QGEM Results & Discussion

Kate Turner & Michael Freeman

Project: Biliverdin production via HO-1 catalyzed breakdown of Heme

Heme and Heme-Oxygenase Effector

Heme is a protein which is crucial for all forms of aerobic life but can also be quite cytotoxic, especially in the presence of oxidants. Due to high concentrations of heme coming from lysed erythrocytes and extracellular hemoglobin, the vasculature is at greatest risk to free heme exposure[1]. Heme, as well as accumulated monocytes and macrophages, generate reactive oxygen species (ROS) which oxidize low density lipoproteins (LDL)[2]. This oxidation is one of the earliest steps in atherosclerotic development. Figure 1 presents the molecular structure of free heme.

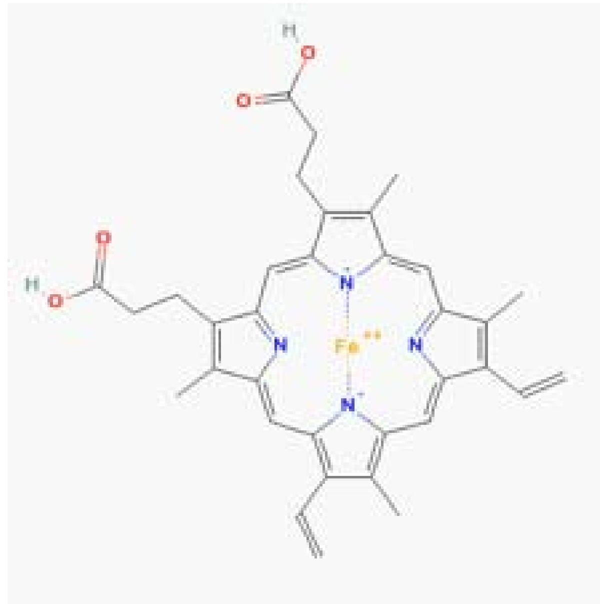

Figure 1[3]: Free heme ($C_{34}H_{32}FeN_4O_4$)

Heme oxygenase (HO) is a protein used by the vasculature in response to oxidative stress. HO is the rate-limiting microsomal enzyme involved in heme breakdown to generate biliverdin IXa (BV), free ferrous iron (Fe^{2+}), and carbon monoxide (CO). All of these help protect the vasculature from radical oxygen species and, in turn, the development of atherosclerosis[4]. Heme oxygenase-1 (HO-1) is a sensitive anti-inflammatory protein induced by various types of oxidative stress, like oxidized LDL[5]. HO-1 is the most prominent player in the conversion of heme to BV, Fe^{2+}, and CO, and the most studied. Figure 2 shows a layout of this catabolic pathway beginning with free heme.

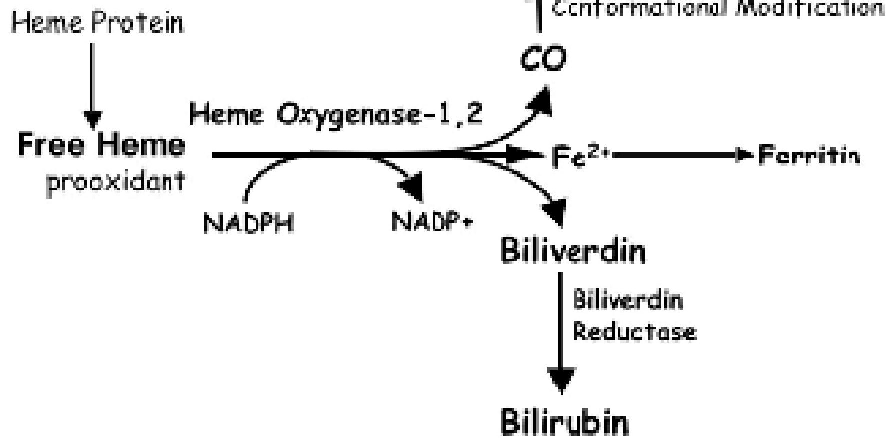

Figure 2 [6]: Catabolism of heme to produce CO, BV (then BR), and Fe^{2+}; all important effectors in the prevention and control of atherosclerosis.

BV is involved in the direct inhibition of monocyte transmigration in the vasculature[7]. This inhibits accumulation of macrophages in the arterial wall, which generate ROS and oxidize LDL. In mammalian systems, BV is converted to BR using biliverdin reductase. Efforts to employ this biological system in bacterial cells is difficult due to the lack of genes encoding mammalian biliverdin reductases, so it is unlikely that BV is further metabolized to BR in engineered bacteria[8].

