Protein Crystallisation at York

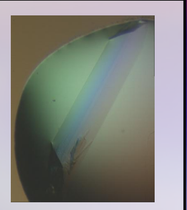
Ongoing developments & personal stories

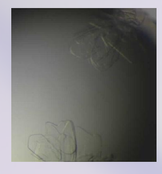
Shirley M Roberts (on behalf of the group)

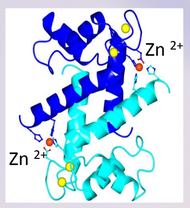
September 11-14 2011 RAMC

York Structural Biology Laboratory

S100A12-zinc complex crystals: avoiding aggregation







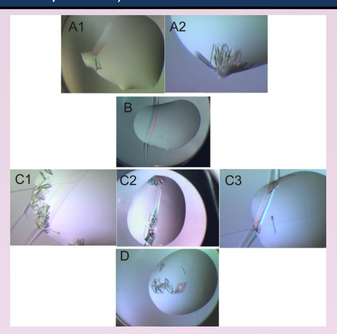
- Human S100A12 (a member of the S100 family of calcium-modulated EF-hand proteins) has a role in inflammation and host parasite responses and is linked to major diseases such as diabetes, cystic fibrosis, rheumatoid arthritis and atherosclerosis. Similar to other S100 proteins, S100A12 binds zinc in addition to calcium, the zinc binding enhances the calcium affinity by a factor of 1500.

- Crystallisation did not occur after 0.5-5mM zinc was added directly to 10mg/ml protein and a range of methods indicated the protein was aggregating at this zinc conc. However, when S100A12 was diluted to 0.1 mg/ml (~10 µM) in buffer + 10 µM zinc acetate and concentrated to 10 mg/ml, crystals were obtained. The procedure was repeated three times to achieve complete saturation with zinc whilst avoiding protein aggregation.

- The protein/zinc complex grew in two crystal forms (P212121 and F222) which diffracted to 1.9 and 1.7 Å resolution respectively. The zinc complex structure suggested an explanation for the zinc-induced 1,500-fold increase in calcium affinity and provided insight into the role of a zinc-calcium interplay in the transition of S100A12 from a dimer through a tetramer to a hexamer.

Olga V Moroz (ref. 1)

Scratching around for crystals



- Nucleation of crystals and improvement of crystal quality was achieved by manually scratching the surface of MRC 96-well plates.

- Initial crystals grew in a 150+150 nl sitting drop (A1). Eventually, after several crystallization trays, another cluster appeared (A2). The crystals appeared to grow from deformations of the borders of the drop, also the drop pH had lowered over time.

- A tray was set up with adjusted lower pH and duplicate crystallization experiments. In one of these a manual scratch was made in the well using standard laboratory tweezers in order to introduce deformations at the edge of the drops (B).

- The scratches had three different effects on the crystallization of the protein. Firstly, the deformations in the borders of the drop promoted nucleation (C1). Secondly, the scratches promoted nucleation in inner parts of the drop (C2). Lastly, single crystals grew in other parts of the drop (C3). In the control drops, where no scratch was introduced, smaller numbers of crystals and fewer single crystals were obtained (D).

- Manual scratches can promote nucleation and improve the crystal quality. Although this is likely to be protein dependent, it is suggested as another trick for the crystallization toolbox.

Javier García-Nafría (ref. 2)

ThermoFluor: an aid in protein/ligand studies

- A fluorescence-based thermal shift assay (ThermoFluor®) is being used to assist with crystallisation and identification of ligands which bind to proteins.

- An environmentally sensitive fluorescent dye, such as SYPRO® orange, is used to monitor the unfolding of the protein as the temperature is increased, using a qPCR machine e.g. Stratagene Mx3005P (ref.3,4). As the temperature increases and the protein unfolds there is an increase in exposed hydrophobic residues which results in an increase in fluorescence. The fluorescence spectra can be used to calculate the melting temperature, T_m, of the protein.

- The binding of ligand to a protein, (Fig.1:TolB), can stabilise the protein and therefore increase the melting temperature. In the presence of Indole-3-propionic acid (IPA), the melting temperature of the protein, TolB, was increased by almost 2°C.

Figure 1

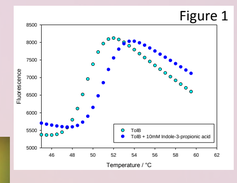


Figure 2

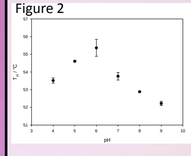


Fig. 2: T_m of TolB in Malonic Acid, Imidazole, Boric Acid (MIB) buffer, at different pH's. Data plotted is the average of duplicates.

