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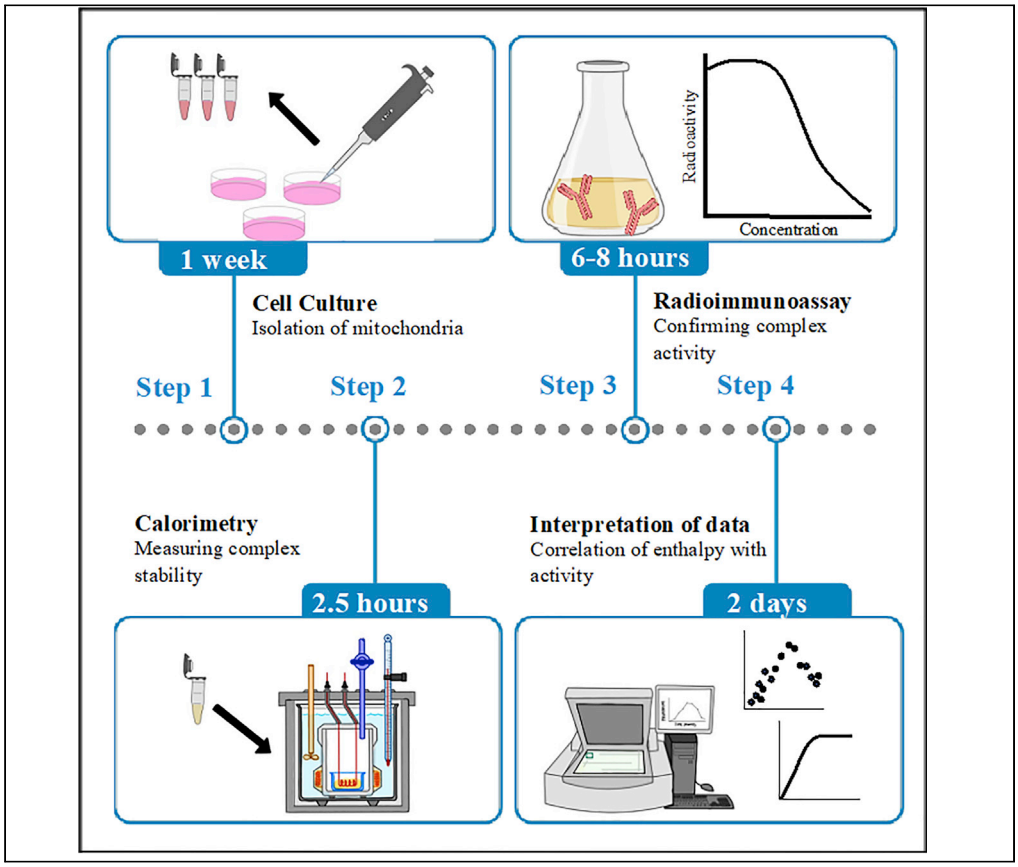
Protocol for direct measurement of stability and activity of mitochondria electron transport chain complex II



Mitochondria electron transport chain (ETC) complex II is essential for steroid metabolism. Here, we present a protocol to measure the stability and activity of mitochondria ETC complex II. We first describe mitochondria isolation from cell lines and tissues. We then detail how to determine the stability of ETC complex II using isothermal calorimetry and quantification of steroidogenesis using activity assays in parallel. Finally, we describe the steps to perform radioimmunoassay (RIA) to confirm the activity of ETC complex II.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Steps to isolate mitochondria from cell lines and tissues

Direct determination of the enthalpy of ETC complex II using isothermal calorimetry

Combined calorimetry and steroidogenic activity assay to measure ETC complex activity

Radioimmunoassay to quantify and confirm enzymatic activity

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Protocol for direct measurement of stability and activity of mitochondria electron transport chain complex II

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SUMMARY

Mitochondria electron transport chain (ETC) complex II is essential for steroid metabolism. Here, we present a protocol to measure the stability and activity of mitochondria ETC complex II. We first describe mitochondria isolation from cell lines and tissues. We then detail how to determine the stability of ETC complex II using isothermal calorimetry and quantification of steroidogenesis using activity assays in parallel. Finally, we describe the steps to perform radioimmunoassay (RIA) to confirm the activity of ETC complex II.

