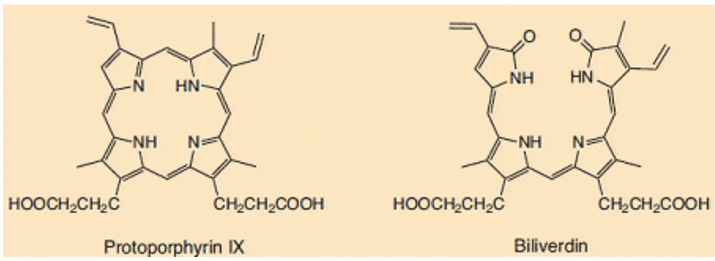
Enhancing the Fluorescence of Wisconsin Infrared Phytofluor—Wi-Phy for Potential Use in Infrared Imaging

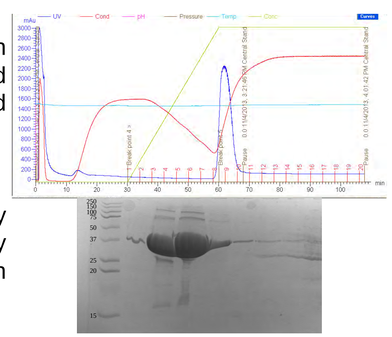
Jerad J. Simmons, Katrina T. Forest
Department of Bacteriology, University of Madison—Wisconsin

Abstract

Infrared fluorescent imaging is ideal as a whole body reporter for noninvasively visualizing deep tissue. Wisconsin Infrared Phytofluor is a promising candidate as an infrared fluorescent protein. Wi-Phy has been engineered from a bacterio-phytochrome isolated from *Deinococcus radiodurans*. However, the phytofluor sometimes nonspecifically binds an incorrect chromophore, reducing its fluorescence. Rather than binding the native biliverdin molecule, Wi-Phy has gained affinity for protoporphyrin. In an effort to prevent nonspecific chromophore binding, Wi-Phy bound to protoporphyrin was assessed and characterized. Although unable to obtain a crystal structure of protoporphyrin bound Wi-Phy, we were able to speculate structural differences and determine what will better enhance future attempts at crystallization.



Above—Figure 1: Comparison of protoporphyrin ix (ppix), the improper ligand with biliverdin, the preferred ligand (chemistryviews.org/).



Right—Figure 2: Purification of apo Wi-Phy by Nickel Affinity Chromatography followed by visualization on an SDS-page gel.

Methods

Protein Purification: Wi-Phy with a C-terminal hexa-His tag was cloned onto a pET21 plasmid and expressed in *E. coli* BL21(DE3) cells induced by IPTG. Cells were lysed using French pressure and incubated with varying concentrations of protoporphyrin ix in DMSO. Wi-Phy was then subjected to Nickel Affinity Chromatography, followed by hydrophobic interaction chromatography to separate ppix-bound Wi-Phy from apo-protein. Alternatively, Wi-Phy was purified before incubation with ppix and subjected to a desalting column to remove excess ppix prior to separation using the hydrophobic interaction column.

Crystallization: Crystallization attempts were made utilizing hanging drop vapor diffusion using both screening kits and prior known crystallization conditions for Wi-Phy.

Zinc Blot: Proteins were incubated with ligand and run on an SDS-Page gel. Zinc was added to cause chromophore fluorescence at protein band.

Goal—Create a new imaging technique for use as a whole body reporter

☑Find a fluorescent protein which emits energy in the infrared range
☑Create a small, efficient version of the protein
☑Shift the emission wavelength to be as long as possible
☑Preliminary testing for use *in vivo*
☐Optimize the brightness of the molecule, including maximizing binding to the proper ligand
☐X-ray crystallography to determine the structure

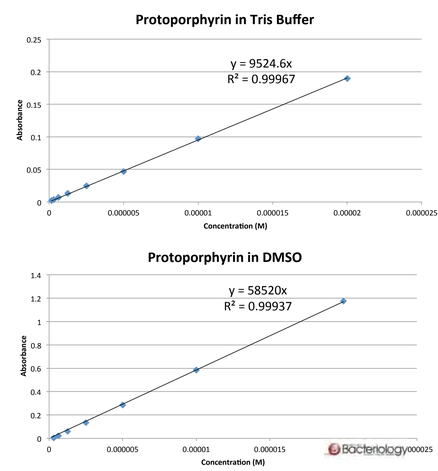


Figure 3: Protoporphyrin extinction coefficients in 30mM Tris buffer compared to DMSO.

Figure 4: Solved structure of Wi-Phy bound to biliverdin (Auldridge, et al., 2012).

Results

Protoporphyrin ix has a different extinction coefficient depending on the buffer it is in as determined by beer's law plot (**Figure 3**). The extinction coefficients are 9524 in 30mM Tris and 58520 in DMSO.