BR is a recognized antioxidant which provides crucial protection against lipid oxidation. By prohibiting LDL oxidation and scavenging oxygen radicals, higher serum levels of BV and BR have been inversely associated with coronary arterial disease[9].

CO has been found to be an effective vasodilator by its actions in activing guanylate cyclase, the enzyme responsible for increasing cyclic guanine monophosphate (cGMP)[10]. These regulate ion channel conductance as well as relax smooth muscle cells, causing vasodilation which increases blood-flow and reduces blood pressure. Both of these results are helpful in treating atherosclerosis by bringing more effectors to the area and by lessening the chance of pressure-caused rupturing of the plaque.

HO-1 releases Fe^{2+} from the core of the heme molecule which has been linked to the rapid production of an iron-binding protein called ferritin. Ferritin sequesters free iron, which is an important catalyst in oxygen-centered radical formation, proving it as an effective preventative anti-oxidant[11].

All of these products of the catalysis of heme by HO-1 are extremely useful in the control and prevention of atherosclerosis. By localizing a concentrated delivery of these effectors to atherosclerotic plaques, results could prove extremely beneficial to the patient involved. Our current efforts are aimed at moving this pathway into a bacterial system and viewing the organism's efficacy breaking down heme.

BioBrick and Construct Legend
BioBrick I716390 - Plasmid containing a composite of Heme A, B, C and D with T7-promoter and RBS. AMP resistance.
BioBrick K098010 - HO and phycocyanobilin:ferredoxin oxidoreductase. KAN resistance.
BioBrick I15008 - HO-1 from *Synechocystis.* AMP resistance.
NLM 350 - Rough *E.coli* strain containing T7-polymerase. Queen’s University Research.

Results

In order to determine whether or not the BioBrick I716390 submitted by the Berkeley iGEM team in 2007 was producing heme, NLM350. Overnight cultures containing 100 µg/mL ampicillin were set up and inoculated with NLM350 containing I716390. The culture was analyzed daily by wavelength scan in a spectrometer. We ran the wavelength scan twice, once with a blank of LB and once with a blank of LB culture containing NLM350 without the BioBrick. Both of the scan results were identical, which indicates that the peaks present are due to the BioBrick plasmid and not resulting from compounds produced by NLM350. The results from day three with a blank of LB are shown in Figure 3.

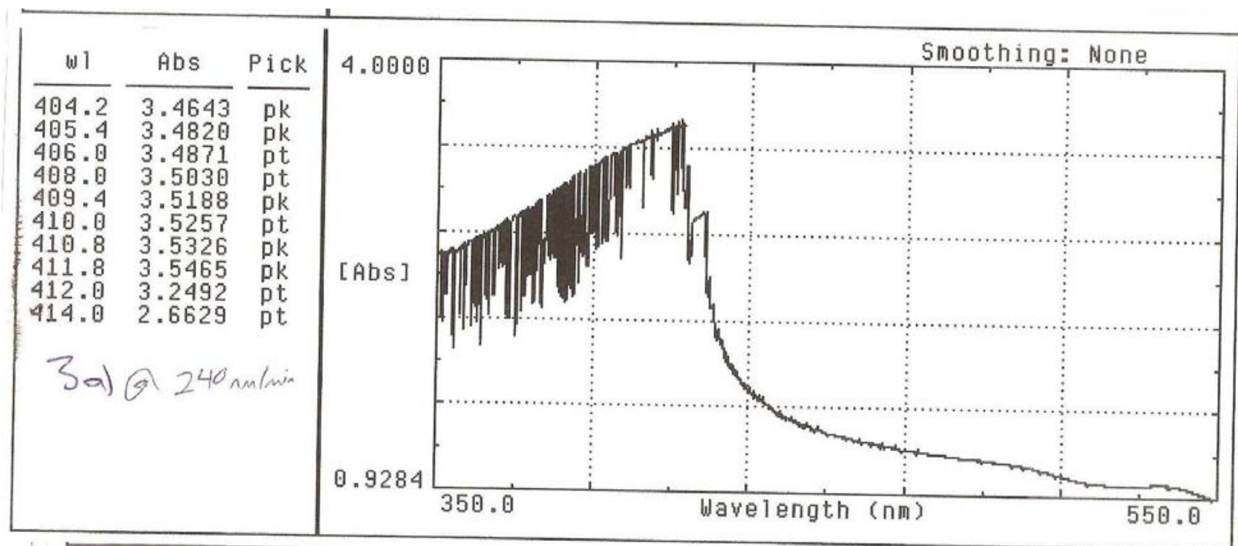

Figure 3: Results of a wavelength scan between 350 and 550nm of the NLM350 + I716390 culture three days after inoculation. Strongest peaks at 412nm.