For complete details on the use and execution of this protocol, please refer to Bose et al. (2020).[1]

BEFORE YOU BEGIN

Adrenals and gonads (ovaries for women) are the main steroidogenic tissues synthesizing all the steroids necessary for survival of all mammals.[2–5] Additionally, the brain synthesizes a meniscal amount of all steroids. The pig adrenal tissues were available from Veterinary School, University of Florida, Gainesville, immediately after the sacrifice. For knockdown experiments, we needed cells which synthesize all the steroids to maintain consistency with the *in vivo* adrenal or gonadal function; therefore, we considered mouse Leydig (MA-10) cells.[6,7] Human adrenal NCI H295[8] cells were considered; however, the extremely low differentiation time in NCI H295 cells solidified the decision to use of MA-10 cells. The cell lines were maintained once thawed and stored at parameters mimicking *in vivo* conditions.

Institutional permission

All animal experiments must be performed in accordance with the ethical guidelines of the institution. Animal tissue used throughout this protocol was provided by University of Florida Vet School, Gainesville. Donation of tissue to researchers was done immediately following animal sacrifice.

Experimental consideration

To avoid any variations matching the data between evaluation of steroids synthesized and the calorimetry experiment, the mitochondrial preparation should be used from the same batch through completion of the protocol. In addition, buffers should be prepared with degassed water. It is essential that the buffers used are degassed and filtered prior to use as this can be a major problem in results. In addition, ensure that there are not any bubbles present in the calorimeter. If the calorimeter being used is not equipped with an automatic injector, manual injections may be a confounding variable skewing data. Efforts must be made to inject substrate consistently and confluently throughout experiment. Given mitochondrial viability is essential for the efficacy of this protocol,

viability should be determined prior to storage in small fractions. Mitochondria must be live, and viability should be determined for each preparation beginning on Page 4 of the protocol prior to storing sample. Each fraction should be thawed once and should not be restored for future use.

Cell culture

Timing: 1 week

The steps for cell culture growth are not required for execution of this protocol unless experiment will be conducted using cells rather than a functional adrenal or gonadal tissue sample. If cell culture is the desired source of mitochondria ETC complex sample, the parameters for growth of cells are below outlining the proper media, storage, growth atmosphere, and materials required.

The following describes growth and maintenance of MA-10 cells.

1. Prepare medium for cell culture using Dulbecco's modified Eagle's medium (DMEM) or Waymouth medium. Mixture of reagents is outlined in "materials and equipment" section for reference.
2. Grow MA-10 cells to a density of 10^5 cells/cm^2 in prepared media, where the 100 mm petri dishes are precoated with 0.2% gelatin for about 30 min.
3. Maintain in a humidified atmosphere of 5% CO_2 at 37°C incubator.

Note: Cells should be cultured in monolayers to ensure proper growth. Mitochondria isolated from cells grown uniformly work more efficiently in comparison to overgrown dishes.

Mitochondria sample preparation

Timing: 2–3 h

This section details how to isolate mitochondrial material from a sample for use throughout the protocol. Preparation of mitochondria samples can be done in two ways, through dicing tissue or through lysis of culture cells. When the experiment was conducted, the functional tissue used was pig adrenal tissue mentioned in the "before you begin" section, while the cell culture was composed of MA-10 mouse Leydig cells. If functional tissue sample is selected for use, tissue cells will be lysed identical to cells collected from cell culture. Reference to "materials and equipment" section will provide instructions on how to prepare buffer mixtures for mitochondria isolation steps in the protocol.

Note: If the sample being used is functional tissue rather than grown via cell culture, begin at step 4 below. If the sample being used was grown via cell culture, begin at step 12 below. An optional step for mitoplast preparation is provided at the conclusion of this section listed as "Optional".

Isolation and purification from tissues

4. Wash tissue with enough 1× PBS (Phosphate Buffer Saline) to submerge the tissue sample. Lightly swirl ensuring all the sample has been cleansed with PBS. Remove and discard supernatant (PBS). Repeat this step 4 times.
5. Transfer washed tissue into a petri dish containing 1–2 mL of mitochondrial isolation buffer. Keep on ice to preserve mitochondrial function.
6. Chop tissues into small pieces using a sterile razor blade on ice. This step does not require chopping of tissue to be in one, uniform direction; however, tissue should be equally diced and thoroughly diced for isolation of complete organelles.

7. Transfer collected tissue fractions (pieces) in hand-held all-glass Dounce homogenizer.
8. Gently homogenize tissue with 10 up and down strokes. Ensure homogenizer is straight to avoid loss of intact organelles.

