Isolation, Purification, and Nanodisc Assembly of Submitochondrial Particles

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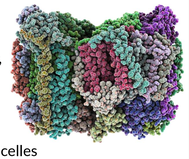
Abstract

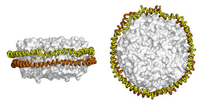
Isolation of mitochondria and submitochondrial particles (SMPs) were performed to begin nanodisc assembly of cytochrome *c* oxidase. Further protocols will allow for examination of cytochrome *c* oxidase function in oxidative phosphorylation. Mitochondria were isolated from bovine myocardium and then sonicated to break the inner mitochondrial membrane to form submitochondrial particles (SMPs). A biuret protein assay using bovine serum albumin (BSA) standard dilutions was performed to develop a standard curve that was used to determine mitochondrial and SMP concentration. A diode-array spectrophotometer set to 540 nm resulted in absorbances corresponding to mitochondrial concentration of 5.13 mg/mL and SMP concentration of 3.41 mg/mL. To closely examine complex IV activity, the assembly of an integral membrane protein was formed into a nanodisc. The sample was washed with bio-beads to remove leftover DDM detergent. The supernatant was then inserted into a DEAE column to help purify the protein at three different pH's, pH 8 - 6. We recorded absorbances at 280 nm and 415 nm to determine that fractions with absorbances above 0.2 potentially contained nanodiscs with cytochrome *c* oxidase. These fractions underwent buffer exchange and were then loaded and ran on a 12% SDS-PAGE to determine the molecular weight. Comparisons of band sizes to a molecular weight marker indicate that SMPs were incorporated into nanodiscs.

Introduction

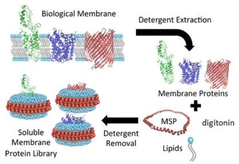
- Mitochondrion is subcellular location for varied metabolic processes and pathways.
- Pathways rely on membrane proteins, whose structure and enzymatic activity can be examined to better understand overall function and role of specific subunits.



- One such enzyme is cytochrome *c* oxidase (complex IV).
  - Integral membrane protein (IMP) and oxidoreductase.
  - Transfers electrons to molecular oxygen from cytochrome *c*, producing H_2O.[1]
  - Still much to be learned about specific subunit activities.
- IMPs challenging to examine with traditional techniques.[2]
- Cytochrome *c* oxidase has been successfully incorporated into bicelles
  - fully reducible by electron donors and exhibiting expected reactions with ligands.[3]
- Bicelles useful because lipid bilayer composition can be adjusted, oriented with magnetic fields, and provide access to both sides of protein.
  - bicelles only stable within narrow composition, temperature, and hydration ranges.[4]



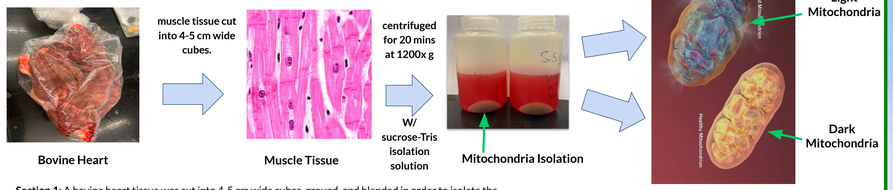
- More beneficial to assemble IMPs into a nanodisc.
  - Self-assembled discoidal fragments of lipid bilayers, stabilized in aqueous solutions by helical membrane scaffold proteins (MSP).[2,5]
  - IMP can be scaffolded into a soluble, phospholipid bilayer structure with full functionality.[2, 5]
- Additional benefits such as native function retention, ability to collect in high yield, and enhanced stability.[4]
  - scaffold proteins required for nanodisc assembly are expensive
- Successful nanodisc assembly of cytochrome *c* oxidase requires multiple semesters.
- First semester's work includes assembly of nanodiscs containing fragments of the inner mitochondrial membrane, or submitochondrial particles (SMPs).



- SMP isolation and nanodisc assembly relies on four specific aims:
  - the isolation and assay of mitochondria and SMPs
  - nanodisc assembly with SMP isolates
  - nanodisc purification using DEAE anion exchange chromatography
  - analysis via SDS-PAGE and Native-PAGE.[6,7]
- We hypothesize that by utilizing our unique protocol, SMPs can be incorporated into nanodiscs
  - SMPs will contain array of IMPs crucial to mitochondrial function.
- Future work will purify and select for nanodiscs containing cytochrome *c* oxidase only.