In order to check for the presence of the heme/HO-1 complex, NLM350 + I716390 cells were made electrocompetent and two sets of electroporations were performed. The BioBricks K098010 and I15008 were taken up by NLM350 + I716390 and the resulting colonies were used to inoculate cultures containing 40 µg/mL kanamycin and 100 µg/mL ampicillin, respectively. Triplicate testing of NLM350 + 176390 + KO98010 as well as NLM350 + 176390 + I15008 was conducted over five days. Both tests produced similar strong peaks for heme/HO-1 complexes, ranging from

407-409 nm. Again, we ran the scans twice with blanks of LB and LB inoculated with NLM350 and found that results were identical. The results from day five with blanks of LB are shown in Figures 4 and 5.

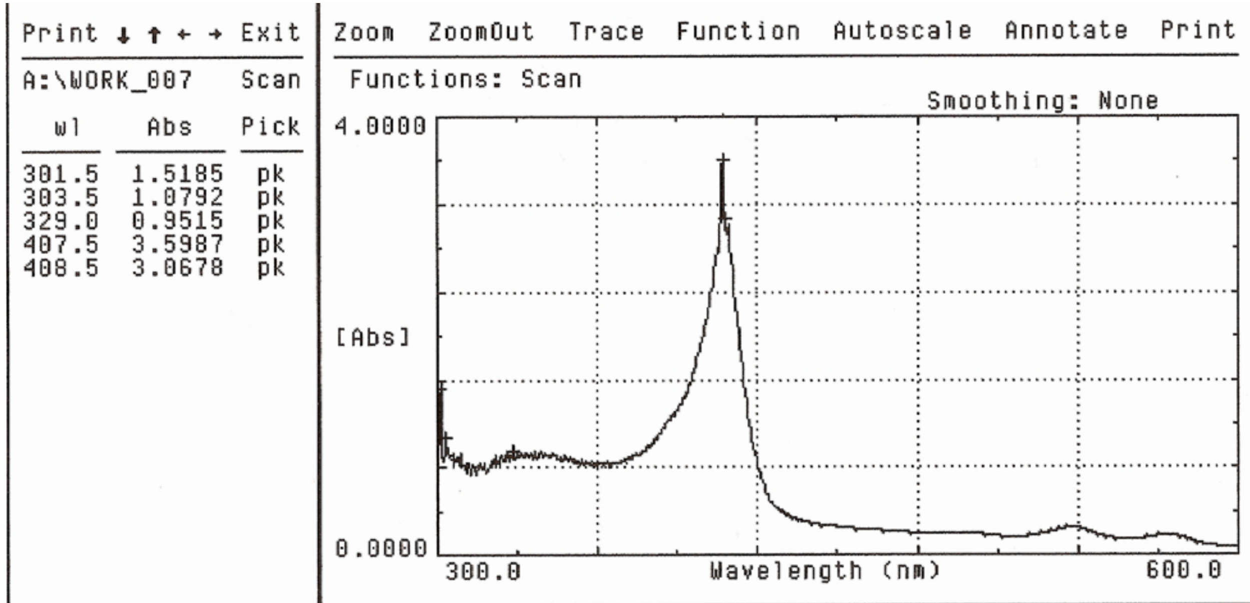

Figure 4: Wavelength scan between 350 and 550 nm of the NLM350 + I716390 + K098010 culture 5 days after inoculation. Strong peak of 407.5 nm.