Note: The purpose for 10 motions in homogenization is to prevent disruption of mitochondrial architecture. If the tissue is homogenized an excessive number, it will result in complete or partial rupture of outer mitochondria membrane and inner mitochondria membrane yielding a metabolically inactive sample.

9. Transfer to labeled centrifuge tubes.
10. Centrifuge at 1,000 × *g* (~3,000 rpm) for 10 min at 4°C.
11. Collect supernatant and discard debris. Proceed to step 19 of "isolation and purification from cell culture" section below for instructions on storing sample.

Isolation and purification from cell culture

12. Carefully remove (aspirate) all media from cell culture petri dishes.
13. Add 10 mL of 1× PBS to each petri dish containing cells and lightly swirl to perform wash. Discard PBS once completed with washing process. Repeat wash 1 additional time and proceed to next step.

Note: Petri dishes should only be washed gently with PBS twice. Over-washing and/or strong washing can result in cells detaching from the bottom of the plate and loss of cells in discarded PBS.

14. Add 500 μL of 10 mM HEPES pH 7.4 buffer to each dish and scrape the cells gently.
15. Collect cells and transfer to a 1.5 mL epp. Tubes. Centrifuge at 1,000 × *g* for 10 min at 4°C.
16. Collect pellet and discard supernatant.
17. Add 800 μL of 1.5× mitochondria isolation buffer to pellet of collected cells in previous step (step 16).
18. Place sample from previous step in all-glass Dounce homogenizer and gently stroke up and down 22 times.

Note: The purpose for 22 motions in homogenization is to prevent full disruption of mitochondrial function. If the sample is homogenized an excessive number of times, it will result in complete separation of outer mitochondria membrane from inner mitochondria membrane yielding a metabolically inactive sample.

19. Further centrifuge the supernatant for 10 min at 10,300 × *g* to isolate the crude mitochondria from the pellet.

Optional: To isolate pure mitochondrial fractions,[9–11] resuspend the crude mitochondrial pellet in isolation medium using a 2.0 mL ultracentrifuge tube and layer the crude mitochondrial suspension on top of a medium containing Percoll density gradient buffer (Prasad et al.[10]). This step is not required but can be done, if necessary.

20. Centrifuge at 95,000 × *g* for 30 min at 4°C in an Ultracentrifuge.
21. Isolate the mitochondrial fraction two-thirds of the way down the tube. Perform isolation using a thin glass Pasteur pipette.
22. Wash to remove the Percoll by first diluting samples with isolation medium. If Percoll density gradient buffer was not used. Disregard this step and proceed to step 23.
23. Centrifuge twice at 6,300 × *g* for 10 min at 4°C.

24. Resuspend final mitochondrial pellet in isolation media and proceed to step 25 below for storing instructions. See troubleshooting 3.

Storing isolated mitochondria protein sample

25. Determine protein concentration of sample. Storing sample at a concentration of 1 μg/μL is more ideal.
    a. Protein concentration was determined via Bradford Protein Assay[12] with BioRad dye reagent. Alternatively, BCA assay is an effective method to determine protein concentration.
26. Store samples at −86°C.

Optional: Mitoplast can be prepared by a hypotonic shock of the purified mitochondria in 1 mM HEPES buffer pH 7.4 for 45 min at 4°C. Vortex for 15 s and purify through centrifugation at 3,500 × *g* for 10 min at 4°C. Collect supernatant containing mitochondria and aliquot as 100 μL (1 μg/μL) and store at −86°C. This preparation may contain endoplasmic reticulum.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CYP11A1 (Cytochrome P450 side chain cleavage enzyme or SCC); Dilution 1:1000	Bon-Chu Chung Academia Sinica, Institute of Molecular Biology Taiwan Personal communication	Ref: Mol Endocrinol 22:915–923, 2008.
COX IV; Dilution 1:2000	Santa Cruz Biotechnology	sc-376731
Calnexin; Dilution 1:1000	Santa Cruz Biotechnology	sc-23954
PDC-α; Dilution 1:3000	Santa Cruz Biotechnology	sc-271534
Biological samples		
Mitochondria	Homemade via isolation	Cell lines listed
Mitoplast	Homemade via isolation	Cell lines listed
Pig adrenals	University of Florida Vet School, Gainesville Meat Processing Facility	Donated following sacrifice
Chemicals, peptides, and recombinant proteins		
Sucrose	Fisher Scientific	84097
HEPES	Sigma	H3375
EGTA	CALBIOCHEM/SIGMA	324628
Na_2HO_4	Fisher Scientific	S375-500
KH_2PO_4	Fisher Scientific	P380-12
NaCl	Fisher Scientific	AC447302500
KOH	Fisher Scientific	501118107
ATP	Sigma	A26209
β-NADPH	Sigma	N7505
Trilostane	Sigma	SML 0141
Percoll density gradient buffer	Sigma	GE17544502
Equine serum	Fisher Scientific	SH3007404
Fetal bovine serum	Fisher Scientific	SH300880340
Dulbecco's Modified Eagle's Medium (DMEM, high glucose) or Waymouth	VWR or Millipore Sigma	10128-210 or W1625
Glutamine	VWR	45000-676
Penicillin-streptomycin	VWR	97063-708
Critical commercial assays		
Radioimmunoassay	MP Biomedicals	07170102*
Quick Start Bradford 1× Dye Reagent	Bio-Rad	5000205
ATP Assay System, ENLITEN	Promega	FF2000

