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## Co-expression of ferrochelatase allows for complete heme incorporation into recombinant proteins produced in *E. coli*

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### Abstract

Over-expression of heme binding proteins in *E. coli* often results in sub-optimal heme incorporation and the amount of heme-bound protein produced usually varies with the protein of interest. Complete heme incorporation is important for biochemical characterization, spectroscopy, structural studies, and for the production of homogeneous commercial proteins with high activity. We have determined that recombinant proteins expressed in *E. coli* often contain less than a full complement of heme because they rather are partially incorporated with free-base porphyrin. Porphyrin-incorporated proteins have similar spectral characteristics as the desired heme-loaded targets, and thus are difficult to detect, even in purified samples. We present a straightforward and inexpensive solution to this problem that involves the co-expression of native ferrochelatase with the protein of interest. The method is shown to be effective for proteins that contain either Cys- or His- ligated hemes.

### Keywords

Heme proteins; Heme incorporation; Free base porphyrin; Ferrochelatase

### Introduction

Heme proteins encompass a wide range of functions that include electron transfer, transport and storage of oxygen, production and sensing of nitric oxide, decomposition of reactive oxygen species, catalytic oxidation of substrates, signal transduction and control of gene expression [1–4]. Under incorporation of heme into recombinant proteins can adversely influence their characterization. Evaluation of enzymatic activity will be systematically underestimated if a significant proportion of the recombinant sample does not contain heme. Furthermore, the proteins devoid of heme may bind another non-native cofactor with a

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**Author contributions:** B.R.C and J.S initiated studies; M.K, S.R.H and D.L.R designed and analyzed resonance raman experiments and discovered free base porphyrin as the contaminant; J.S and B.R.C designed and analyzed the heme-incorporation experiments using Ferrochelatase; B.P and M.V.A verified the method for BP450 and HBPAS; J.S and B.R.C wrote the manuscript.

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contaminating activity of its own. A pure protein sample is usually essential for structural characterization by techniques such as X-ray crystallography, as this method often requires a single, well-folded species for crystallization. Other methods of heme protein characterization are less sensitive to the level of heme incorporation, particularly if those methods directly detect the metallocofactor (e.g. UV/Vis, EPR, and Mossbauer spectroscopy) or rely on enzymatic activity. This is both good and bad in that under incorporation may not greatly affect the analysis, but heterogeneity in the sample may go undetected.

Incomplete heme incorporation into recombinant proteins has been a frequently encountered problem [5–10] and thus, techniques have been developed to improve heme loading [8–14]. During induction of recombinant protein expression from highly active vectors, such as those that employ the T7-polymerase, a population of protein will fold without the heme co-factor under conditions where folding outpaces heme delivery [15]. Supplementing the growth media with  $\delta$ -amino levulinic acid ( $\delta$ -ALA), a precursor in the C5 heme biosynthesis pathway, increases levels of heme biosynthesis and thereby heme incorporation into the target protein [8, 16, 17]. Increased heme biosynthesis rates through  $\delta$ -ALA supplementation allows for complete heme incorporation into some proteins, but not others [11, 12, 18, 19].

Another technique for increasing heme incorporation into recombinant proteins involves supplying the bacteria with hemin in the growth media. However, most *E. coli* strains do not possess an efficient heme transport system, and thus uptake of hemin relies on diffusion through the cell membrane. As a result, hemin feeding is much more effective with strains that co-express heme transport genes from other gram-negative bacteria, along with the heme-protein of interest [10, 13, 14]. For example, co-expression of the heme transport system from *P. shigelloides*, which consists of the proteins Hug A/B/C/D, TonB, and Exb B/D, while also supplementing the growth media with hemin, results in higher amounts of the target holoprotein (in this case hemoglobin) [10]. A similar method [13] involves the co-expression of the heme receptor ChuA from *E. coli* strain O157:H7 to enhance hemin. This latter method also shows a significant increase in the amount of heme-loaded protein generated, although in both cases, the ratio of holoprotein:apoprotein was not evaluated. Another approach utilizes the heme-permeability of *E. coli* strain RP523, which has the *hem B*, porphobilinogen synthase gene disrupted to prevent native heme synthesis. All heme and/or heme analogs are procured by the cells from the growth media and incorporation is nearly stoichiometric for proteins expressed in the cytoplasm (0.8–1.0 heme / heme analog per protein)[20].

