Investigating the Calcium Binding Site in *S. cerevisiae* Supercomplex III_2IV_2

Aims of the Project

My project was focussed on yeast cytochrome *c* oxidase (CcO) and its supercomplex with complex III (III_2IV_2). CcO is a vital mitochondrial membrane protein for oxidative phosphorylation and its absence or malfunction would be detrimental to any cell's survival. It is therefore an important protein to characterise to understand its functionality.

An area of interest is the calcium binding pocket located in COX5 of C*c*O. It is hypothesised that calcium ions alter the redox potential of heme A (1). However, limited research had been conducted on this pocket in yeast. Previous research investigated calcium binding to bovine CcO using ATR-FTIR, however *S. cerevisiae* C*c*O signals were much weaker and inconclusive (1). After the discovery of the supercomplex III_2IV_2 and the incorrect folding of C*c*O without complex III, it opens the hypothesis whether the yeast supercomplex would provide stronger calcium binding signals than complex IV alone.

The project was therefore a continuation of previous research, now working with the supercomplex. I would first learn how to culture yeast, extract the mitochondrial membranes then practise ATR-FTIR spectroscopy on bovine oxidase. The supercomplex would then be purified from wildtype mitochondrial membranes and prepared for perfusion-assisted ATR-FTIR spectroscopy.

Impact

Mitochondrial diseases affect 1 in 5,000 people and any research advancements would prove valuable for the field of bioenergetics (2). Complex IV is extensively studied, yet the role of calcium binding is still unknown. This role could be essential structurally or functionally to the working of complex IV, so characterisation is necessary.

Infrared spectra generated from calcium binding is useful for other researchers studying complex IV using different characterisation methods to investigate calcium binding function. The combination of specialised methods, each with their own strengths, produces more concrete results.

This research assists the Biochemistry Society in achieving its strategy due to the promotion and sharing of knowledge. It has supported my career development by enhancing my laboratory confidence, expertise and transferable skills.

Work Undertaken

One of the difficulties of working with a supercomplex is its instability in strong detergents (3). Thus, the first task of the project would be to identify weak detergents that maintain the stability of the supercomplex whilst being readily removed when preparing an ATR-ready sample. In order to form a protein layer, the supercomplex must be hydrophobic thus detergent must be stripped from the protein. Weak detergents, such as digitonin, GDN and LMNG preserve the supercomplex but have a low CMC, which provides difficulty in removal by dilution.

In order to test the possibility of GDN removal, wildtype mitochondrial membranes were solubilised in 2% GDN, centrifuged to remove insoluble material, diluted in buffer and centrifuged again at a higher speed to pellet out the membrane proteins. The pellet was then loaded onto the Si prism, dried with argon gas, rewetted and an ATR-FTIR spectrum recorded. After repetitions, it was concluded that GDN can be removed using this method.

Wildtype mitochondria membranes were first washed with magnesium and calcium salts to inhibit the F1 head of ATP synthase. Complex V contributes to a large proportion of protein in the membrane. This is important for solubilisation as the detergent:protein ratio is determined from complex III concentration by UV/vis redox spectroscopy. After solubilisation of the mitochondrial membranes, the supercomplex was purified by immobilised metal ion chromatography, concentrated, then aliquots were flash-frozen. By experimenting with different conditions, the supercomplex was then exposed to several dilution and centrifugation steps before loading onto the prism to observe layer formation.

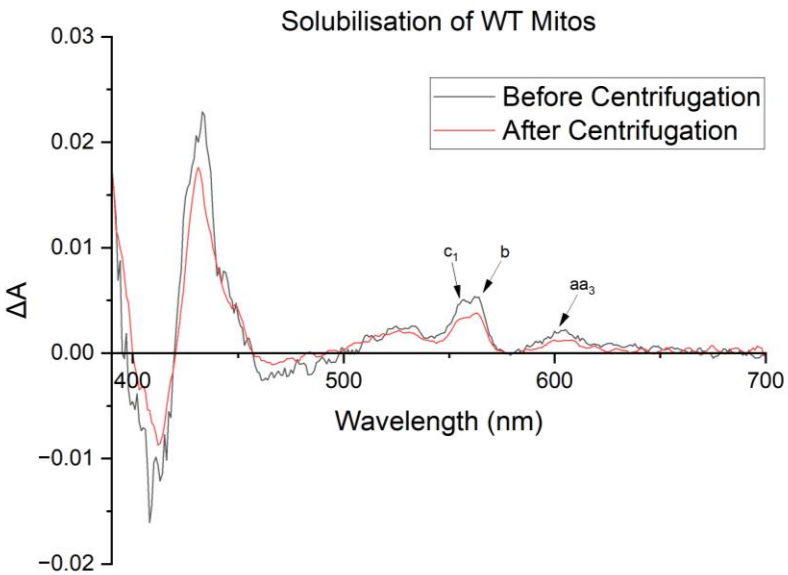

Figure 1. Baseline-corrected UV/vis redox spectra of WT mitochondrial membranes. 300 µL of sample was taken before and after centrifugation and diluted by 2. Bands arising from heme light absorption are labelled.

