by the Tau equation (inset) to calculate lag before steady state[1]. *Left:* A progress curve (green) for N943Y fit with the Tau equation (dashed line). *Right Lower:* Table showing calculated lag phases.

Conclusions and References

- T348Y, S607Y, D778Y, D779A, A987Y behave similar to WT
- D779Y and D779W show significantly diminished NADH formation in channeling assays. P5CDH activity is nearly absent in steady-state assays
- D779Y binds NAD^+ similarly to WT, suggesting a properly folded P5CDH domain
- N943Y has a 6.5 minute lag before steady state NADH formation occurs
- Mutating the adjacent residue, D778 (D778Y) did not impact channeling activity, indicating that D779 is orientated into the channel. Thus, placing bulkier side chains within the channel has a specific effect on the channel.
- Removing the carboxylic group of D779 (D779A) does not impact channeling, suggesting the charge of D779 is not critical.
- Substituting D779 or N943 with a larger residue such as Try (D779Y) or Trp (D779W) disrupts channeling activity possibly by obstructing the travel of P5C/GSA in the channel.

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References

1. D. Srivastava, J. P. Schuermann, T. A. White, N. Krishnan, N. Sanyal, G. L. Hura, A. Tan, M. T. Henzl, D. F. Becker and J. J. Tanner: Crystal structure of the bifunctional proline utilization A flavoenzyme from Bradyrhizobium japonicum. Proc Natl Acad Sci U S A, 107(7), 2878-2883 (2010)
2. B. W. Arentson, N. Sanyal and D. F. Becker: Substrate channeling in proline metabolism. *Front. Biosci.* (2011)