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Report by Dr Hernan Alonso

2012 Churchill Fellow

To study the structure of an oil-degrading protein using cryo-electron microscopy - Japan

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Signed Dr Hernan Alonso Dated 4th October 2013

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INTRODUCTION

As part of my 2012 Churchill Fellowship I travelled to Tokyo (Japan) to learn how to use Cryo-Electron Microscopy to determine the three dimensional structure of proteins. This is a cutting edge technique that allows the determination of high-resolution protein structures without the need of 3D protein crystal. There are only a few groups in the world that have mastered this technique, and none in Australia.

This fellowship allowed me to visit the laboratory of Dr Kaoru Mitsuoka at the National Institute of Advanced Industrial Science and Technology (AIST). I was able to learn, from one of the experts in the field, the steps involved in the preparation of the protein sample, data collection, and data analysis. I worked on two different samples: AlkB, a protein from *Pseudomonas putida;* and the glideosome from *Toxoplasma gondii*.

Many aquatic and terrestrial environments have been severely damaged by oil spills caused by human activity. Petrochemicals are very resilient to natural degradation and, therefore, remain in the environment for very long periods. The only clean up strategy currently in use is the dispersion of the contaminating oils using detergents. This approach does not remove the problem, but simply reduces the impact by spreading and diluting the contaminant. Therefore, by studying the oil-degrading protein AlkB we hope to help in the design of future environmentally friendly clean-up strategies.

Toxoplasma gondii, the causative agent of toxoplasmosis, is one of the most ubiquitous pathogens, chronically infecting 30-80% of the world's population. Although most healthy individuals quickly recover from infection, pregnant women and immune-compromised patients are at risk. The parasite is able to move and infect cells thanks to a unique molecular motor, unique to the apicomplexan parasites. The detailed understanding of this motor and its function will allow us to design new and more potent drugs for the treatment of infections.

Acknowledgments

I would like to thank the Winston Churchill Memorial Trust for sponsoring my trip to Japan and providing me with the unique opportunity to develop my skills in Cryo Electron microscopy. This trip has allowed me to bring back to Australia a state-of-the-art technique that will allow us to study the structure of many important proteins in a way that was not possible before.

I thank Dr Kaoru Mitsuoka for opening his lab to me and for his unreserved guidance and support. I would also like to thank Dr Takeshi Yokoyama for his friendship and technical advice and Dr Kazuhiro Mio for his help and support.

I would have not received this fellowship without the help and encouragement of Dr Anna Roujeinikova (Monash University), who is the principal investigator behind the AlkB project. Finally, I would like to extend my thanks to Dr Chris Tonkin (Walter and Eliza Institute of Medical Research), my current supervisor and the driving force behind the *Toxoplasma gondii* project.

EXECUTIVE SUMMARY

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Project description

Proteins function as molecular machines in all living organisms. They are in charge of performing many tasks at the molecular level, and this is why it is so important to understand their structure. The purpose of this project was to travel to Japan to learn cyo-electron microscopy, an emerging and powerful technique for the study of protein structure. I was interested in discovering the structure of two different molecular machines:

- AlkB, an oil-degrading protein from the bacteria *Pseudomonas putida*. Understanding how some bacteria can grow in oil-contaminated environments is of great importance to design and plan effective and environmentally-friendly clean-up strategies.
- The glideosome, a protein complex from the parasite *Toxoplasma gondii* responsible for motility and invasion. Understanding how these parasites move and burrow themselves into cells is of critical importance to design new and more effective therapies.

Highlights

The Churchill Fellowship allowed me to travel to Tokyo and spend one moth as a visiting Fellow at the laboratory of Dr Kaoru Mitsuoka. During this time I was able to work on different aspects of cryo-electron microscopy, including sample preparation, optimisation, screening, data collection, and initial analysis. Unfortunately, the 2D crystals of the AlkB protein did not survive the trip, and I was not able to collect data. However, the glideosome motor complex of *Toxoplasma gondii* arrived in good condition, and I was able to spend time optimising sample preparation and data collection.

Recommendations, implementation and dissemination

The work I started in Japan is ongoing, and I will continue to process and analyse the data collected on the glideosome complex in Australia. I expect this work will result in a scientific publication and will be presented in seminars and conferences. Furthermore, these new set of skills will allow me to become an active member of the new Ramaciotti Centre for Structural Cryo-Electron Microscopy at Monash University, to be completed by mid 2014. With state-of-the-art technology, the technical knowledge to use it, and strong international collaborations, I will be able to contribute to the establishment and development of cryo-electron microscopy in Australia.