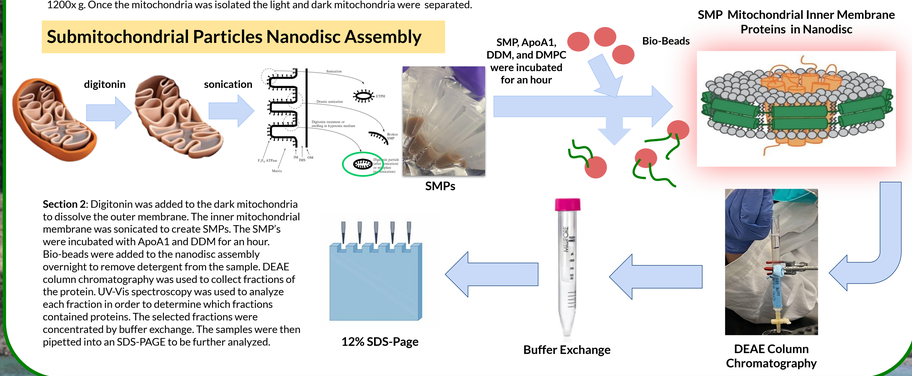
Methods

Isolation and Purification of Submitochondrial Particles



Section 1: A bovine heart tissue was cut into 4-5 cm wide cubes, ground, and blended in order to isolate the mitochondrial tissue. The tissue was suspended and centrifuged in sucrose-Tris isolation solution for 20 mins at 1200x g. Once the mitochondria was isolated the light and dark mitochondria were separated.

Submitochondrial Particles Nanodisc Assembly



Section 2: Digitonin was added to the dark mitochondria to dissolve the outer membrane. The inner mitochondrial membrane was sonicated to create SMPs. The SMP's were incubated with ApoA1 and DDM for an hour. Bio-beads were added to the nanodisc assembly overnight to remove detergent from the sample. DEAE column chromatography was used to collect fractions of the protein. UV-Vis spectroscopy was used to analyze each fraction in order to determine which fractions contained proteins. The selected fractions were concentrated by buffer exchange. The samples were then pipetted into an SDS-PAGE to be further analyzed.

Results

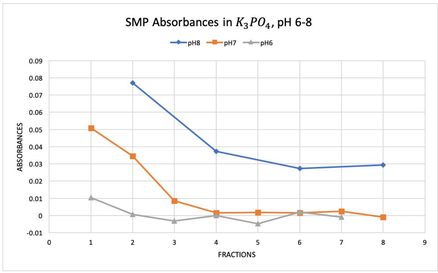


Figure 3. SMP elution absorbances from different pH values. Different pH values of K_3PO_4 were used with the DEAE column to purify the protein. Absorbances above 0.2 potentially contained nanodiscs with cytochrome c oxidase.

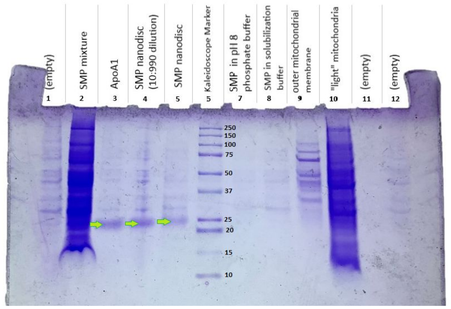


Figure 4. SDS-PAGE of samples collected throughout the mitochondrial and SMP isolation and nanodisc assembly. Lane 3 was loaded with the ApoA1 used to scaffold the nanodiscs. The characteristic band of ApoA1 at about 23 kDa also appears in the bands of wells 4 and 5 containing assembled nanodiscs of SMPs. This band is absent from samples collected prior to nanodisc assembly. Wells 1, 11, and 12 were empty, but show bands from overloaded neighboring wells.

Results

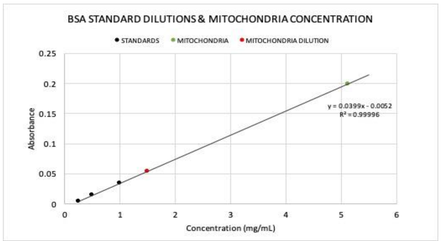


Figure 1. Bovine Serum Albumin (BSA) standard dilutions and isolation of mitochondrial absorbance and concentration values. A diode-array spectrophotometer was used to record the absorbance values to form a linear slope.

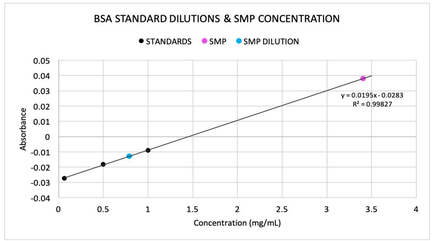


Figure 2. Bovine Serum Albumin (BSA) standard dilution and isolation of submitochondrial particles absorbance and concentration values.ode-array spectrophotometer was used to record the absorbance values to form a linear slope.

Table 1. Absorbance and concentration of mitochondrial and diluted mitochondrial samples.

	Absorbance	Concentration (mg/mL)
Mitochondria	0.1995	5.13
Mitochondria 1:5	0.0542	1.49

Table 2. Absorbance and concentration of SMP and diluted SMP samples.

	Absorbance	Concentration (mg/mL)
SMP	0.03810303	3.40528359
SMP 1:10	-0.0128	0.0340528359

Conclusions

- ❏ Isolation of mitochondrial fractions and submitochondrial particles were successful.
- ❏ Successfully determined concentrations of mitochondrial fractions and submitochondrial particles using a Biuret Protein Assay.
- ❏ Successfully assembled nanodisc structures into submitochondrial particles.
- ❏ Determined molecular weight of samples using a 12% SDS-PAGE.

Future Directions

- ❏ Further analysis involves the measurement of oxygen consumption in submitochondrial particles and nanodiscs, Native-PAGE, and additional spectroscopy.

Acknowledgments

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