Deciphering Pyocyanin Biosynthesis: Investigating PhzS/PhzM Enzyme Dynamics and Substrate Interactions

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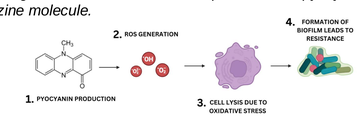
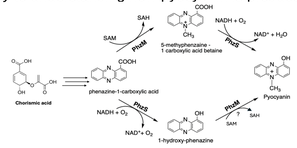
Background and Significance

- *Pseudomonas aeruginosa, a common Gram-negative bacterium, is notorious for causing severe infections due to its production of pyocyanin, a potent phenazine molecule.*



- Pyocyanin biosynthesis in Pseudomonas aeruginosa involves two enzymes: PhzS, a flavin-dependent monooxygenase, and PhzM, a SAM-dependent methyltransferase. Scheme 1 shows two proposed pathways: one where PhzS first hydroxylates PCA to 1-HP, and another where PhzM first methylates PCA to form 5-MPCA. Clarifying the correct sequence of these steps is key to understanding how pyocyanin is produced and regulated.



Scheme 1. The enzymatic formation of phenazine-1-carboxylic acid (PCA) to pyocyanin facilitated by enzymes PhzM and PhzS.

Structure and Mechanism of PhzS, PhzM, and Complex

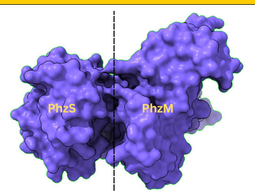


Figure 2: The complex was modeled using AlphaFold, with structures derived from PDB codes 2RGJ (PhzS) and 2IP2 (PhzM). PhzS, located on the left, and PhzM, located on the right, are shown bound together, potentially forming a tubular structure connecting two central cavities between the enzymes.

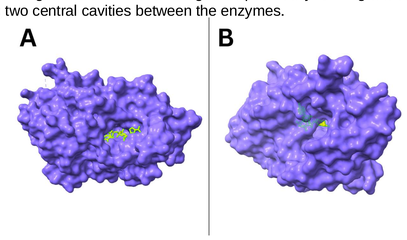


Figure 3: Structural representation of PhzS showing key catalytic features. **(a)** The globular structure highlights the FAD cofactor (yellow) in an open, solvent-accessible pocket, positioned for NADH interaction. **(b)** The substrate-access tunnel guides PCA toward the active site near FAD, ensuring proper alignment for hydroxylation and decarboxylation.

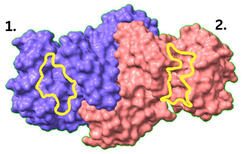


Figure 4: Globular structure of PhzM, a SAM-dependent methyltransferase involved in pyocyanin biosynthesis, shown as a homodimer.

Hypothesis & Research Objectives

Hypothesis: Based on previous studies and fluorescence binding data, PhzS acts first to hydroxylate PCA into 1-HP, which then serves as a preferred substrate for PhzM. Cooperative binding in the PhzS/PhzM complex enhances reaction efficiency and intermediate stabilization.

1. **Determine the substrate binding affinities** of PhzS and PhzM for PCA, NADH, and SAM using fluorescence spectroscopy.
2. **Investigate whether PhzS and PhzM form a functional complex** that enhances substrate binding and catalytic efficiency.
3. **Distinguish between the hydroxylation-first and methylation-first pathways** in pyocyanin biosynthesis using sequential fluorescence assays.
4. **Assess the formation and stability of proposed intermediates** (1-HP and 5-MPCA) via HPLC analysis.

What is the binding affinity of PCA to PhzS?

Fluorescence quenching PhzS upon titration with PCA

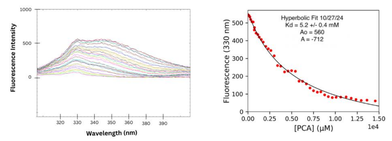


Figure 6A: (Left) The fluorescence emission spectrum observed during the titration. General fluorescence intensity decreases progressively as ligand is added to a cuvet containing PhzS. Saturation is reached slowly for both PCA and NADH, reflecting low intrinsic affinity. (Right) Fluorescence intensity monitored at **330 nm** and fit by nonlinear regression using the hyperbolic binding equation ($Y = \frac{B_{max} \times [L]}{K_d \times [L]}$) indicates a K_d (PCA) = **5.2 ± 0.4 mM.**

What is the binding affinity of NADH to PhzS?

