BIVMiB003 BB/L013711/1 1 February – 31 July 2015
POCMiB002 BB/L013711/1 1 August 2015 – 31 May 2016
Funded by a Metals in Biology BBSRC NIBB Business Interaction Voucher and Proof of Concept Funding
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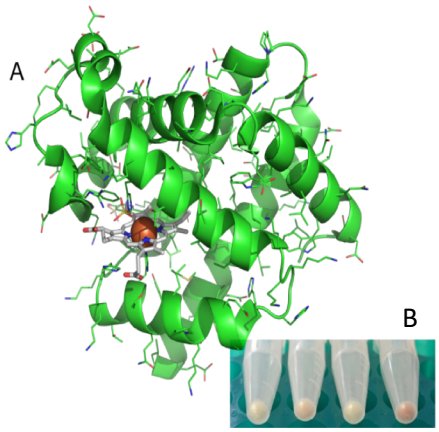
Enhancing *E. coli* for optimal cofactor insertion into heme-containing proteins

"This work showed that the P450 enzyme was sub-optimally loaded with heme. Further enhancement of heme incorporation could increase the commercial yields of this enzyme, so we support further investigations into this and are pleased to continue our collaboration with Professor Le Brun's laboratory." **Biocatalysts Ltd**

Nick Le Brun, Myles Cheesman, Jonathon Todd & Jason Crack, University of East Anglia; Mark Blight & Jon Wood, Biocatalysts Ltd

OUTCOMES: Our initial set of studies showed that the amount of cytochrome $P450_{BM3}$ recovered from *E. coli* cell lysates was low and that only 56% of the P450 contained heme. Moreover, $P450_{BM3}$ existed as truncated protein; most likely due to proteolysis in the cell lysates. We then explored several different strategies to improve heme incorporation into several heme-containing proteins, including over-expression of parts of the heme biosynthetic pathway and the expression of membrane-bound importers capable of taking up intact heme from the growth medium. Using *E. coli* (a workhorse for protein production), we generated a system that gives ~3-fold enhancement of heme incorporation into two very different 'test' heme-containing proteins (*E. coli* bacterioferritin and human myoglobin). Preliminary results with a third 'test' protein indicated that the effect is reproducible for other heme-containing proteins. To date, we have not seen any negative effects on yield of protein. These are all important properties for a process that could be commercially useful.

A) Cartoon structure of human myoglobin showing the heme group with an iron atom at its centre. **B**) *E. coli* cell pellets. The two tubes on the left show pellets of cultures without the heme enhancement system; the two on the right with it. Each pair shows before and after induction of heme protein production.



INITIAL AIMS: Many metalloproteins have the potential to be used as biocatalysts in the synthesis of useful materials or medicines. However, to exploit this the metalloprotein must be purified with its metallo-cofactor fully incorporated to avoid major inefficiencies in the production process. A strategy to improve cofactor insertion is to more carefully match protein synthesis with cofactor synthesis/insertion. This should be achievable by engineering cell factories to increase their capacity to produce and incorporate the cofactor. In these projects, we will first analyse the level of heme insertion in a commercially relevant metalloprotein, cytochrome P450. We will then generate *E. coli* strains of that have increased capacity to incorporate heme into cytochrome P450, which, together with optimised growth protocols, will be tested for the production by the commercial partner.

- Awarded funding (£12k) from UEA to continue the project
- Aim to submit application to BBSRC Follow on Funding Pathfinder scheme
- Aim to test increased heme incorporation into wider range of proteins

BIVMiB041 BB/L013711/1
1 September – 31 October 2017
Funded by Metals in Biology BBSRC NIBB Business Interaction Voucher
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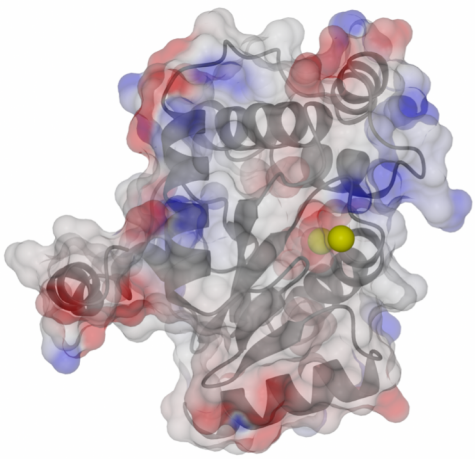
Exploiting a copper-dependent chaperone system to improve bioprocessing of therapeutic antibodies

"NIBB meetings have brought me into contact with a range of industrial contacts. This BIV sparked a new project with Diosynth Biotechnologies to investigate the potential of bacterial copper-tolerance machinery to facilitate assembly of protein targets of biotechnological importance." Mark Shepherd, University of Kent

Mark Shepherd, University of Kent; Christopher Lennon, FujiFilm Diosynth Biotechnologies



Structure of ScsC, a thioredoxin-like protein of Salmonella with a potential role in disulphide folding of therapeutic proteins. Negative and positive surface charge are shown in red and blue, respectively. Sulphur atoms of active site cysteines are shown as yellow spheres.