Figure 3

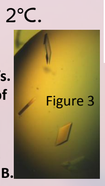


Fig. 3: TolB/ligand crystals.

Figure 4

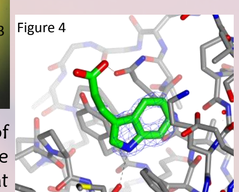


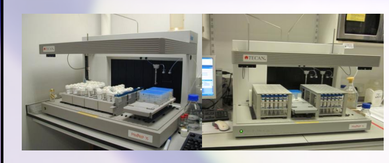
Fig. 4: X-ray structure of IPA bound within a pocket of TolB.

- The technique can also assist with crystallisation of proteins if problems occur. The melting temperature is measured for protein plus a screen of different buffers, pH's and additives to identify conditions in which the protein is most stable (ref.4).

Abigail K Bubb

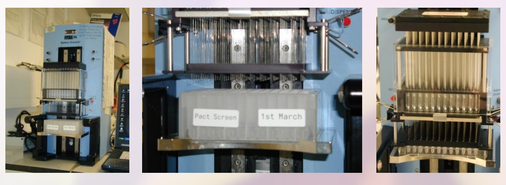
Structural Biology at York: various robotics facilitate the work flow!

Preparation of crystallisation screens:
Tecan Miniprep robots (Tecan UK Ltd)

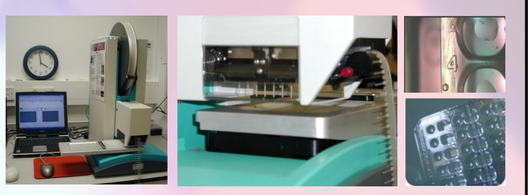


Crystallisation Screens used in YSBL:
Index from Hampton Research (HR)
Silver Bullet and additive screens (HR)
Crystal Screen 1 and 2, mixed in York (York)
Peg-Ion 1 and 2 (York)
Pact from Molecular Dimensions (MD)
Clear Strategy Screen 1 and 2 (MD)
PGA (MD)
Morpheus (MD)
Variation of JCSG (York) and JCSG+ (MD)
Stura Footprint and 24 buffer screen (York)
MPD screen (York)
Ammonium Sulphate Screen (York)

Transfer of screen solutions from deep-well storage plates to crystallisation plate:
Reconditioned Hydra (αlpha βiotech, London)

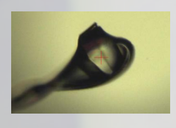


Setting up the crystallisation trays:
Mosquito (TTP Labtech, Cambridge)



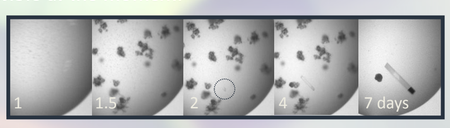
Favourite crystallisation trays are the MRC plates (2-drop 96 well and maxi 48-well) from SWISSCI.

In the last year, our Rigaku Actor sample changer robot has become fully operational. Once conditions have been optimised for cryoprotection, many crystals can be fished and loaded. The robot will test and rank crystals and, if wanted, proceed to collect data on the best one. It has made testing crystals ready for synchrotron trips a lot less time consuming!



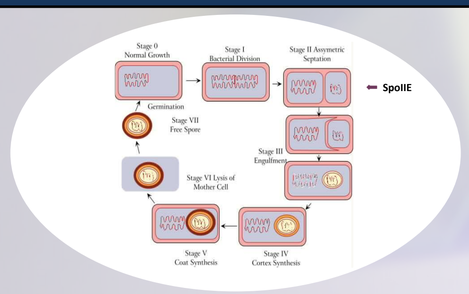
Examination of Trays

Unfortunately our BioStore Imager robot finally died a while ago so we are only able to examine our trays using microscopes and observing crystal growth in retrospect (images below) and the use of crystalPIMS is no longer possible at the moment.



A home-made sticker chart is used to indicate which screens and conditions give protein (and salt!) crystals

A counterintuitive solution to a Mn problem

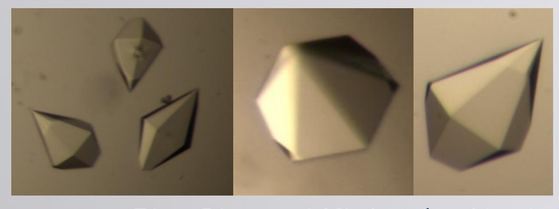


- SpoIIE from *B. Subtilis*, a key protein in sporulation, is an 872 residue protein thought to consist of three domains. It has 10 putative membrane-spanning segments at its amino terminus, a FtsZ-binding domain and a PP2C-type phosphatase domain at its C-terminus.