Fluorescence quenching PhzS upon titration with NADH

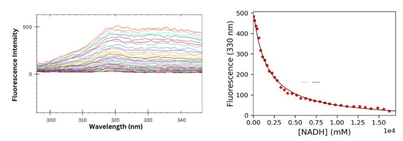


Figure 6B (Left) The fluorescence emission spectrum observed during the titration. General fluorescence intensity decreases progressively as ligand is added to a cuvet containing PhzS. Saturation is reached slowly for both PCA and NADH, reflecting low intrinsic affinity. (Right) Fluorescence intensity monitored at 330 nm and fit by nonlinear regression using the hyperbolic binding equation indicates a K_d (NADH) = **1.62 ± 0.08 mM**.

What is the binding affinity of SAM & PCA to PhzM?

Fluorescence quenching PhzS upon titration with NADH

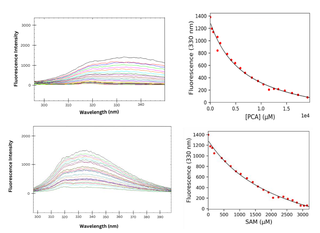


Figure 7 PhzM binding of PCA (top) and SAM (bottom). (Left) Emission spectra show gradual quenching with increasing ligand, indicating weak binding. (Right) Fluorescence at 330 nm fit to a hyperbolic binding model yielded K_d values of **6.8 ± 0.5 mM** (PCA) and **2.4 ± 0.3 mM** (SAM).

Does **PhzS–PhzM complex** formation affect substrate binding and intermediate processing in pyocyanin biosynthesis**?**

Quenching of protein fluorescence of a solution containing both PhzS and PhzM by the substrates.

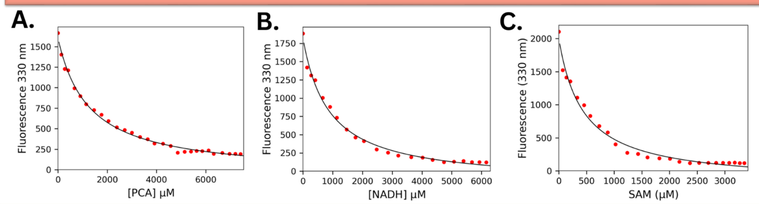


Figure 8 (A) Hyperbolic fit of a titration with **PCA** results in a K_d **of 1.2 ± 0.01 mM**. **(B)** Hyperbolic fit of a titration with **NADH** yields a K_d **of 0.81 ± 0.07 mM**. **(C)** Hyperbolic fit of a titration with **SAM** yields a K_d **of 0.44 ± 0.05 mM**.

In each case the measured K_d for the complex is smaller than the K_d measured for the titration of either enzyme titrated alone.

Does **the order of substrate addition** affect the fluorescence behavior and activity of the PhzS–PhzM complex?

Hydroxylation 1st

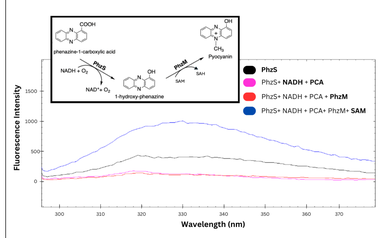


Figure 9: Sequential fluorescence quenching of PhzS (1 μM) upon substrate and PhzM addition. NADH and PCA (each 22.2 μM) cause full quenching, indicating binding and possible hydroxylation. PhzM addition shows no change, while SAM (22.2 μM) causes a fluorescence increase, supporting a hydroxylation-first mechanism in pyocyanin biosynthesis.