Full incorporation of heme in recombinant proteins is also important for commercial applications. For example, the feasibility of employing recombinant human hemoglobin as an oxygen delivery pharmaceutical is limited by the yield of holoprotein that can be made in *E. coli* [10]. Some of the methods discussed above, while effective, require co-expression of several heme transport proteins, which could limit yields, and/or require addition of the heme cofactor. Herein, we put forth a straightforward and inexpensive method for high fidelity incorporation of heme into heme proteins that are over-expressed in *E. coli*. Co-expression of just one native protein, ferrochelatase (FC), in the presence of 60  $\mu$ M  $\delta$ -ALA (10 mg/L, ~\$0.50 per liter of cell culture) is sufficient for 100% heme incorporation into three unrelated heme proteins derived from different organisms.

## Materials and Methods

### Co-expression of Ferrochelatase with gsNOS

FC and gsNOS were expressed from the same pACYCduet vector (Novagen). To clone FC, genomic DNA was extracted from *E. coli* BL21 (DE3) cells with the genomic DNA extraction kit from Epicenter. The FC gene was then PCR-amplified (Phusion polymerase, New England Biolabs) from the genomic DNA with primers that generated *NdeI* and *XhoI* sites at the 5' and

3' ends of the gene, respectively. A stop codon was introduced into the 3' primer before the *XhoI* site to prevent C-terminal attachment of the vector-supplied S-tag. The amplified FC gene was then cloned into the *NdeI* and *XhoI* sites in Multiple Cloning Site-2 of the pACYCduet vector. The gene for gsNOS was derived from a previous pET28a-gsNOS plasmid [21] by digesting the vector with *NcoI* and *XhoI* so as to include the His-tag and the thrombin cleavage site along with the coding sequence for gsNOS in the excised fragment. The *NcoI*-*XhoI* fragment was then cloned into the pACYCduet-FC plasmid between the *NcoI* and *Sall* sites. *Sall* and *XhoI* produce compatible cohesive ends and thereby allow the His-tag, thrombin cleavage site and gsNOS fragment to be cloned between the *NcoI* and *Sall* sites of Multiple cloning site-1 of the pACYCduet-FC plasmid. The resulting pACYCduet plasmid allows us over-expression of gsNOS with a cleavable His-tag and FC with no tag. GsNOS was expressed and purified as reported before [21]. Co-expression of gsNOS and FC was also performed similarly to expression of gsNOS alone, although, a lesser amount of  $\delta$ -ALA was added at the time of induction (10mg/L versus 25 mg/L for gsNOS), and the growth media was supplemented with 100  $\mu$ M FeCl<sub>3</sub>. The antibiotics, chloramphenicol (34  $\mu$ g/L) and kanamycin (50  $\mu$ g/L) were added to the growth media of pACYCduet-gsNOS-FC and pET28a-gsNOS plasmids respectively.

### Co-expression of Ferrochelatase with BP450 and HBPAS

For co-expression of BP450 and HBPAS the same procedure was used. BP450 (NCBI: CBG70284) was cloned into pET151/D-TOPO (Invitrogen), a directional cloning vector with a N-terminal 6x His-Tag followed by a TEV cleavage site and an ampicillin selectable marker (provided by Sarah Barry, University of Warwick, UK). HBPAS (NCBI: NP\_248866) was cloned into pET28a (Novagen) using *NdeI* and *HindIII* restriction sites, which included a N-terminal 6xHis-Tag followed by a Thrombin cleavage site and a kanamycin selectable marker. Competent *E. coli* BL21 (DE3) cells containing FC/pACYCduet were transformed with either BP450/pET151/D-TOPO or HBPAS/pET28. Cells were grown at 37°C in Luria broth containing 20  $\mu$ g/mL Cm and 100  $\mu$ g/L Amp (BP450) or 50  $\mu$ g/L Kan (HBPAS) to an OD = 0.6–0.8. Prior to induction with IPTG, the temperature was reduced to 17°C and 25 mg/L  $\delta$ -ALA was added to the growth media. Cells were harvested 18–20 hrs after induction. An identical procedure with cells lacking the FC plasmid was used to express BP450 and HBPAS without FC. Both proteins were purified using Ni-NTA (Qiagen) chromatography techniques following the manufacturer's protocol. Furthermore, the proteins were purified to >95% purity using size exclusion chromatography after the removal of 6xHis.