PROGRAME

July 29th 2013: Departed Melbourne.

July 30th 2013: Arrived in Tokyo, Japan (via Sydney).

July 30th to August 23rd: visiting Fellow at the laboratory of Dr Kaoru Mitsuoka, National Institute of Advanced Industrial Science and Technology (AIST, Tokyo, Japan).

August 23rd 2013: Departed Tokyo for Australia.

August 24th 2013: Arrived in Melbourne (via Sydney).

MAIN BODY

Background

A clear understanding of the biological function and mechanism of proteins requires atomic-resolution knowledge of their structure. There are several approaches available to look at protein structure, with each having their own advantages and limitations.

The first protein structures to be solved were hemoglobin and myoglobin, by Perutz and Kendrew, respectively, in 1958 by single-crystal X-ray crystallography. This technique has seen mayor advances during the last 50 years, including sample preparation, crystallization, crystal handling, data collection, synchrotron sources, phasing approaches, and high-speed computing and visualization (Egli, 2010). However, all these advances do not change the fact that X-ray crystallography is still a complex technique. One of the main practical obstacles is the generation of protein crystals, which may take months or even years. Membrane protein, in particular, are very hard to crystallize and alternative approaches are often required to look at their structure.

Electron microscopy (EM) and cryo-electron microscopy (cryo-EM) can be used to provide information on the structure of purified molecules, including membrane proteins, without the need of 3D crystals. Cryo-EM enables the visualization of the protein embedded in amorphous ice, which preserves its native state. Two different techniques are available (Egli, 2010):

- Electron diffraction: in this technique the diffraction of a beam of electron by a crystalline sample can be used to determine its structure. It is particularly useful for certain membrane proteins that may form 2D (thin layers) but not 3D crystals. This two-dimensional protein arrays are formed in thin lipid bilayers, preserving the protein native environment and possibly its structure and function. An electron diffraction pattern is collected and processed to obtain the structure of the individual proteins within the 2D crystal.

- Single particle analysis: when it is not possible to generate crystals, single-particle analysis is the method of choice. This technique allows the determination of the 3D structure of relatively large proteins and macromolecular complexes from a large set of images, assuming that all the particles have the same shape. A single protein molecule produces a weak and poorly defined image in the electron microscope. Increasing the signal by using beams with higher intensity or by using longer exposure times only leads to rapid degradation of the sample. Therefore, thousands of particles need to be processed and combined together to produce an average image of high resolution. Images of random oriented particles are collected and cluster together based on their similarity. Angles are then assigned to the different views and a 3D projection is generated.

As part of my Fellowship I used two different protein samples to learn both of these techniques: AlkB, an oil-degrading membrane protein from *Pseudomonas putida*; and the glideosome, the motility protein complex from *Toxoplasma gondii*.

AlkB, an oil-degrading membrane protein

AlkB is an alkane hydroxylase that allows the bacteria *Pseudomonas putida* to use alkanes as the sole source of carbon and energy, making it able to grow and survive in oil-contaminated environments (Whyte *et. al.* 2002). It is versatile biocatalyst that inserts an oxygen atom derived from O_2 into otherwise inert alkanes with great regio- and stereoselectivity. This selective oxidation under mild conditions offers tremendous potential to produce a large number of commercially valuable precursors for the chemical, pharmaceutical and fragrance industries using green chemical approaches (van Beilen, Duetz, Schmid and Witholt, 2003).

AlkB is an integral membrane non-heme diiron alkane hydroxylase with a n-alkane substrate range limited to C3-C12 (gasoline-range alkanes) (van Beilen and Funhoff, 2007). During the catalytic reaction AlkB transfers one oxygen atom from O_2 to the alkane molecule, while the other oxygen is reduced to H_2O using the electrons provided by soluble NADH-rubredoxin reductase (RR) via soluble electron-transfer protein rubredoxin (Rd) (Figure 1). In addition to the terminal hydroxylation of linear alkanes, *P. putida* AlkB catalyses the hydroxylation of branched alkanes, alicyclic, and alkylaromatic compounds, oxidation of terminal alcohols to the corresponding aldehydes, demethylation of branched methyl ethers, sulfoxidation of thioethers and epoxidation of terminal olefins and allyl alcohol derivatives (Ramos and Levesque, 2004).

