Characterization of pII Family (GlnK1, GlnK2, GlnB) Protein Uridylylation in Response to Nitrogen Availability for *Rhodopseudomonas palustris*

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OVERVIEW

- Affinity Purifications used to isolate GlnK1, GlnK2, and GlnB proteins from *R. palustris* grown under nitrogen and non-nitrogen fixing conditions.
- Top-down mass spectrometry was used to examine affinity purifications of the GlnK1, GlnK2 and GlnB affinity purifications.
- The high mass accuracy and mass resolution afforded by FT-ICR-MS allowed for the identification of multiple isoforms and uridylylation status of the proteins by top-down analysis.
- The data obtained from the top-down analysis was correlated with bottom-up data to confirm isoform identifications and location of uridylylation sites.

INTRODUCTION

- The GlnK and GlnB proteins are a member of the pII signal transduction protein family.
- pII family plays a pivotal role in nitrogen metabolism regulation.[1]
- Sense internal cellular ammonia concentrations
- GlnK1, GlnK2, and GlnB each function as a trimer in *E. coli*.[2]
- GlnK has been shown in *E. coli* to have a compact barrel structure.[2]
  - 50Å in diameter and 30Å high
  - unstructured T-loop
- Heterotrimers between GlnB-GlnK are thought to play a role in regulating enzymatic activity of the AmtB ammonium transporter and glutamine synthase (glnA) (Figure1).
- GlnK2, GlnK1, and GlnB are uridylylated at tyrosine 51.
- In *R. palustris* there are three annotated forms of pII proteins; GlnK1, GlnK2, and GlnB (Figure 2).
- There are two levels of regulation in R. *palustris*. The first level is the regulation of AmtB ammonium transporter by GlnK. The second level is the regulation of glutamine synthase by GlnB.

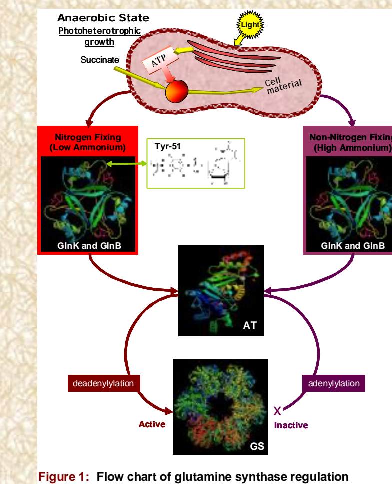

Figure 1: Flow chart of glutamine synthase regulation

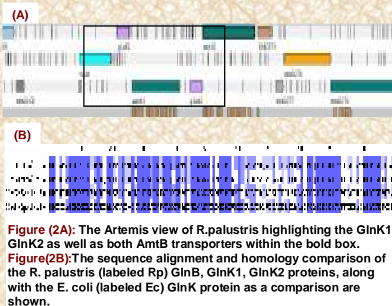

Figure (2A): The Artemis view of R.palustris highlighting the GlnK1, GlnK2 as well as both AmtB transporters within the bold box. Figure(2B):The sequence alignment and homology comparison of the R. palustris (labeled Rp) GlnB, GlnK1, GlnK2 proteins, along with the E. coli (labeled Ec) GlnK protein as a comparison are shown.

EXPERIMENTAL

- Affinity Purifications
  - The R. palustris wild type strain (CGA009), harboring the pBBR5-DEST/42 modified Gateway expression plasmid (Invitrogen, Carlsbad, CA) with the RPA0274 open reading frame (ORF) was generated at Oak Ridge National Laboratory. The ORF was cloned into the expression plasmid with the V5 and 6xHis affinity tags fused at the C-terminus of the protein. The presence of two tags (6X His-tag and V5 antibody tag) within the plasmid allowed the use of a dual affinity purification strategy to "capture" the GlnK2, GlnK1, and GlnB complexes.
- ESI-FTICR Mass Spectrometry
  - All ESI-FTICR mass spectra were acquired with an IonSpec (Lake Forest, CA) 9.4-Tesla HiRes electrospray Fourier transform ion cyclotron resonance mass spectrometer. A Harvard syringe pump (flow rate of 1.75 µL/min) was used for direct infusion into an Analytica electrospray source (Analytica of Branford, CT).
- 1D LC-MS-MS
  - Bottom-up experiments were performed using trypsin digestion. For all peptide samples, one-dimensional (1D) LC-MS-MS experiments were performed with a Famos/Switchos/Ultimate HPLC System (Dionex, Sunnyvale, CA) coupled to an LCQ-DECA XP Plus quadrupole ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with a nanospray source. Peptide searching was performed with SEQUEST.