### Spectroscopy

Resonance Raman and UV-Visible spectra were recorded as described previously [22].

### Materials

Sodium Chloride was obtained from Mallinkrodt, Ferric Chloride, IPTG and TRIS were from Fisher Scientific, Kanamycin and Chloramphenicol from USBiological.  $\delta$ -ALA was obtained from Sigma-Aldrich.

### Results and Discussion

GsNOS (*Geobacillus stearothermophilus* Nitric Oxide Synthase) is a thermophilic enzyme that forms a highly stable heme-oxygen complex [21] that has helped in identification of catalytic intermediates responsible for L-arginine oxidation to nitric oxide [23]. In the over-expression of heme proteins we routinely add the heme precursor  $\delta$ -ALA to the growth media when protein production is induced. Such  $\delta$ -ALA supplementation results in complete heme incorporation for two other bacterial NOS proteins: *B. subtilis* NOS [24] and *D. radiodurans* NOS [25]. However, in what follows, we show that gsNOS over-expressed and purified from

*E. coli* consists of two species: native heme-containing gsNOS, and gsNOS with protoporphyrin IX (free-base porphyrin) bound instead of heme. We hypothesized that co-expression of ferrochelatase, the enzyme that metallates porphyrin would ameliorate this problem.

### UV-Vis spectroscopy and SDS-PAGE analysis of gsNOS

A UV-Vis spectroscopic analysis of gsNOS over-expressed in *E. coli* shows that the amount of heme incorporated with the protein changes from batch to batch, with the ratio of Soret peak height (403 nm) to protein peak height (280 nm) ( $Abs_{403}/Abs_{280}$ ) varying between 0.25 – 0.40. Co-expression of FC with gsNOS increased  $Abs_{403}/Abs_{280}$  to 0.6 (Fig 1) in several (>3) different protein expression trials. Thus, gsNOS co-expressed with FC increases heme content of the protein in a consistent fashion.

Purified gsNOS, when over-expressed in *E. coli* with 25 mg/L  $\delta$ -ALA added at the time of induction, results in two bands of ~42 kDa on SDS-PAGE (Fig 1, Inset, lane A). Both bands shift on His-tag cleavage (Fig 1, Inset, lane B), which indicates that both species represent recombinant gsNOS, with an intact N-terminus, but different gel mobilities. MALDI mass spectrometry of the sample shows only one sharp peak at a mass consistent with that of full-length gsNOS, which rules out proteolysis as the factor distinguishing the proteins represented in the two bands. Thus, the apparent difference in molecular weight on the gel of the two species (~3 kDa) may rather stem from a net charge difference. GsNOS, when co-expressed with FC from *E. coli* BL21 (DE3) cells, results in only a single species on SDS-PAGE (which corresponds to the lower band of the two observed previously; Fig 1 Inset, lane C). On His-tag cleavage, this band shifts as a single species (Fig 1, Inset, lane D). Protoporphyrin IX is the penultimate product in the heme biosynthesis pathway. FC catalyzes the last step in heme biosynthesis i.e insertion of an iron atom into protoporphyrin IX. Thus, the presence of two bands in the absence of FC co-expression suggests that protoporphyrin IX, rather than heme, has been incorporated into a substantial fraction of the sample.