This project explored the impact of copper and the Salmonella Scs proteins upon the assembly of Herceptin and Lucentis, therapeutic antibodies used to treat breast cancer and macular degeneration respectively. First we developed systems for expression of Herceptin and Lucentis in *E. coli*. After this we used state-of-the-art mass spectrometry approaches to perform quantitative proteomics measurements on *E. coli* strains grown in the presence and absence of copper to assess total protein and antibody abundance. We showed that copper elevates protein levels in *E. coli*. Copper diminished the expression of Herceptin, an effect that was reversed by expression of ScsABCD. We also showed that the native disulphide-folding machinery in *E. coli* is essential for Herceptin production.

INITIAL AIMS: The production of biotherapeutics has a total market value of around £100 billion per year. As well as therapeutic uses, antibodies have applications as research tools, in diagnostics and in consumer healthcare products. We have previously investigated the potential of copper-dependent protein folding catalysts (Scs proteins) to improve the production of antibody fragments of Herceptin (Trastuzumab) in *E. coli (*BIVMiB014). This project expands the repertoire of that system; we will build upon work done so far with Herceptin and also study Lucentis (Ranibizumab).

- Future studies may focus on the effects of copper and ScsABCD on Lucentis yield

BIVMiB001 BB/L013711/1
16 February – 15 August 2015
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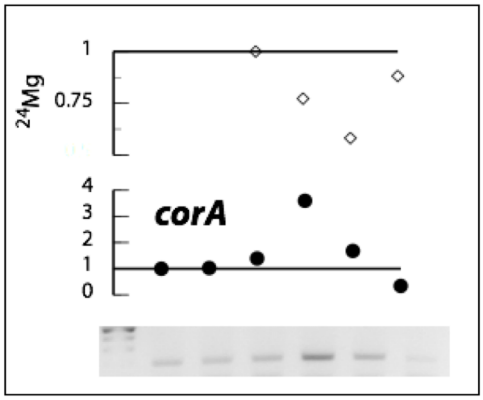
Metal demands during protein overexpression in bacteria

"For Biocatalysts Ltd, this study highlighted the importance of metal supplementation in commercial fermentation processes to maximise enzyme activity and yield."

Peter Chivers, Durham University; Mark Blight, Biocatalysts Ltd



Cellular Mg content is inversely correlated with changes in transcript levels of the *corA* gene, which is associated with Mg uptake.

OUTCOMES: Transcript levels and metal content of *E. coli* cells were measured at different time points post-induction during a representative fermenter run. Increased transcript levels of genes important for Mg, Fe, Mn, and Ni acquisition were observed in the latter half of the protein overexpression time course (≥ 9 h post-induction). These increases correlated with decreases in total cellular metal content for each metal, consistent with metal deficiency sensed by metal-responsive transcriptional regulators. These deficiencies have potential effects on translational efficiency (Mg), synthesis of non-natural amino acids that affect the fidelity of tRNA charging (Ni), and posttranslational processing of newly synthesized polypeptides (Fe and Mn). No evidence for Zn-deficiency or Cu-stress was detected based on transcript levels and metal content. These results suggest straightforward strategies — namely metal supplementation — to ensure metal supply is maintained during the protein overexpression time course.

INITIAL AIMS: Protein overexpression is a major facet of industrial biotechnology, yet the capacity of host organisms to overexpress proteins is not naturally optimized. Transition metals are key components of the cellular protein synthesis machinery. This project will explore the changes in metal demands brought about by protein overexpression in *Escherichia coli*, a widely used platform for biologics production. Metal allocation and use will be determined by transcript analysis to monitor changes in the expression of metal-regulated genes and measurement of cellular metal content to establish links between changes in gene expression and metal supply.