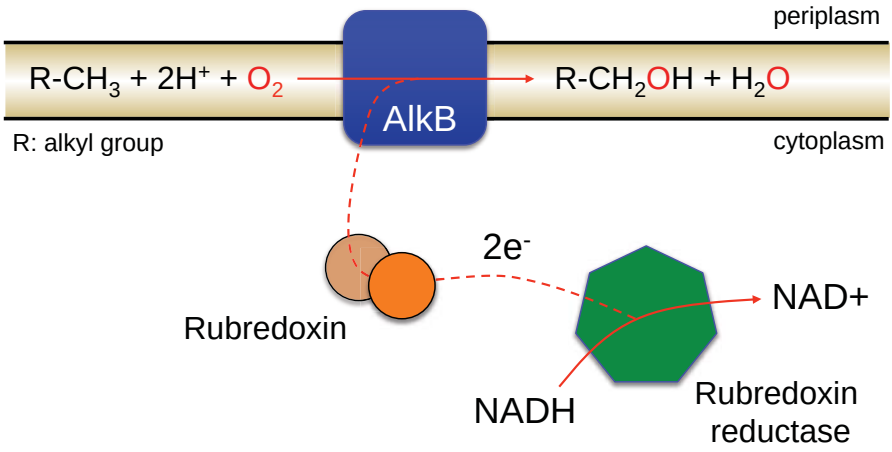

Figure 1: Schematic representation of the alkane hydroxylase system of the bacteria *Pseudomonas putida*.

Although the potential of AlkB is well recognised, very little is known about its 3-dimensional structure and the locations of the catalytic residues, the substrate binding pocket and the docking sites for its redox partners, RR and Rd. The protein is likely to have six transmembrane (TM) segments, with the amino terminus, two hydrophilic loops, and a large carboxy-terminal domain located in the cytoplasm (van Beilen, Penninga and Witholt, 1992).

The main obstacle in the structural characterisation of AklB has been the difficulty in isolating this integral membrane protein in a stable and active form. Recently, I was able to develop a new purification protocol that allows isolation of milligram amounts of recombinant, in a folded, catalytically active form, to purity levels above 90% (Alonso and Roujeinikova, 2012).

Using detergent-solubilised AlkB I performed 2D crystallisation trials reconstituting the membrane protein into a lipid bilayer by slow-detergent dialysis for several weeks at 37°C. I was able to produce several 2D crystals of 1 µm or more (Figure 2A) (Alonso and Roujeinikova, 2012). These were analysed using Transmission Electron Microscopy (TEM) images of negatively stained crystals.

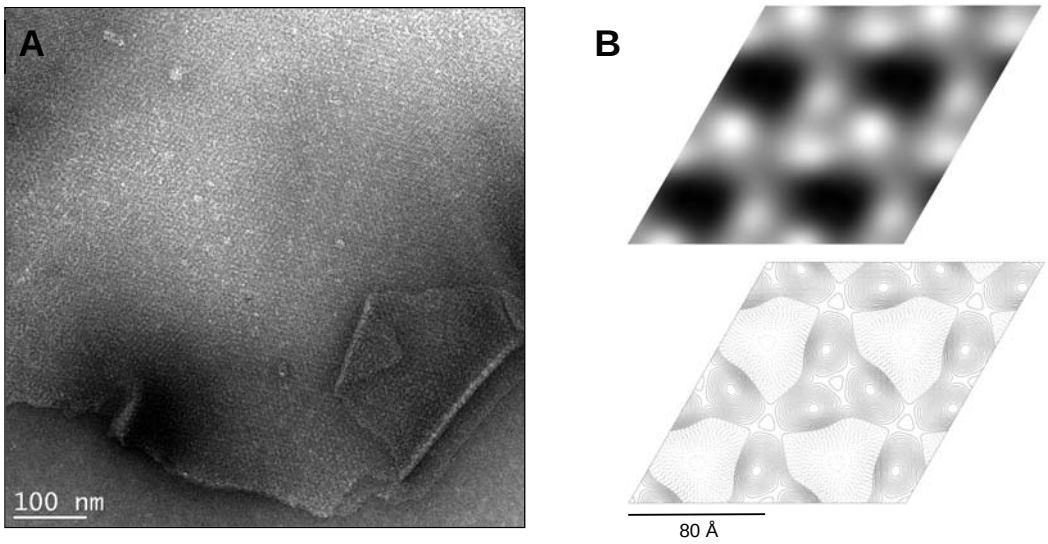

Figure 2: A) Electron micrograph of a 2D crystal of the oil-degrading protein AlkB. B) Reconstruction of the 2D crystal, map showing 2 x 2 unit cells (protein is seen as white over dark background) and topology map after application of p3 symmetry

Merging and processing 5 images from different regions of a single negatively stained crystal allowed me to generate the projection map shown in Figure 2B, which shows a trimeric protein complex with a three-fold rotational symmetry. Although novel, the low resolution of this structure is not good enough to look at the molecular detail of the protein and its function.