RESULTS

Non-Nitrogen Fixing

- GlnK2
  - Top-down Non-Nitrogen Fixing Conditions (Figure 3)
    - Unmodified Glnk1 was measured at 12,360.824 Da as compared to a calculated value of 12,360.776 Da, giving a 4 ppm mass error.
    - Unmodified tagged GlnK2 was measured at 16,318.980 Da with a calculated value of 16,318.859 Da provides a 7 ppm mass error.
  - Results consistent with Non-Nitrogen fixing growth state
    - High levels of ammonium within the cell leads to an inactive AmtB transporter.
    - The forced over-expression of tagged GlnK2 under non-nitrogen fixing conditions explains why this affinity purification yields only the tagged version of this protein.
  - Bottom-up Non-Nitrogen Fixing Conditions
    - Unmodified GlnK1 was observed at 89.3% sequence coverage with 16 unique peptides.
    - Unmodified tagged GlnK2 was observed at 94.6% sequence coverage with 25 unique peptides.
    - GlnB was present in low levels in the bottom-up analysis at 30% sequence coverage and 2 unique peptides.

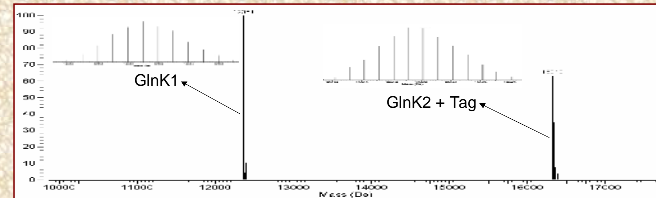

Figure 3: ESI-FTICR mass spectrum of GlnK2 affinity purification from *R. palustris* grown under non-nitrogen fixing conditions. Only unmodified GlnK1 and unmodified tagged (tag refers to 6X His-tag and V5 antibody tag) GlnK2 proteins are present in the growth state.

- GlnK1 and GlnB
  - Top-down Non-Nitrogen Fixing Conditions (Figure 4)
    - GlnK1 (Figure 4A)
      - Unmodified GlnK1
      - Unmodified tagged GlnK1
    - GlnB (Figure 4B)
      - Unmodified GlnB
      - Unmodified tagged GlnB
  - Results consistent with Non-Nitrogen fixing growth state
    - High levels of ammonium present within the cell.
    - The 6X His tag and V5 antibody tag used for affinity purifications are inserted within the plasmid DNA. The untagged version of the protein is coming from the bacterial chromosomal DNA.
    - Trimeric from = tagged and untagged versions
  - Bottom-up Non-Nitrogen Fixing Conditions
    - GlnK2 seems to be expressed at a significantly lower level in the cell as compared to GlnK1 in the non-nitrogen fixing growth state.
    - GlnK1 was observed at 35.7% sequence coverage with 7 unique peptides while GlnK2 was observed at 21.4% sequence coverage with 1 unique peptide. GlnB was present at 30% sequence coverage and 4 unique peptides.