- Supplementing growth media with metals could improve the quality and/or quantity of protein synthesis

BIVMiB013 BB/L013711/1
1 February 2016 – 31 July 2016
POCMiB022 BB/L013711/1
27 November 2016 – 26 May 2016
Funded by a Metals in Biology BBSRC NIBB Business Interaction Voucher and Proof of Concept funding
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New routes for the expression of heme protein targets

"Production of enzymes in quality and quantity sufficient for biophysical and structural analysis has consistently been a major bottleneck for drug discovery efforts. This scheme is hugely welcomed, to help overcome these bottlenecks, not just for my company but for the entire pharmaceutical sector." Andreas Kuglstatter, Roche Innovation Centre

Emma Raven, University of Leicester
Andreas Kuglstatter, Roche Innovation Centre

OUTCOMES: In this project, new expression methods were developed for a range of different heme enzymes that are not readily expressed using conventional methodologies in *E. coli.* Targets from Emma Raven's laboratory were used as a 'test bed' for other heme systems. Human CLOCK protein, which is important in the control of circadian rhythm and thus an important drug target, was included amongst the targets.

INITIAL AIMS: Heme-containing enzymes are a mainstay of industrial biotechnology, and the industry depends on fundamental improvements in methodology emerging from academic groups to harness the potential of their investments in biopharmaceuticals, bioenergy, biocatalysis and drug design. For a number of complex reasons, the interactions between industry/biotechnology and academic laboratories are often less facile and less extensive than they could be, so that new (often specialist and/or unpublished) information is not transferred fluently to industrial partners.

Our overall objective is to use this project to develop new refolding methodologies for expression of difficult (insoluble) heme protein targets, and to set up an on-going dialogue between industrial and academic partners with mutual cognate interests in specific heme enzyme targets. The methodologies that we develop will open up new avenues for industry partners in cases where they have intractable (insoluble) protein targets.

- New expression methods may overcome bottlenecks in drug discovery

Kent and UCB partner on new strategy to boost antibody production

A new partnership between the University of Kent and biopharmaceutical company UCB — funded by a business interaction voucher from Metals in Biology BBSRC NIBB — has identified a potential new way to improve the production of protein-based drugs.

Many drugs that are used to treat cancer and autoimmune disease, as well as insulin, are proteins, and as such need to be made in cells, for example bacteria, mammalian cells or yeast. To function as drugs, proteins must be correctly folded, a process that depends on the formation of disulphide bonds.

Mark Shepherd from the University of Kent, who was the principle investigator on this project, has previously characterised a protein called ScsC (survival of *Salmonella* under copper stress protein, see figure). This enzyme catalyses the formation of disuphide bridges and its activity is influenced by copper.

"The ability of ScsC to facilitate disulphide folding in the *E. coli* periplasm has huge potential for the production of proteins of therapeutic importance."

In this project with UCB, he tested whether the the Scs system and copper could used in *E. coli* to improve the yield of a therapeutically relevant antibody fragment through its effects on disulphide bridge formation.

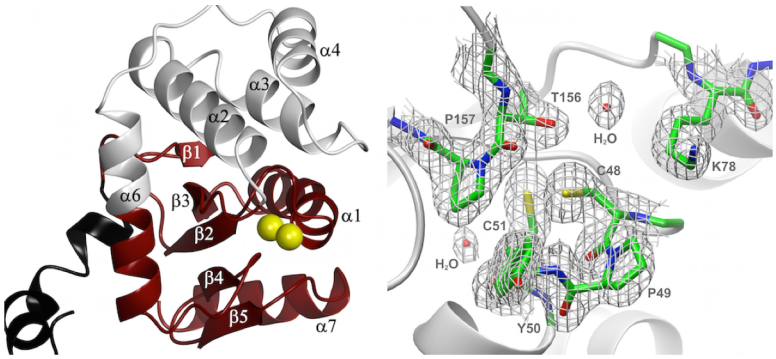
"Improving the yield of correctly-folded antibody fragments (and other high-value proteins) is of clear benefit to UCB," says David Humphreys, industrial partner on this project.

The study used Fab (fragment antigen-binding region) fragments of the breast cancer drug trastuzumab (Herceptin). Compared to full length antibodies, Fab fragments have advantages that include improved specificity, increased delivery options and more economical production systems.

Results from the study showed that Fab fragments of trastuzumab were successfully expressed in *E. coli* periplasm.

"This project has sparked a new relationship with UCB to investigate the potential of our disulphide-folding machinery to assemble a range of protein targets of biotechnological importance," says Mark. Indeed, the partners hope to continue their work to investigate the interaction of ScsC with other protein targets.

"The ability of ScsC to facilitate disulphide folding in the *E. coli* periplasm has huge potential for the production of proteins of therapeutic importance," concludes Mark.



Structure of ScsC, a thioredoxin-like protein of *Salmonella* with a potential role in disulphide folding of therapeutic proteins. Overall protein fold and active site S atoms (yellow spheres) are shown on the left, and the local environment of the CPYC active site is depicted on the right.

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University of Kent
UKRI Biotechnology and Biological Sciences Research Council