In order to obtain a higher resolution structure the AlkB protein, I was awarded this Churchill Fellowship to travel to Japan and conduct high-resolution cryo-electron microscopy analysis on the 2D crystals.

The glideosome, motility machine of *Toxoplasma gondii*

Toxoplasma gondii is a member of the apicomplexan parasites. This phylum also includes opportunistic pathogens such as *Plasmodium falciparum*, the causative agent of the deadliest form of malaria, and *Cryptosporidia*, a cause of severe gastrointestinal diseasae. All apicomplexan parasites use a unique form of substrate-mediated motility to migrate to the appropriate location and invade their host cells (Figure 3A).

The gliding machinery involves a network of actin filaments, which form the tracks, the motor, a myosin-based protein complex anchored to the inner membrane complex, and a series of adhesive proteins that are released by the parasite to the media (Figure 3B). These adhesive proteins bind to the surface of host cells, and are used as anchor points by the parasite to glide through the cell surface.

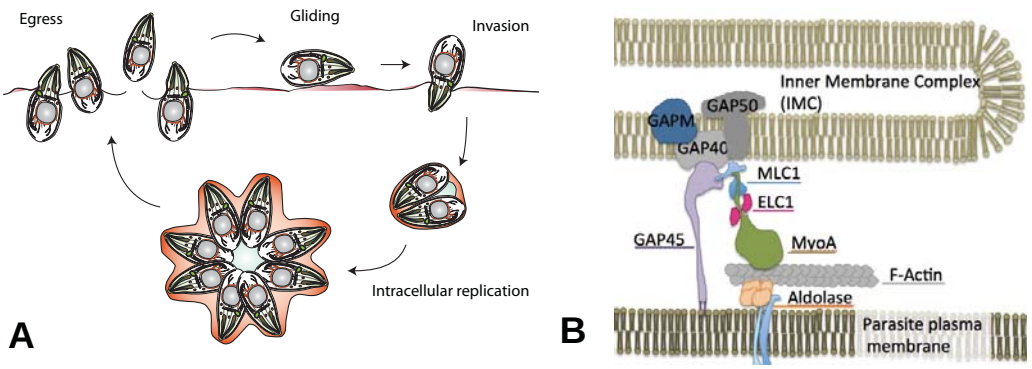

Figure 3: A) Life stages of *T. gondii* parasites during asexual reproduction, motility is crucial during egress, gliding and invasion. B) Schematic representation of the glideosome motility complex.

The motor complex involves a series of proteins including myosin A (MyoA), a class XIV myosin are only found in apicomplexan parasites, the myosin light chain (MLC1) and the essential light chain (ELC1), which are thought to play a regulatory role, and several glideosome-associated proteins (GAP40, GAP45, GAP50) which act as anchor points to the outer side of the inner membrane complex (IMC), which is a series of flattened cisternae joined together, and the plasma membrane (Keeley and Soldati, 2004) (Figure 3B).

From a mechanistic point of view, MyoA is the power generator, it uses ATP to pull rearwards actin filaments connected to aldolase, which in turn interacts with transmembrane adhesins that attach to host-cell receptors, thus driving the parasite forward (Figure 3B). Despite this general understanding of the glideosome, its assembly and detailed molecular structure remain unknown. Therefore, as part of this Fellowship I decided to look at the structure of this actomyosin motor in a bit to understand how the force generated by the myosin motor is transduced to the underlying cytoskeleton to move the parasite forward.

National Institute of Advanced Industrial Science and Technology (AIST), Tokyo, Japan.

AIST is a public research institution funded by the Japanese government. Its main role is to contribute to advances in technology and support the growth and development of the Japanese industry.

The headquarters of AIST are located in Tsukuba and Tokyo, and it has more than 40 autonomous units in various research fields located all over Japan. AIST was ranked 9th in the “Top 20 Japanese Research Institutions for All Field 2002-2012”.

As part of my Churchill Fellowship I joined the laboratory of Dr Kaoru Mitsuoka, member of the Electron Microscopy (EM) group based at the Biomedical Research Institute. The research focus of the institute is the development of new technology for drug discovery and medical treatment.