### Resonance Raman and fluorescence analysis of gsNOS

Confirmation of porphyrin incorporation into gsNOS came from resonance Raman studies of gsNOS in the presence of substrate L-arginine. A sample of gsNOS (expressed without FC) in the presence of substrate L-arginine, shows vibrational frequencies at 662  $cm^{-1}$ , 738  $cm^{-1}$ , 783  $cm^{-1}$ , 1360  $cm^{-1}$  and 1543  $cm^{-1}$ , apart from the typical vibrational frequencies that have been previously observed [26, 27] for other NOSs (Fig 2b). These additional bands disappear after exposure to laser (Fig 2c) and the subsequent difference spectrum (before and after laser exposure) highlights the original, additional resonances (Fig 2d). The frequencies of these vibrations indicates the presence of free-base porphyrin in the protein sample [28, 29]. Furthermore, the enhanced photosensitivity of porphyrin compared to heme explains why the bands disappear after exposure [28, 29]. The presence of free base porphyrin is corroborated by the fluorescence spectrum of gsNOS (Fig 2e), which when measured with excitation at 397 nm shows definitive characteristics of un-metallated heme [30]. Iron-bound heme is not fluorescent when excited at this wavelength. Incorporation of free-base porphyrin suggests that the last step of heme biosynthesis i.e Fe metallation to heme, which is performed by FC, cannot keep pace with protein folding and porphyrin incorporation. Co-expression of FC from *E. coli* with gsNOS and addition of 10 mg/L of  $\delta$ -ALA of (60  $\mu M$ , ~ \$0.50 per liter) generates a sample that is non-fluorescent and whose resonance Raman spectrum shows no evidence of porphyrin (Fig 2a). Thus, under these conditions, the protein is fully heme incorporated.

We also tested if foregoing the addition of  $\delta$ -ALA, while co-expressing FC would result in complete heme incorporation of gsNOS. The purified protein, which was checked for heme content by both SDS-PAGE and by UV-Vis spectroscopy had a higher degree of heme

incorporation than under conditions of only adding  $\delta$ -ALA (heme:protein ratio ~0.5); however, heme incorporation was not complete. Addition of a small amount of  $\delta$ -ALA, (10 mg/L) is sufficient to make up for the slow rate of  $\delta$ -ALA biosynthesis and produce fully incorporated protein in the presence of FC.

### FC-assisted heme incorporation for a bacterial P450 (BP450) and a heme-binding PAS protein (HBPAS)

In addition to gsNOS, FC also increases heme content to saturating levels in two other unrelated proteins: BP450, a Cys-ligated heme protein and HBPAS: a His-ligated heme protein. Both of these proteins, when over-expressed in *E. coli*, are produced with partially heme incorporation. UV-Vis spectra of purified BP450 and BP450 co-expressed with FC are strikingly different, with the increased intensity of the Soret peak indicative of greater heme content in the material produced along with FC (Fig 3). When analyzed by SDS-PAGE, BP450 again also shows two bands (Fig 3, Inset, lane A), which both shift on His-tag cleavage (Fig 3, Inset, lane B). Like gsNOS, on co-expression with FC (Fig 3, Inset, lane C) BP450 produces only one band, which shifts on His-tag cleavage (Fig 3, Inset, lane D). In contrast, HBPAS always results in a single band on a SDS-PAGE gel (not shown), but shows absorption for four Q-bands in the UV-Vis spectrum when produced without FC (Fig 4). Pure heme proteins show only two such bands. The extra band(s) are representative of protein bound protoporphyrin IX [30]. Co-expression of FC results in an increase in the  $Abs_{(Soret)}/Abs_{(280)}$  and the extra Q-bands disappear (Fig 4). The fluorescence spectrum of HBPAS without co-expression of FC is similar to that of gsNOS expressed without FC (data not shown), but this fluorescence, attributable to protoporphyrin IX, also disappears on co-expression with FC.