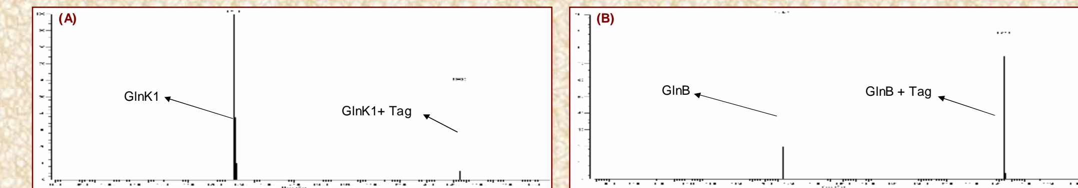

Figure 4: (A) ESI-FTICR mass spectrum of GlnK1 affinity purification from R. palustris grown under non-nitrogen fixing conditions. The tagged and un-tagged versions of the protein are present. (B) ESI-FTICR mass spectrum of GlnB affinity purification from R. palustris grown under non-nitrogen fixing conditions. The tagged and un-tagged versions of the protein are present.

Nitrogen Fixing

- GlnK2
  - Top-down Nitrogen Fixing Conditions (Figure 5)
    - Unmodified tagged GlnK2
    - Uridylylated tagged GlnK2
    - GlnK2
    - Uridylylated GlnK2
    - GlnK1 from HPLC-FTICR-MS (Figure 6)
  - Results consistent with nitrogen fixing growth state
    - Low levels of ammonium within the cell activates the AmtB transporter.
    - Trimeric from = tagged and untagged versions
  - Bottom-up Non-Nitrogen Fixing Conditions
    - GlnK2 was observed at 100% sequence coverage with 20 unique peptides.
    - GlnK1 was observed at 92.9% sequence coverage with 15 unique peptide.
    - The MS/MS spectra of the 48-GAEY*AVSFLPK-58 peptide with the uridylylation on tyrosine 51 is shown in Figure 7.

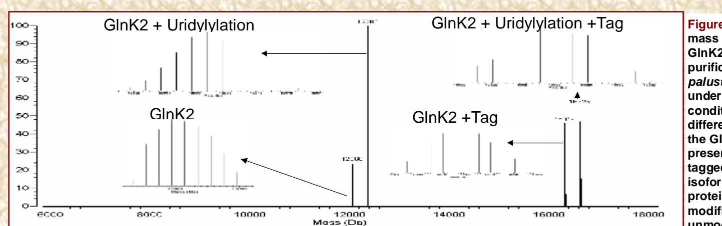

Figure 5: ESI-FTICR mass spectrum of GlnK2 affinity purification from *R. palustris* grown under nitrogen fixing conditions. Four different isoforms of the GlnK2 protein are present, including the tagged and untagged isoforms of the protein as well as the modified and unmodified isoforms.

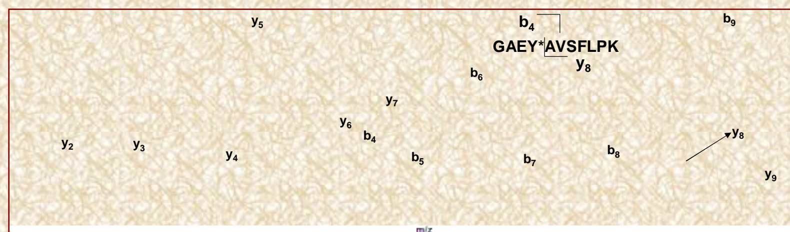

Figure 7: MS/MS spectrum of uridylylated peptide 48-GAEY*AVSFLPK-58. The spectrum has the b and y ions labeled showing the uridylylation on tyrosine 51 (y8 and b4 ions).

- GlnK1 and GlnB
  - Top-down Nitrogen Fixing Conditions (Figure 8)
    - Unmodified tagged GlnK1
    - GlnK2
    - Uridylylated tagged GlnK1
    - Uridylylated GlnK2
  - Mixture of GlnK1 and GlnK2 isoforms may indicate GlnK2 plays a more primary role in AmtB regulation
  - GlnB (Figure 9)
    - Unmodified tagged GlnB
    - GlnB
    - Uridylylated tagged GlnB
    - Uridylylated GlnB
  - Bottom-up Non-Nitrogen Fixing Conditions
    - GlnK1 was observed at 73.2% sequence coverage with 14 unique peptides. GlnK2 was observed at 92% sequence coverage with 19 unique peptide.
    - The Bottom-up analysis also confirmed the presence of two unique uridylylated peptides (48-GAEY*AVSFLPK-58 and 41-GHTEIYRGAEY*AVSFLPK-58) for GlnK1 as well as the unique uridylylated peptide 48-GAEY*AVSFLPK-58 from GlnK2.
    - Within the affinity purification of GlnK1, it was observed at 50.7% sequence coverage with 11 unique peptides.