Figure 4: AIST Tokyo Waterfront building

The EM group is located at the AIST Tokyo Waterfront building (Figure 4) in Daiba, a large artificial island in Tokyo Bay, across the Rainbow Bridge from central Tokyo. This island was originally built for defensive purposes in the 1850s, but has developed since the 1990s as a major commercial, residential and leisure area.

During my time in Japan I lived in a furnished apartment in Shimbashi, a district of central Tokyo that is a significant business centre and commercial area. I travelled to the laboratory every day using the elevated Yurikamome train line, Tokyo's first fully automated transit system, controlled entirely by computers with no drivers on board.

Electron microscopes

The EM group has 3 JEOL electron microscopes, a JEM-3000SFF (300 kV), a JEM-1010 (100 kV) (Figure 5) and a 200 kV TEM.

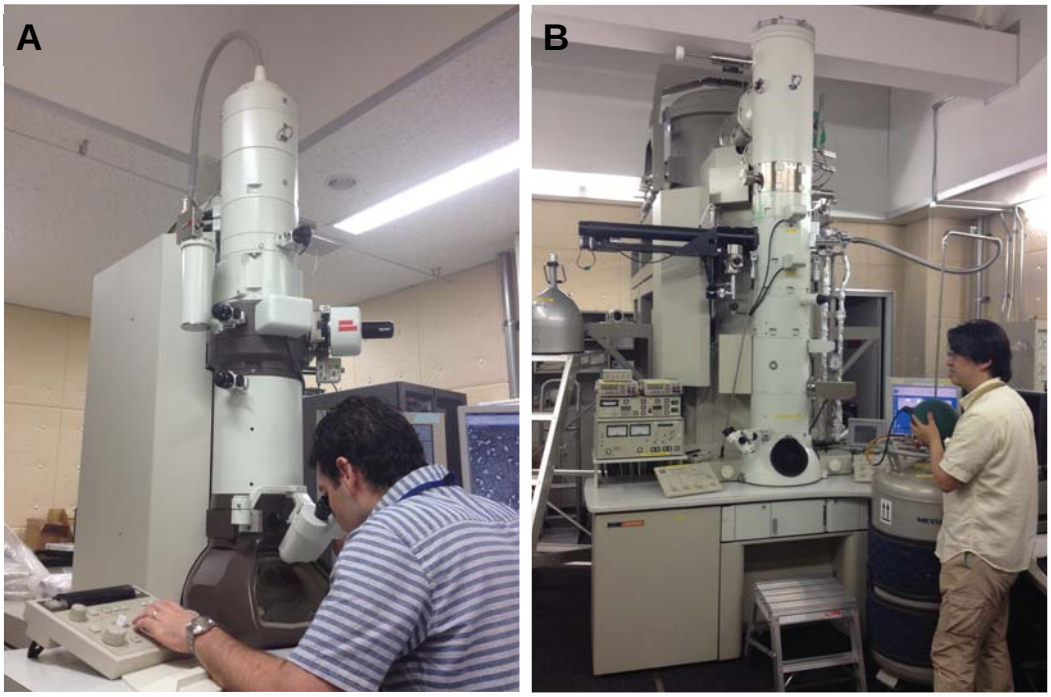

Figure 5: A) Dr Hernan Alonso working with the JEM-1010 and B) Dr Kaoru Mitsuoka filling the JEM-3000SFF with liquid helium.

Analysis of AlkB crystals

The crystals of the oil-degrading AlkB protein were carried over to Japan on wet ice. It is not possible to keep them at room temperature as protein and lipids run the risk of degradation, and it not possible to freeze them as the formation of ice crystals are likely to disrupt the crystalline lattice. Therefore, the only option available was to transport them on ice.

Carbon sandwich sample preparation

The 2D crystals of the AlkB protein were prepared for inspection under the cryo-electron microscope following the carbon sandwich technique, as described by Gyobu *et. al.* (2004) (Figure 6):

(A) A piece of carbon film (approximately 4mm×4 mm) was floated onto buffer solution and picked up with a molybdenum grid.
(B) The buffer was exchanged for a trehalose solution by transferring the grid to the surface of a drop of trehalose with the carbon film facing up.
(C) The grid was turned over and 2.5 µl of AlkB 2D crystal solution were deposited on the side opposite the carbon film and mixed. After several ~30 seconds, the excess liquid is removed.