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Cell lines		
MA-10 cells	ATCC	CRL-3050 Range of passage: 4–12
COS-1	ATCC	CRL-1650 Range of passage: 4–12
Oligonucleotides		
siRNA (MDH silencing plasmid)	Bose et al.[1] (2 out of 5 sequences provided are functional)	Open Biosystem/Santa Cruz (Sequence information not provided)
Other		
Scintillation counter	Beckman Model LS6500	SKU8043-30-1194
Calorimeter	MicroCal (Currently sold by Malvern, HL)	VP-ITC
Phosphoimager	GE HealthCare	Storm & Typhoon
ATP measurement microplate reader	Turner Biosystems	Model 9100-002
Ultracentrifuge	Beckman	TL100

Alternatives: Radioimmunoassay (RIA) kit sold from MP Biomedicals for ^{3}H-pregnenolone has been replaced by kit ^{125}I-progesterone. Given that progesterone is a downstream product of pregnenolone in steroid synthesis pathway, substitution with above alternative kit will provide means for replicable data.

MATERIALS AND EQUIPMENT

Throughout the procedure, it is necessary to use a calorimeter, phosphoimager, and scintillation counter. Without the listed equipment and materials below, the protocol may not be fully productive. Later in the protocol, radioactive reagents are used for to perform a radioimmunoassay. The complete step by step is available with RIA kit from the manufacturer, MP Biomedicals.

Mitochondria Isolation Buffer					
Reagent	Stock concentration (M)	Final concentration (mM)	1× (mL)	1.5 × (mL)	2.0 × (mL)
Sucrose	1.3 M	225 mM	384.6	577	769
HEPES	1.0 M	25 mM	50	75	100
EGTA	0.2 M	1.0 mM	10	15	20
Water	N/A	N/A	1585.4	1,378	1,171
Total (mL)	N/A	N/A	2,000	2,000	2,000

Note: Mitochondria isolation buffer must be adjusted to pH 7.4. It is suggested to prepare a stock of 500 mL or 1L and to make a multiple of small aliquots stored at −20°C. Buffer is viable for up to 1 week in −20°C freezer; however, −86°C is more ideal for storage up to 6 months.

Stock Solutions					
Reagent	MW	Measured (grams)	Final concentration (M)	Final volume (mL)	pH
HEPES	238.3	23.8 g	1.0 M	100 mL	7.4
Sucrose	342.3	342.3 g	1.3 M	1,000 mL	N/A
EGTA	380.35	76 g	0.2 M	1,000 mL	7.4
KOH	56.1	28 g	5.0 M	100 mL	N/A

Note: pH must be adjusted for HEPES solution and EGTA solution. Mixture provided should yield proper pH; however, titration with KOH may be necessary. Buffer will remain viable for up to 6 months when stored at −20°C.

DMEM for cell culture		
Reagent	Final concentration	Final amount
Dulbecco's Modified Eagle's Medium (high glucose)	N/A	839 mL
Glutamine	2 mM	1 mL
Penicillin-streptomycin	100×	10 mL
Equine Serum	10% [Vol/Vol]	100 mL
Fetal Bovine Serum	5% [Vol/Vol]	50 mL
Total	N/A	1000 mL

Note: Media must be adjusted to pH of 7.4 with titration of KOH. If powdered media is used, sterilization must be done through filtration with 0.22 μm and then adjust media pH. Use of already prepared commercial media and listed mixture results in pH to be 7.4. DMEM will remain viable for up to 6 months if stored immediately at −20°C. To avoid increased time in thawing media, any DMEM bottle can be kept at 4°C for no more than 1 month.