Crystallized Wi-Phy bound to biliverdin has been obtained and characterized (**Figure 4**). Wi-Phy consists of the monomerized *Deinococcus radiodurans* with only the chromophore binding domain remaining. It has two mutations and is denoted as *Dr*CBD$_{mon}$-D207H/Y263F (Auldridge, et al., 2012).

Apo Wi-Phy binds the endogenous ppix, and adding additional ppix increases the signal found in the zinc blot assay (**Figure 5**). The assay indicates that ppix is covalently bound without any added exogenous ligand.

Using *in silico* studies, we were able to predict the binding pocket of ppix in Wi-Phy as compared to that of biliverdin (**Figure 6**).

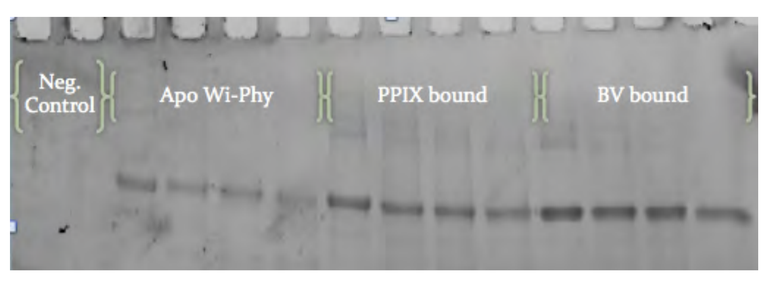
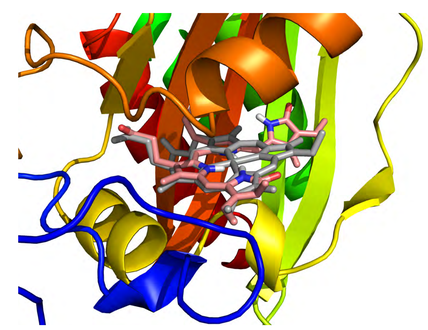


Figure 5: Zinc blot assay visualizing the chromophore bound protein. The negative control was run using lysozyme to ensure binding was specific, and biliverdin-bound Wi-Phy was used as a positive control.

Conclusions

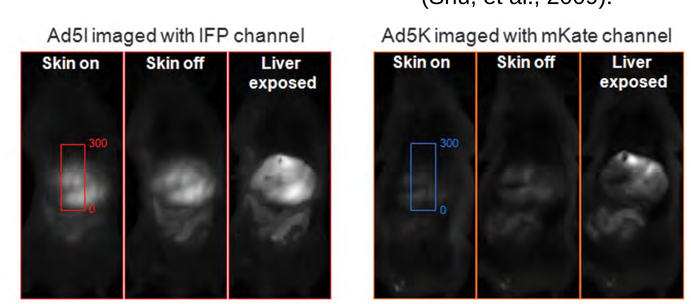
In attempts to crystallize the protein for structural analysis followed by directed mutagenesis, we encountered some problems. Protoporphyrin ix is highly hydrophobic and has a buffer-dependent extinction coefficient, both of which resulted in need for a change in protein purification strategy. Pure, ppix-bound Wi-Phy has not yet been obtainable, so crystallization attempts are to be continued.

The *in silico* study shows the likely binding site for protoporphyrin. With a crystal structure, the exact distances and residues that allow the endogenous ppix binding could be determined. Directed mutagenesis could be performed in order to prevent protoporphyrin from binding in the chromophore binding domain, thus increasing the overall fluorescence of the Wi-Phy phytofluor.



Left—Figure 6: *In silico* overlay of Wi-Phy bound protoporphyrin (gray) and biliverdin (pink) in the binding pocket.

Below—Figure 7: *In vivo* imaging using live mice comparing infrared fluorescent proteins with far-red fluorescent proteins (Shu, et al., 2009).



Practical Use

Infrared Fluorescent Proteins (IFPs) have benefits over other imaging methods. Wavelengths in the infrared range (650-900 nm) are absorbed less by hemoglobin, water and lipids, in addition to undergoing less light scattering. IFP can be visualized noninvasively through the skin, unlike mKate, a far-red fluorescent protein (**Figure 7**) (Shu, et al., 2009).

References

1. Auldridge ME, et al. 2012. Structure-guided Engineering Enhances a Phytochrome-based Infrared Fluorescent Protein. *J Biol Chem*. **287(10):** 7000-7009.
2. Shu X, et al. 2009. Mammalian expression of infrared fluorescent proteins engineered from a bacterial phytochrome. *Science*. **324(5928):** 804–807.

Acknowledgements

Funding provided by the NIH and the Hilldale Undergraduate Research Fellowship. Additional support was provided by all members of the Forest laboratory, especially Shyamosree Bhattacharya, Anna Baker, Kenneth Satyshur, and Katrina Forest.