(D) A second piece of carbon film (approximately 2mm×2 mm) floated on buffer solution was picked up with a loop and deposited on the side of the grid that was not covered by the first carbon film.
(E) Excess liquid was blotted away from the side of the grid with filter paper.
(F) The final carbon-sandwiched specimen was frozen by plunging into liquid nitrogen.

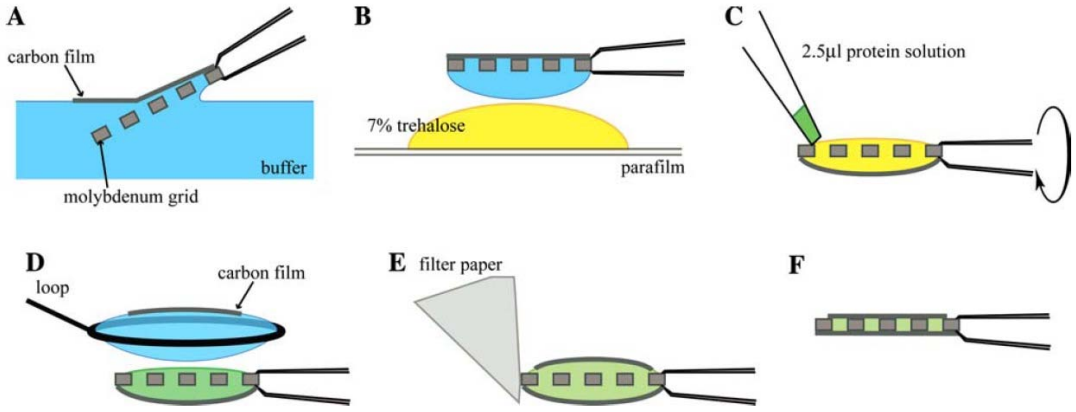

Figure 6: Carbon sandwich sample preparation as described in Gyobu *et. al.* (2004).

Cryo-electron microscopy analysis of AlkB crystals

Frozen specimens of the AlkB crystals were then transferred into a JEOL JEM-3000SFF electron microscope equipped with a superfluid helium stage and a field emission gun. The microscope was operated at a specimen temperature of 4K and an acceleration voltage of 300 kV.

Unfortunately, the images of the AlkB crystals revealed that the samples had suffered severe damage during transportation from Australia. There were no intact crystals left, and I was not able to collect any diffraction data.

Analysis of *T. gondii* glideosome complex

The purified glideosome particles were carried over to Japan in dry ice. By freezing the samples the protein complexes were more likely to arrive intact. Freezing of the glideosome was possible as there were no crystal structures to be preserved as in the AlkB protein sample.

Electron microscopy of negative stained sample

After the disappointing experience with the degraded AlkB crystals, I decided to check first the integrity of the glideosome by preparing a negatively stained sample before setting up grids for cryo-electron microscopy.

A solution of purified Glideosome (0.2 mg/ml, 3 µl) was placed on top of a glow-discharged carbon-coated cupper grid. After 30s, the excess solution was blotted out, and the grid washed three times over water droplets to remove all detergent. Finally, the grid was stained 5 µL of 2% uranyl acetate for 30 s and subsequently examined at room temperature using a JEM-1010 transmission electron microscope (JEOL) operated at 100 kV.

The preparation showed several protein aggregates (Figure 7A), probably as the result of transport from Australia. In order to conduct single-particle cryo-electron microscopy, the particles in the sample must be as homogeneous as possible. Therefore, we decided to further purify the glideosome using size-exclusion chromatography. The final sample was quite homogeneous and adequate for cryo-EM analysis (Figure 7B).