- Difficulties in soluble expression of SpoIIE proved an obstacle to biophysical characterisation and structural studies. A set of soluble fragments of SpoIIE were identified, overproduced and characterised enabling the crystal structure of SpoIIE (590-827, the PP2C phosphatase domain) to be solved.

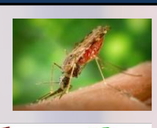
- It is known that Mn ions are required for the phosphatase activity of the protein and it's PP2C domain in particular. Initial SpoIIE 590-827 crystals used for structure determination did not contain Mn ions. Interestingly, crystals grown in the presence of 10-50 mM $MnCl_2$ only in the well solution were found to be free of manganese.

- A preliminary incubation of the protein with the metal ions was required to obtain crystals with Mn bound. The protein was incubated with a small amount (0.1-2mM) $MnCl_2$ for 30 minutes before setting up the hanging drop crystallisation tray mixing protein and well solutions in a 1:1 ratio. A similar small amount of Mn was added to the well during crystallisation.

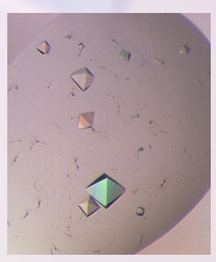


Elena Blagova & Vladimir Levdikov (ref.10)

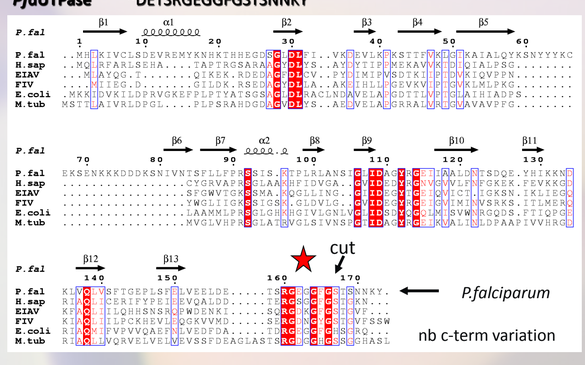
Pf dutpase c-terminus cut aids crystallisation



Trimeric *pf*dutpase plus trityl inhibitor (ref. 5)

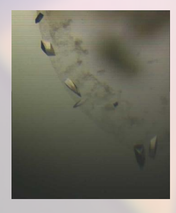


Crystals of *pf*dutpase



Shirley M Roberts, Jim Brannigan & Jean L Whittingham (ref. 6)

Aids to obtaining crystals and data in two projects



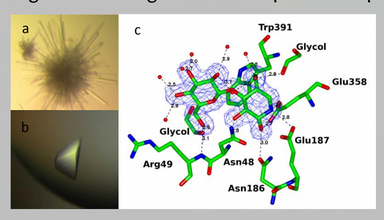
- The crystal growth conditions for a target protein involved iso-propyl alcohol or tertiary butanol. Crystal growth needed repeating for new ligand soaks.

- Gel Filtration in the York laboratory at 21 C:- protein degraded, working at 4 C was crucial.

- His-tag + cleavage:- no crystals. Untagged protein needed expressing for crystal growth.

- Crystallisation and fishing were all done at 4°C.

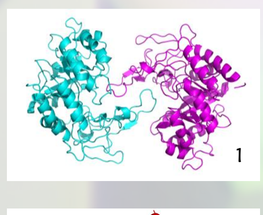
- Oil (paratone + mineral oil mix) next to the crystal drop during fishing helped to stop crystals dancing around and was an effective cryoprotectant. Often no cryo was used which made fishing easier, the faint ice-rings on the images did not impede data processing.

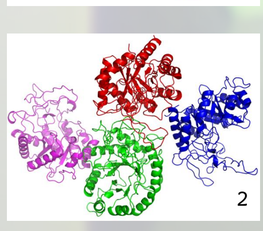


- A Xyloglucanase required a double amino acid mutation to obtain crystals of the enzyme for ligand studies (figures b,c). Previously the native enzyme from various species was screened but only poor crystals were obtained if any (figure a).