### Conclusions

The production of  $\delta$ -ALA production is rate-limiting step for heme biosynthesis [31–34] and  $\delta$ -ALA synthesis is itself slowed by heme feedback inhibition. Thus, as has been well recognized [31–34], feeding with  $\delta$ -ALA greatly aids recombinant heme protein production in *E. coli*. However, we have shown here that under conditions of augmentation with  $\delta$ -ALA, ferrous iron insertion into protoporphyrin IX becomes rate-limiting. Co-expression with Ferrochelatase along with the addition of a small amount of  $\delta$ -ALA, is sufficient to produce fully incorporated heme protein. This method is applicable for both Cys-ligated and His-ligated heme proteins. In the case of the two Cys-ligated proteins, porphyrin substitution could be observed on an SDS-PAGE gel as two closely spaced bands and also by fluorescence spectroscopy. In the one His-ligated heme protein example, UV-Visible and fluorescence spectra were effective indicators of insufficient porphyrin metallation, but only one band was observed by SDS-PAGE even with less than full porphyrin content. This is probably because the heme or porphyrin does not remain associated with the PAS protein during electrophoresis, unlike the other two cases. In conclusion, the simple and inexpensive method of co-expressing Ferrochelatase is effective at producing fully incorporated heme proteins in *E. coli*.

### Abbreviations used

$\delta$ -ALA	$\delta$ -aminolevulinic acid
FC	Ferrochelatase
gsNOS	<i>Geobacillus stearothermophilus</i> Nitric Oxide Synthase
BP450	Bacterial P450 protein from <i>S. turgidiscabies</i>
HBPAS	Heme binding PAS domain containing protein.