Methylation 1st

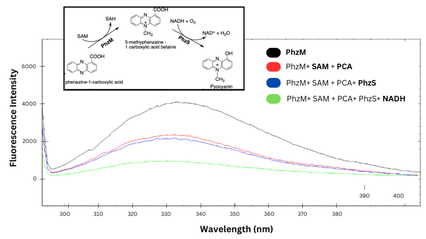


Figure 10: Sequential fluorescence quenching of PhzM (1 μM) upon stepwise addition of PCA and SAM (22.2 μM each), showing partial quenching. Addition of PhzS (1 μM) causes minimal change, while NADH (22.2 μM) leads to full quenching, supporting a hydroxylation-dependent reaction sequence.

Is there any identifiable **intermediate** formation based on our **sequential experiments?**

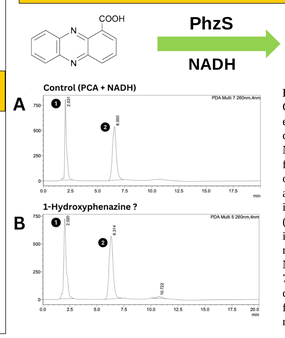


Figure 11: HPLC Chromatograms showing the elution profiles of a mixture containing PhzS, PCA and NADH to assess for 1-HP formation. (A) Control reaction of PCA and NADH monitored at 260 nm, where Peak 1 is NADH and Peak 2 is PCA. (B) Reaction mixture after PhzS incubation (5 min) and enzyme removal, showing a decrease in NADH peak height (800 to 700), suggesting partial NADH consumption and possible 1-HP formation, though no distinct new peak was detected.

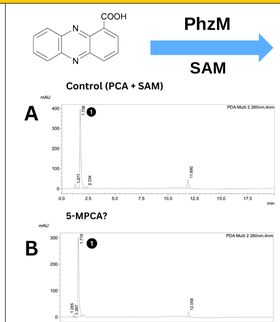


Figure 12: HPLC chromatograms of PhzM, PCA, and SAM reactions to assess 5-MPCA formation. (A) Control shows SAM peak at 400. (B) PhzM reaction shows reduced SAM peak (~300), suggesting partial consumption. No 5-MPCA peak detected; PCA was absent, likely due to column retention or elution inefficiencies.

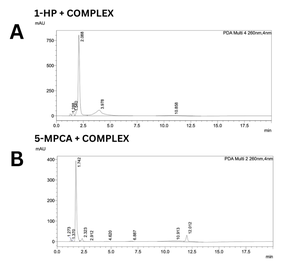


Figure 13: HPLC chromatograms of reactions with the PhzS/PhzM complex. (A) PCA + NADH reaction shows NADH peak and a new peak at 3.978 min, possibly NAD^+, suggesting cofactor oxidation. No 1-HP peak detected. (B) PCA + SAM reaction shows SAM peak but no 5-MPCA or PCA, likely due to degradation or column retention

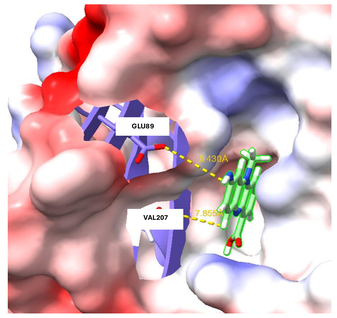
Comparison of Kinetic Results

Table 1: Comparison of Dissociation Constants (Kd) for Substrate Binding to PhzS, PhzM, and the PhzS/PhzM Complex

Enzyme	Substrate	K_d (mM)
PhzS	PCA	5.2 ± 0.4
PhzS	NADH	1.62 ± 0.08
PhzM	PCA	6.8 ± 0.5
PhzM	SAM	2.4 ± 0.3
PhzS/PhzM Complex	PCA	1.2 ± 0.01
PhzS/PhzM Complex	NADH	0.81 ± 0.07
PhzS/PhzM Complex	SAM	0.44 ± 0.05

Future Research

- Use mass spectrometry to confirm and characterize transient intermediates like 1-HP and 5-MPCA.
- Optimize HPLC or alternative chromatographic conditions to improve detection of tightly bound or unstable phenazine intermediates.
- Perform site-directed mutagenesis of PhzS and PhzM to identify residues essential for intermediate stabilization and catalysis.



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