Shirley M Roberts (ref.9)

Re-routing around a crystallisation dead-end

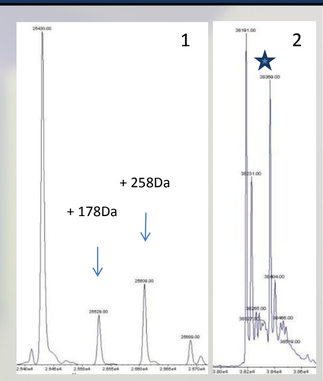




- Enzyme A was selected for crystallisation and kinetic studies. It proved easy to express and purify and was amenable to kinetic study using various synthetic substrates and ligand compounds.
- Crystallisation trials and subsequent optimisation yielded small rod-like crystals belonging to the crystallographic spacegroup P1. Structure solution revealed a dimeric model where two molecules of the protein formed a "loop-swapped" dimer (1).
- Unfortunately this arrangement blocked both active sites. Further optimisation, rescreening for new crystallisation hits, seeding and co-crystallisation with high affinity ligands all failed to produce crystals with altered molecular packing.
- However, during this time new sequence information was released for enzyme B from a closely related organism which was functionally homologous to A. Protein B was also easy to express and purify and was immediately setup in crystallisation trials. Crystals of Protein B from two conditions belonged to spacgroups $P6_122$ and I4 respectively. Structure solution revealed four protein molecules in a monomeric conformation for the former (2) and an asymmetric unit harbouring only a single molecule for the latter. The active site of protein B appeared solvent accessible in both crystal forms, thus allowing successful structure solution for a variety of ligand complexes.

Andrew J Thompson

Protein heterogeneity uncovered by ESI-MS



- Routine characterisation studies of proteins at YSBL using electrospray ionisation mass spectrometry (ESI-MS) have demonstrated the presence of 178 Dalton adducts.

- His-tagged proteins grown in LB media and expressed in *E.coli*BL21(DE3) can undergo modification by gluconic acid derivatives. The incidence of modification is dependant on the N-terminal amino acid sequence adjacent to the His-tag (ref.7) with polypeptide sequences of MGXXHHHHHH, where XX is AS, SA, AA or SS, being most prone to modification.

- The additional mass of either 178Da or 258Da, represents the addition of glucolactone or 6-phosphogluconolactone and leads to protein heterogeneity. This ranges from low levels of adduct formation (Fig.1) to an almost even distribution between adducted and non-adducted forms (Fig.2★). N-terminal phosphogluconoylation can inhibit protein crystallisation (ref. 8)).

- Should crystallisation difficulties occur, growing cells in a high nutrient media (e.g. Terrific Broth), deleting the His-tag or using a different expression vector could yield unmodified proteins more amenable to crystallisation.

Simon J Grist

THE ROYAL SOCIETY · EPSRC Engineering and Physical Sciences Research Council · wellcome trust · British Heart Foundation · bbsrc biotechnology and biological sciences research council · novozymes · hefce

THE UNIVERSITY of York · Structural Biology Laboratory

Department of Chemistry - University of York - York - YO10 5YW - www.ysbl.york.ac.uk

The York Structural Biology Laboratory is headed up by a team of senior staff and supported by the various grant awarding bodies pictured. My thanks to all those in YSBL and the Biology Graphics Office who have helped me with advice and content for this poster and for providing such a pleasant and stimulating working environment! Finally my thanks to Allan D'Arcy and Bob Cudney for organizing the excellent series of RAMC meetings and for aiding me tin attending RAMC 2011. Finally my very best wishes got to Allan D'Arcy for his future retirement.

References:
1) Moroz, OV, et al. (2009) The crystal structures of human S100A12 in apo form and in complex with zinc: new insights into S100A12 oligomerisation. J Mol. Biol. 391(3):536-51
2) García-Nafría, J, et al. (2010) The *Corynebacterium glutamicum* aconitase repressor: scratching around for crystals. Acta Cryst. (F) (2010) Struct Biol Cryst Commun. 66(9)1074-7
3) Pantoliano, MW, et al. (2001) High-density miniaturized thermal shift assays as a general strategy for drug discovery. J Biomol. Screening **6**(6): 429-440.
4) Niesen, FH, et al. (2007) The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability. Nature Protocols **2**(9) 2212-2221.
5) Whittingham, JL , et al. (2005) dUTPase as a Platform for Antimalarial Drug Design: Structural Basis for the Selectivity of a Class of Nucleoside Inhibitors. Structure, Vol. 13, 239-338
6) Hampton, SE, et al. (2011) Design, Synthesis, and Evaluation of 5'-DiphenylNucleoside Analogues as Inhibitors of the *Plasmodium falciparum* dUTPase. ChemMedChem 6, 1–17
7) Geoghegan , et al. (1999) Analytical Biochemistry, 267, 169-184
8) Kim, et al. (2001) Acta Cryst. D57, 759-762
9) Ariza, A, et al. (2011) Structure and Activity of *Paenibacillus polymyxa* Xyloglucanase from Glycoside Hydrolase Family 44. J Biol. Chem. (in press)
10)Levdikov, VM, et al. (submitted) Structure of the Phosphatase Domain of the Cell Fate Determinant SpoIIE from *Bacillus subtilis*. J. Mol. Biol. 15