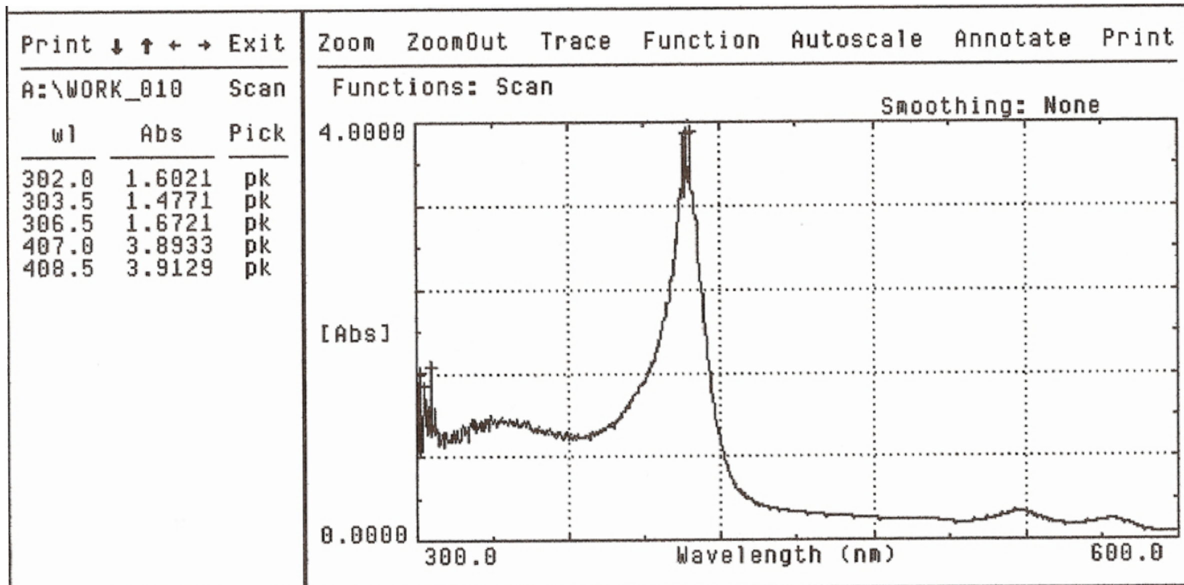

Figure 5: Wavelength scan between 350 and 550 nm of the NLM350 + I716390 + I15008 culture 5 days after inoculation. Strong peak of 407.5 nm.

Discussion

As can be seen from our results above, the peaks in the cultures containing heme and HO-1 producing BioBricks become clearer and occur at a lower absorbance, around 408nm, than the peaks from cultures containing only heme-producing BioBricks. Cultures containing only the heme-producing BioBrick has the strongest peaks at 412nm, which corresponds to the peak found by Moffet et al. in 2003 [12]. Since HO-1 does not have an absorbance peak of its own [8,13,14], the presence of HO-1 can only be inferred by identifying the heme/HO-1 complexes via spectroscopy. According to several authors [8, 14], Soret bands for the oxy-bound form of the heme/HO-1 complex occur at 410nm. The peaks seen in Figures 4 and 5 are slightly blue-shifted compared to the literature values, which may be the result of different biochemical subunits present in the heme protein[15]. However, the difference between the spectra of Figures 3, 4, and 4 indicates that HO-1 is being produced and is binding with heme to form a complex which peaks at a different wavelength than heme alone.

It was expected to see a peak for biliverdin as well, which would have occurred between 440 – 460nm according to the literature [16, 17], though we did not. This might have been due to the fact that the cultures containing heme and HO-1 producing BioBricks had some exposure to light. Researchers have noted in their methods that they had avoided light when handling biliverdin [18, 19]. If biliverdin is photosensitive, this might be why these peaks did not appear in our wavelength scans.

Further Research

In order to continue on this project track, the genetic effector system would have to be streamlined and optimized. More thorough kinetics testing for the production of heme as well as its metabolism by HO-1 would be required to maximize effector quality and concentration. Though many of the products of heme metabolism were likely produced, bilirubin was almost certainly not.

As mentioned previously[8], there is a lack of biliverdin reductases available for cloning into bacterial systems, especially mammalian. Recently the entire genome of *Synechocystis sp*. PP6803 was sequenced, along with a cyanobacterial biliverdin reductase homologue. This enzyme is used in the production of light-harvesting pigments called phycobilins which is a product in the breakdown of heme[20]. With more research into extraction of this gene from blue-green algae, a very useful biliverdin reductase BioBrick could be contributed the registry, allowing full degradation of heme to bilirubin. This would enable the microbe as well as the system to which it is contributing, to reap the strong antioxidant properties of this molecule.