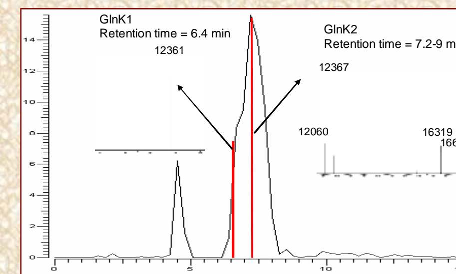

Figure 6: LC-FTICR-MS total ion chromatogram showing the GlnK1 protein as well as all four forms of the GlnK2 protein. The first peak in the chromatogram (tr = 4.5min) is ubiquitin which was used as an internal standard for the chromatography.

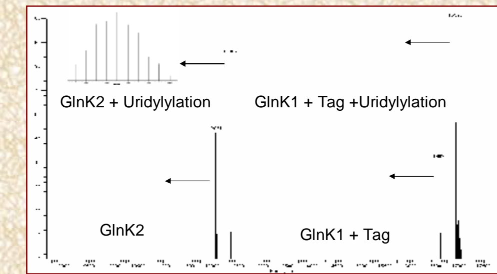

Figure 7: ESI-FTICR mass spectrum of GlnK1 affinity purification from R. palustris grown under nitrogen fixing conditions. The unmodified and modified tagged (tag refers to 6X His-tag and V5 antibody tag) GlnK1 isoforms are present in the growth state as well as the unmodified and modified isoforms of GlnK2

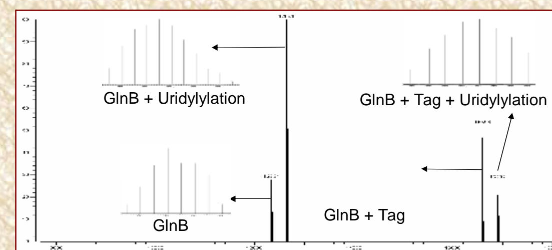

Figure 8: ESI-FTICR mass spectrum of GlnB affinity purification from R. palustris grown under nitrogen fixing conditions. Four different isoforms of GlnB proteins are present in the growth state, including the tagged and untagged isoforms of the protein as well as the modified and unmodified isoforms.

Endogenous GlnK1, GlnK2, and GlnB

- To ensure the addition of the tags inserted on the C-terminal did not affect endogenous expression, wild type cells were grown under nitrogen and non-nitrogen fixing conditions.
- From the wild type cells GlnK1, GlnK2, and GlnB were examined by top-down and bottom-up analysis.
- Results were consistent with the results obtained from affinity purifications of all three proteins.
  - Nitrogen fixing conditions
    - GlnK2
    - GlnK2 + Uridylylation
    - GlnB
    - GlnB +Uridylylation
  - Non-nitrogen fixing conditions
    - GlnK1
    - GlnB

CONCLUSION

- Affinity purifications allowed for the isolation and measurements of the functional state for Glnk1, Glnk2, and GlnB.
- Uridylylation of Glnk1, Glnk2, and GlnB identified under nitrogen fixing conditions tightly coordinated by nitrogen availability.
- Multiple Isoforms of all three proteins from affinity purifications identified. This confers the presence of a multi-mer type structure.
- Endogenous protein purifications under both nitrogen and non-nitrogen fixing growth conditions show that C-terminally inserted tags and over-expression did not alter normal state of complex.
- GlnK2 seems to be a key regulation site for AmtB under nitrogen fixing conditions in *R. palustris*.

ACKNOWLEDGMENTS

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