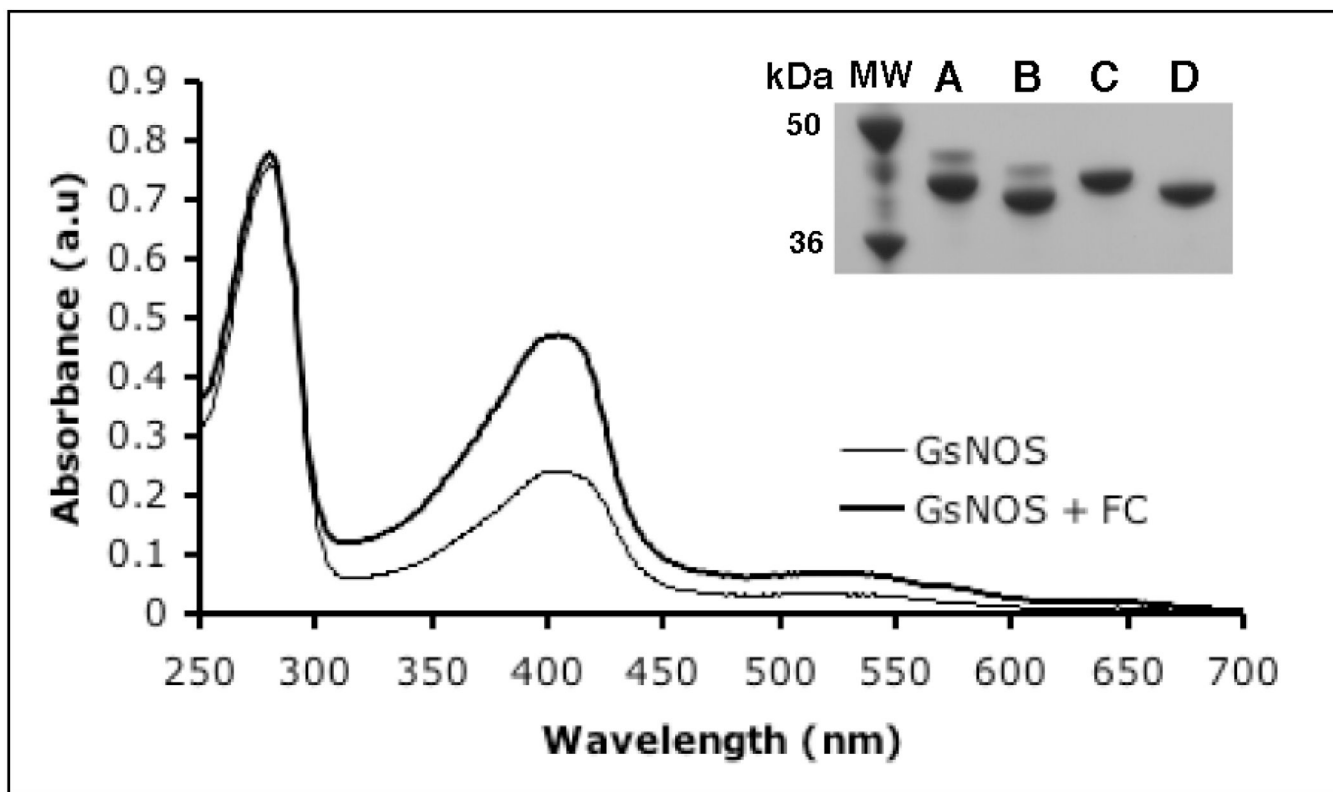
## Acknowledgments

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