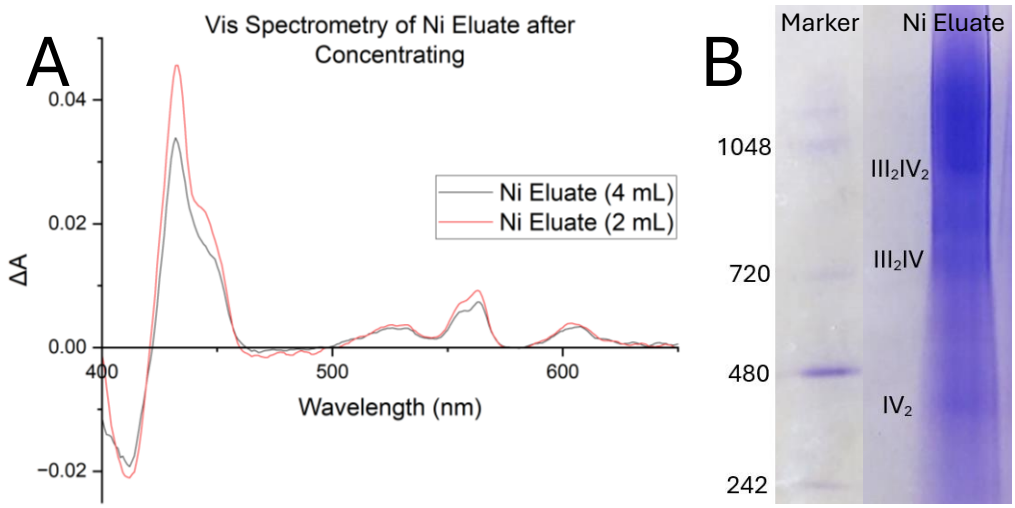

Figure 2. Baseline-corrected UV/vis redox spectra of the Ni Eluate after concentrating to 4 and 2 mL (A). BN-PAGE of Ni eluate after concentration to 4 mL (B). Labelled bands identify the species that produce them.

References

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2. Ng, Y. S., and Turnbull, D. M. (2016) Mitochondrial disease: genetics and management. *J Neurol*. **263**, 179

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Results and Outcomes

Yeast mitochondrial membranes were solibilised in 1% GDN and the results are shown in Figure 1. The concentration of complex III can be calculated from the absorbance change between 560 nm and 578 nm, given a molar absorptivity of 28000 $mol^{-1}cm^{-1}$. The calculated bc_1 concentration was 327 nM in 155 mL before centrifugation, and 239 nM in 180 mL after centrifugation. This resulted in a solubilisation yield of 85%, which is successful.

Furthermore, the yeast supercomplex was purified from WT mitochondrial membranes with a yield of 20-27%, based on the starting complex IV concentration of 151 uM in 15 mL. Panel A of Figure 2 shows the UV/vis spectra of the final products and was used to calculate the final complex IV amount of 4.46-6.04 nmol. The identify of the supercomplex is shown by panel B. Other species may be present due to denaturation, however the bands are not clear enough to make valid conclusions.

A sufficient protein layer is required for ATR-FTIR redox spectroscopy and calcium binding to ensure an appropriate signal-to-noise ratio. The Amide II band amplitude must be above 0.1 when a dried protein layer is rewetted. Unfortunately, this amplitude could not be achieved with the yeast supercomplex thus further experimentation was halted. This was due to the difficulty of GDN removal, resulting in gel-like appearances on the prism. The most successful result is shown in Figure 3, where a stable layer was formed overnight. Having achieved six repeats of the experiment and plenty of purified protein remaining, there are sufficient resources and data for the research team or myself to continue this project in the future.

Transferable Skills and Future Prospects

This research experience has been invaluable for developing my skills and confidence in the laboratory. I enjoyed collaborating in a research team and the frequent presentations have strengthened my communication skills. It has not only developed subject-specific skills such as using UV/vis and infrared spectrometers, but enhanced transferable skills such as making buffers, good practises of centrifugation, running electrophoretic gels and running purification columns. The insight into academia research I experienced has solidified my future aspirations.

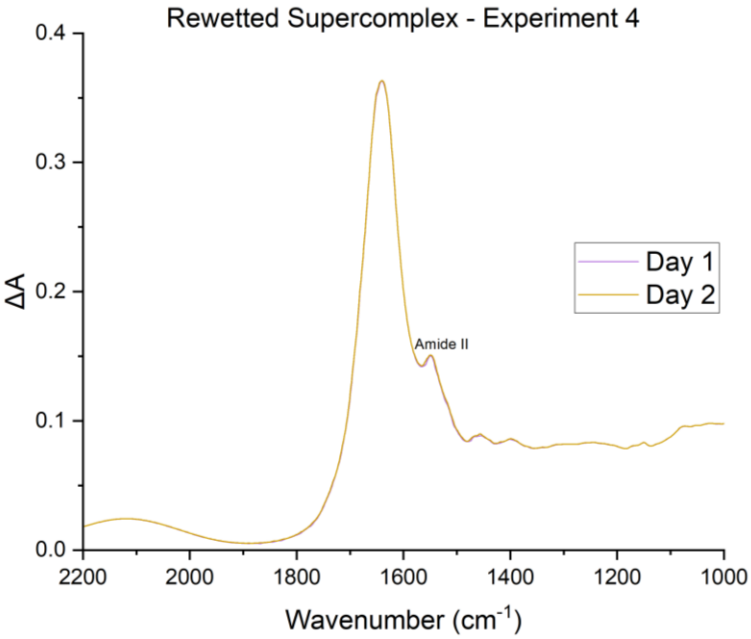

Figure 3. ATR-FTIR spectrum of rewetted yeast supercomplex. The layer was left to hydrate overnight and a second spectrum recorded (Day 2). The Amide II band labelled has an amplitude of ~0.07.

Acknowledgments

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