References

1. Parhami F., Fang Z.T., Fogelman A.M., Andalibi A., Territo M.C., Berliner J.A. Minimally modified low density lipoprotein-induced inflammatory responses in endothelial cells are mediated by cyclic adenosine mono- phosphate. *J Clin Invest*. 1993;92:471– 478
2. Vincent S.H., Grady R.W., Shaklai N., Snider JM, Muller-Eberhard U. The influence of heme-binding proteins in heme-catalyzed oxidations. *Arch Biochem Biophys*. 1988;265:539 –550.
3. PubChem Compound. Heme. Compound ID: 444098. <http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=444098>. Retrieved July 21, 2009.
4. Maines M.D. The heme oxygenase system: a regulator of second messenger gases. Annu Rev Pharmacol Toxicol. 1997; 37:517–554.
5. Wang L.J., Lee T.S., Lee F.Y., Pai R.C., Chau L.Y. Expression of heme oxygenase-1 in atherosclerotic lesions. *Am J Pathol*. 1998;152:711–720.
6. Morita, T. Heme Oxygenase and Atherosclerosis. *Atherioscler Thromb Vasc Biol*. 2005;25:1786-1795.
7. Morita T., Imai T., Yamaguchi T., Sugiyama T., Katayama S., Yoshino G.. Induction of heme oxygenase-1 in monocytes suppresses angiotensin II-elicited chemotactic activity through inhibition of CCR2: role of bilirubin and carbon monoxide generated by the enzyme. *Antioxid Redox Signal*. 2003;5:439 – 447.
8. Frankenberg-Dinkel, N. Forum Review: Bacterial Heme Oxygenases. Antioxidants and Redox Signalling 2004;6:825 - 834.
9. Stocker R., Yamamoto Y., McDonagh A.F., Glazer A.N., Ames B.N. Bilirubin is an antioxidant of possible physiological importance. *Science.* 1987;235:1043–1046.
10. Morita T., Perrella M.A., Lee M.E., Kourembanas S. Smooth muscle cell-derived carbon monoxide is a regulator of vascular cGMP. *Proc Natl Acad Sci U S A*. 1995;92:1475–1479.
11. Fogg S., Agarwal A., Nick H.S., Visner G.A. Iron regulates hyperoxia-dependent human heme oxygenase-1 gene expression in pulmonary endothelial cells. *Am J Respir Cell Mol Biol*. 1999; 20:797– 804. 92.
12. Moffet, D.A., Foley J., Hecht M.H. Midpoint reduction potentials and heme binding stoichiometries of de novo proteins from designed combinatorial libraries. *Biophys Chem* 2003;105:231-239.
13. Huber W., and Backes W. Quantitation of heme oxygenase 1: Heme titration increases yield of purified protein. *Anal. Biochm.* 2008;373:167-169.
14. Migita C.T., Zhang X., and Yoshida T. Expression and characterization of cyanobacterium heme oxygenase, a key enzyme in the phycobilin synthesis. *European Journal of Biochemistry.* 2003;270:687 – 698.

15. Wang N., Zhao X., and Lu Y. Role of Heme Types in Heme-Copper Oxidases: Effects of Replacing a Heme b with a Heme to Mimic in an Engineered Heme-Copper Center in Myoglobin. *Journal of American Chemical Society* . 2005;127:16541 – 16547.

16. Yoshida T. and Kikuchi G. Purification and Properties of Heme Oxygenase from Rat Liver Microsomes. *The Journal of Biological Chemistry.* 1979;254:4487 – 4491.

17. Ding Z.K., and Xu Y.Q. Purification and characterization of biliverdin IXalpha from Atlantic salmon (Salmo salar) bile. *Biochemistry.* 2002;67:927 – 932.

18. Ollinger R., Yamashita K., Bilban M., Erat A., Kogler P., Thomas M., Csizmadia E., Usheva A., Margreiter R., and Bach F. H. Bilirubin and Biliverdin Treatment of Atherosclerotic Diseases. *Cell Cycle.* 2007;6:39 – 43.

19. Ishikawa K., Navab M., Leitinger N., Fogelman A.M., and Lusis A. J. Induction of heme oxygenase-1 inhibits the monocyte transmigration induced by mildly oxidized LDL. *J. Clin. Invest.* 1997;100:1209-1216.

20. Cornejo J., Willows R. D., and Beale S. I. *Phytobilin biosynthesis: cloning and expression of a gene encoding ferredoxin-dependent heme oxygenase from* Synechocystis *sp. PCC 6803. The Plant Journal* 1998;15:99 – 107.