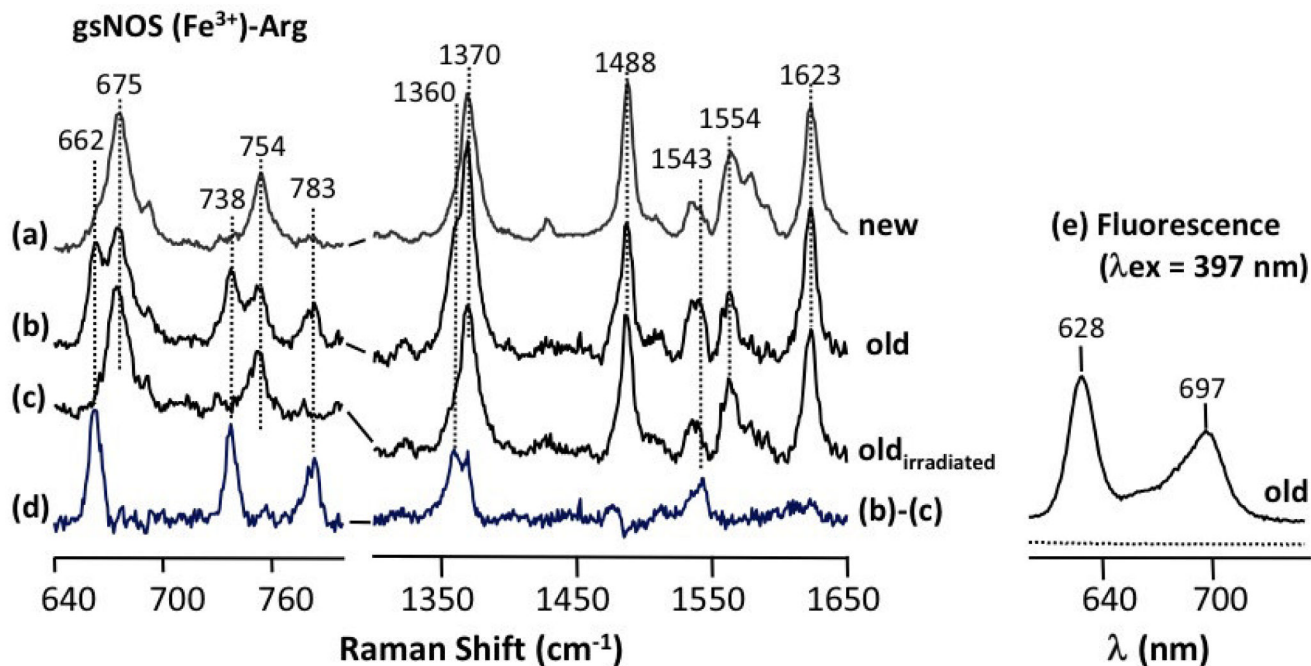
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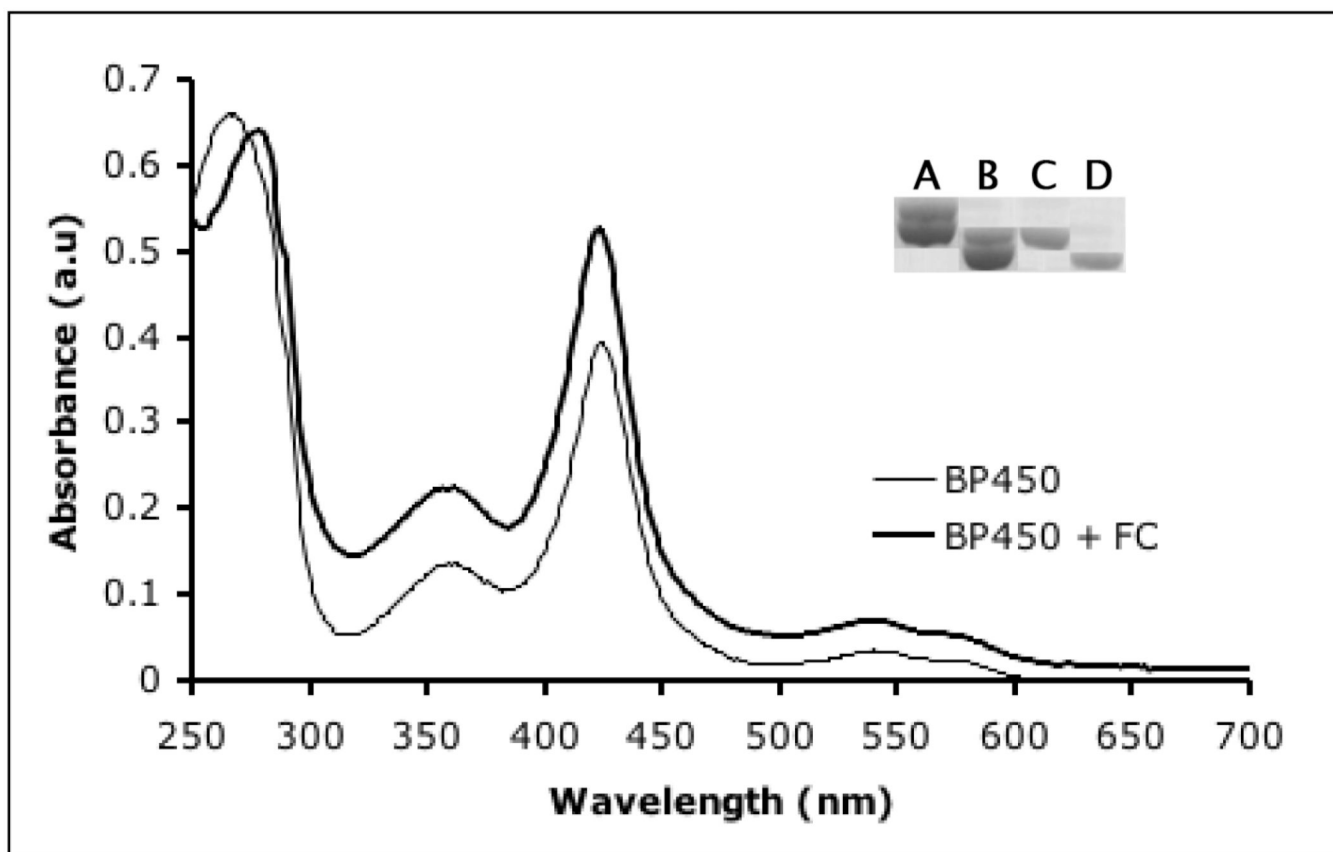
**Fig 1.**