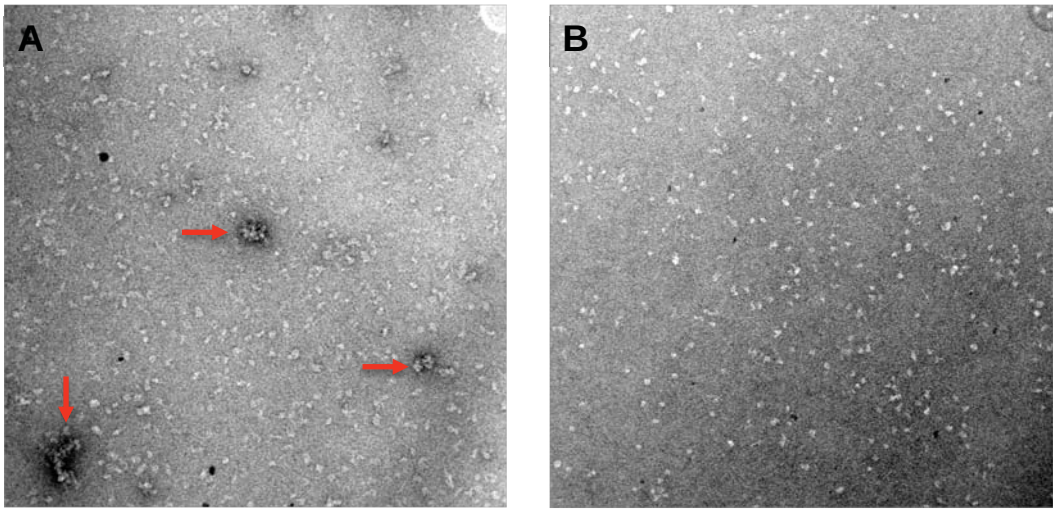

Figure 7: Negative stained images of the glideosome sample. Individual particles are seen as white 'dots' over a dark background. A) Non-homogeneous sample with aggregates (indicated by red arrows) after the trip to Japan. B) Final homogensous sample after further purification using size-exclusion chromatography.

Grid preparation for single-particle cryo-EM

Quantifoil R 2/2 grids, containing closely spaced 2 µm diameter holes, were washed three times for 1 hour in milli-Q water. Then, the girds were completely submerged in ethyl acetate three times to remove the plastic support film. Carbon was then deposited onto the grids using a JEOL JEE-400 carbon evaporator.

The carbon-coated grids were irradiated intensely overnight (total dose ~100e/Å^2; 100 kV electrons) in the projection chamber of a JEOL JEM-1010 transmission electron microscope. This treatment was done to improve the conductivity of the carbon and to strengthen and clean the support film. This leads to greater reproducibility in the thickness and degree of spreading of the ice films produced by the subsequent blotting and freezing steps.

Finally, carbon support films were prepared on freshly cleaved mica using a JEOL JEE-400 carbon evaporator in a vacuum better than 2×10^{-6} torr. A thin piece of carbon film (approximately 4mm×4 mm) was floated onto water and picked up with the irradiated grid.

Sample preparation for single-particle cryo-EM

Vitrification, or rapid cooling of the sample to solidify water without forming ice crystals, was done using a Vitrobot (Figure 8). This automatic vitrification robot controls the process of plunging, blotting and vitrification. The device allows accurate control of the humidity, temperature, the number of blots, and a number of critical time settings.

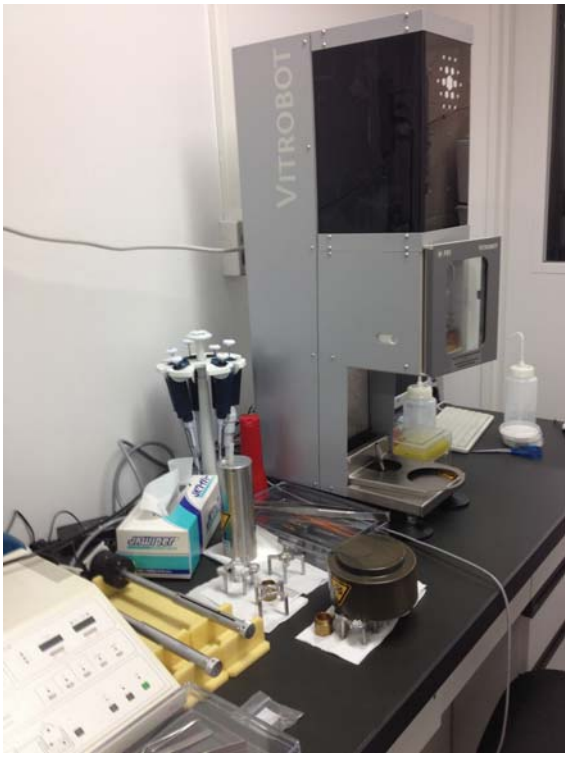

Figure 8: Vitrobot used for the vitrification of the glideosome protein sample.

To prepare the glideosome samples, the humidity was always kept at 100% and the temperature at 4°C. I tested blotting times from 5 to 30 seconds, and offset position of the blotting paper from -3 to -4.

The glideosome sample (4 µL) was applied to a glow-discharged grid inside the Vitrobot chamber on the side of the carbon film. After a waiting time of 20 seconds the grid was automatically blotted according to the set protocol and dropped by free-fall into liquid nitrogen-cooled ethane (-160°C). Grids were then stored in liquid nitrogen until examination.