UV-Vis spectra of gsNOS expressed alone (thin line) and gsNOS expressed with FC (**thick line**). Co-expression of FC results in a substantial increase in heme content of gsNOS as measured by the  $Abs_{403}/Abs_{280}$  ratio. This value saturates at 0.6, which indicates nearly complete heme incorporation. Inset: GsNOS expressed by itself results in two bands on SDS-PAGE (lane A), both of which shift on His-tag cleavage (lane B). GsNOS co-expressed with FC results in just one band (lane C), that shifts on His-tag cleavage (lane D) as expected.

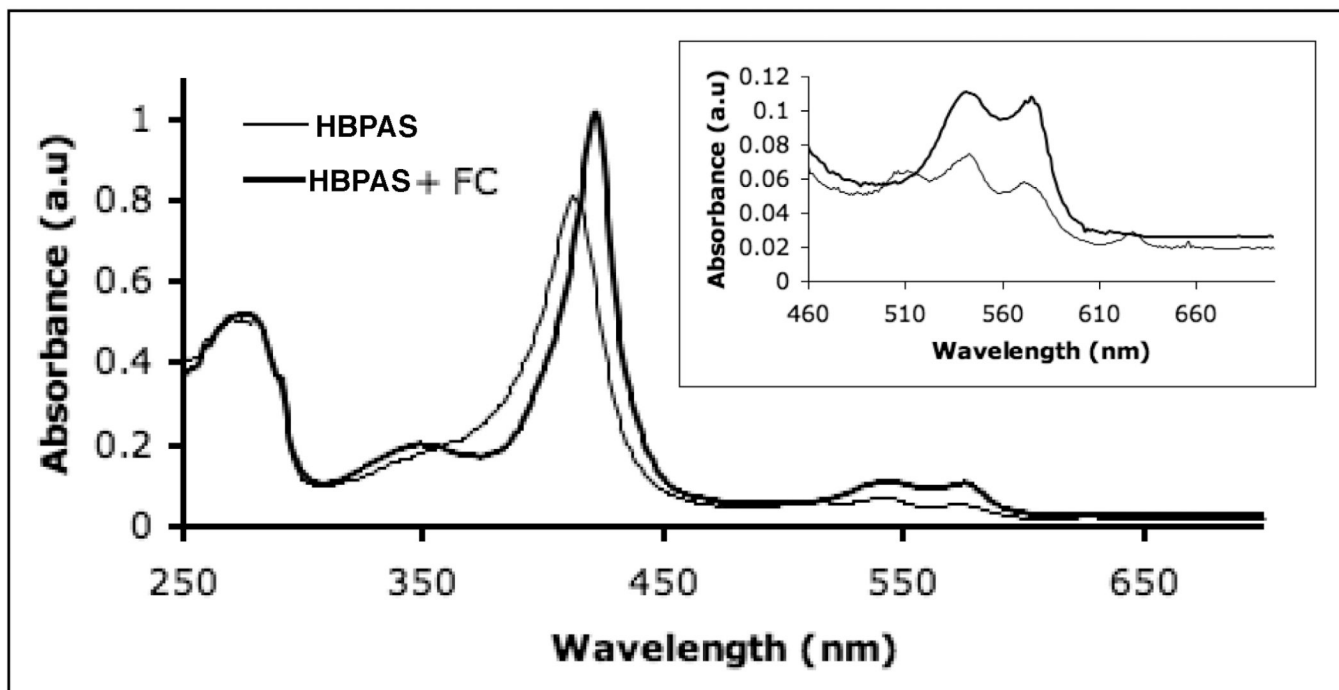




**Fig 2.** Resonance Raman spectra of gsNOS from gsNOS co-expressed with FC (a), gsNOS expressed by itself (b) and (c). The spectrum in (b) is obtained with 3 mW of 413.1 nm laser excitation with an acquisition time of 5 min. The spectrum in (c) is for the same sample as (b), but obtained after prolonged irradiation with 42 mW of 413.1 nm laser for two hours. (d) shows the difference spectrum (b) – (c), which highlights spectral lines resulting from contamination by photosensitive protoporphyrin IX. The fluorescence spectrum in (e) obtained after a 397 nm excitation is representative of that of protoporphyrin IX bound to protein (see text) and is from same sample in (b).



**Fig 3.** UV-Vis spectra of BP450 expressed by itself (thin line) and BP450 expressed with FC (thick line). Co-expression of FC results in a substantial increase in heme content of BP450 ( $Abs_{Soret}/Abs_{280}$ ). Inset: BP450 expressed alone also results in two bands (lane A), both of which shift on His-tag cleavage (lane B). BP450 co-expressed with FC results in one band (lane C), which shifts on His-tag cleavage as expected (lane D).



**Fig 4.** UV-Vis spectra of a Heme Binding PAS domain (HBPAS; His-ligated) when expressed by itself (thin line) shows four Q-band absorption peaks (inset). Co-expression with FC (thick line) increases the heme content and results in only two Q-bands (inset).