Results

After screening several freezing conditions, the images with the best contrast were obtained using 20 seconds blotting, -4 offset and 4 µL of sample (Figure 9).

Unfortunately, after I found the right conditions to prepare the samples, the valve that controls the CCD camera on the JEOL JEM-3000SFF electron microscope broke down, and as I not able to collect the hundred of images needed for particle reconstruction. Nevertheless, I left several frozen samples in liquid nitrogen, and Dr Kaoru Mitsuoka kindly offered to do the data collection once the electron microscope is fixed.

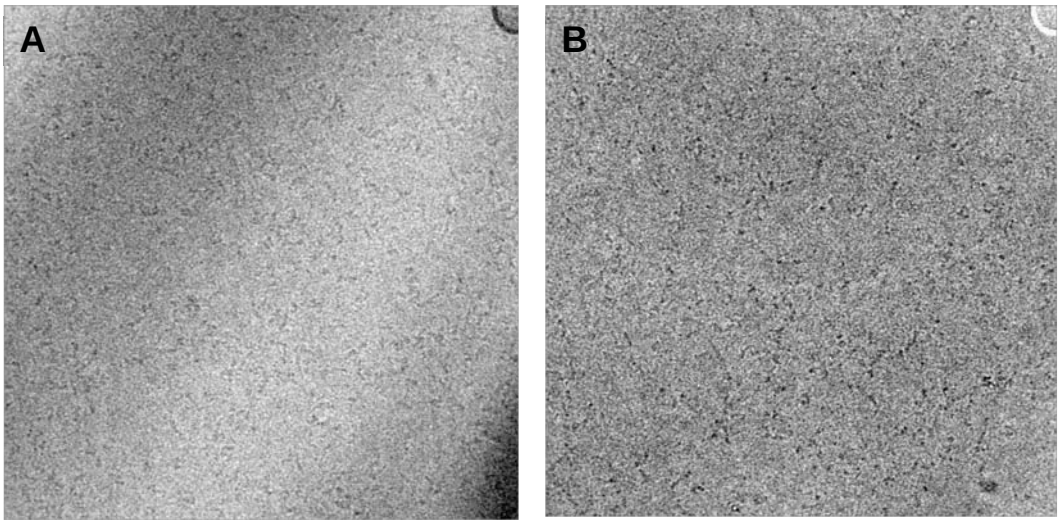

Figure 9: Cryo-EM image of the glideosome sample, the protein particles appear as dark spots over a clear background. A) Area of the grid where the ice is think and the contrast is low. B) Area where the ice is thin and the contrast is high, allowing for particle identification and downstream processing.

CONCLUSIONS AND RECOMMENDATIONS

My Churchill Fellowship allowed me to travel to Japan to study protein samples using cryo-electron microscopy. I was able to learn directly from the experts in the field how to prepare the samples, screen for optimal conditions, troubleshoot problems and collect data for structural determination analysis.

The membrane protein AlkB allows the bacteria *Psudomonas putida* to grow in oil-contaminated environments. I was able to purify enough active protein to produce 2D crystals for structural analysis. These crystals were taken to Japan to conduct electron diffraction analysis. Unfortunately, these crystals broke down during the trip from Australia, and no information could be collected. Therefore, in future studies the crystals will have to be grown at the place of analysis, to avoid the problems associated with handling and transport.

The second protein sample I studied was the glideosome complex from *Toxoplasma gondii*. Although there was some degradation of the sample during the trip, further purification allowed me to obtain a homogeneous preparation suitable for cryo-EM single particle analysis. After a long optimization process, I was able to find the right conditions to produce grids with thin vitreous ice and high image contrast. This time, the electron microscope broke down before I could collect enough data. However, I left several samples in liquid nitrogen that Dr Kaoru Mitsuoka will image for me once the microscope is repaired.

With the new technical skills and knowledge of cryo-EM I brought back to Australia I will be able to keep working on protein structure determination

largely on my own. Furthermore, with the new Ramaciotti Centre for Structural Cryo-Electron Microscopy to open at Monash University next year, I will be able to have access to state-of-the-art cryo-electron microscopes in Melbourne that will allow me to continue the studies I started in Japan. This will allow me to put into practice what I have learned and contribute to develop in Australia the expertise to conduct new and exiting research projects in the field of protein cryo-electron microscopy.

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