Additional reagents

Below are detailed instructions for the preparation of the following.

NADPH reagent

- β-NADPH is soluble in 10 mM NaOH to a final concentration of 50 mg/mL yielding a clear, light-yellow solution.

Note: Stored at −86°C in small aliquots, as single use aliquot and should be made fresh in two weeks. Maximum storage time at this parameter is no more than 1 year.

ATP reagent

- Weigh 551.1 mg of ATP and add 1.2 mL H_2O.
- Neutralize with 5 M KOH (approx. 400 μL). ATP dissolves after addition of KOH.

Note: Stock concentration of 500 mM should be aliquoted should be stored at −86°C in small aliquots and each aliquot should be used once. Maximum storage time should not exceed 1 year.

Equipment

Each instrument is listed in the KRT on Page 6 with the brand, vendor, and model utilized by the authors; however, alternate vendors/brands produce comparable instruments that will reproduce similar results. For research purposes, brand name/vendor may be subject to substitution. Calorimeter used was equipped with an autoinjector. Use of autoinjector may prevent variations in data.

Alternatives: Beckman Scintillation Counter, Calorimeter, and Phosphoimager vendors can vary. The medium mixtures provided were proven successful in this protocol; however, for research purposes may be subject to substitution.

⚠ CRITICAL: All media and solutions must be sterilized prior to use/storage via autoclave at 15 pounds per square inch at 125°C for 30 min.

STEP-BY-STEP METHOD DETAILS

Assessment of mitochondrial complex stability

Timing: 2 h

The following steps utilize calorimetry to conclude mitochondria complex stability for confirmation of steroidogenic ability. To determine whether the desired mitochondrial complex is viable, it will be confirmed via isothermal calorimetry to evaluate the enthalpy of the initiated reaction. Using the knowledge acquired pertaining to the mitochondrial ETC complex of interest, defining the stability of the complex is the first step in determining the ETC complex's vital role in metabolism.

⚠ CRITICAL: The samples tested in the following steps must be from the same, uniform isolated mitochondrial preparation. Use of a confluent sample throughout the protocol is essential to eliminate potential bias or discrepancies in supporting data.

⚠ CRITICAL: All buffers should be prepared in degassed water and prefiltered through 0.22 μm to reduce inconsistencies in calorimetry data.

Note: Mitochondrial preparation must be kept on ice between steps to preserve full metabolic activity.

1. Prepare mitochondrial samples for calorimetric experiment.
   a. Thaw all samples at 4°C for 15 min.
   b. Transfer samples to calorimeter chamber.

   Note: Within calorimeter, there are 2 chambers that will contain mitochondrial protein sample, one that will be tested and one for a constant allowing for comparison in heat production.

2. Resuspend 100 μL of mitochondrial sample (1 μg/μL) in 1.5 mL of mitochondria isolation buffer containing 1 mM ATP.
3. Place 20 μL of mitochondrial protein suspension into each enclosed chamber of the isothermal calorimeter (ITC).

   Note: Isothermal calorimeter has two independent, enclosed cells (also called chambers) and is equipped with an automatic injector. The reason for utilizing both chambers is to have a control data point used when calculating change in heat. All steps following that involve addition of substrates will pertain to tested chamber.

4. Gradually increase heat in calorimeter every 15 min until the sample has reached 37°C prior to proceeding to next step.
5. Inject 5 μL of succinate at a speed of 0.5 μL/s into calorimeter in 7-min intervals.
   a. Measure heat content reading following each interval.
6. Continue step above describing 5 μL succinate injection every 7 min until the heat content (ΔH) readings show minimal variation in data points resembling a plateau.
   a. Repeat measurements 3 times for all data points acquired.

   Note: Measurements will show a resemblance in the pattern of data points, independent to data values, depicted in Figure 1. The amount of succinate injected, heat content readings, and number of data points required to reach plateau phase may vary depending on samples used. The graph provided in Figure 1 exemplifies the correct data trend supporting the point of full enzymatic saturation and stability. The amount of succinate required to reach plateau phase will be used in later steps as a guide assess ETC complex metabolic capacity. See troubleshooting 1.

7. Repeat measurements three times for all data points acquired.
8. Calculate enthalpy (ΔH) for each interval by dividing power generated by the reaction with the corresponding concentration. $\Delta H = \Delta G - T\Delta S$.

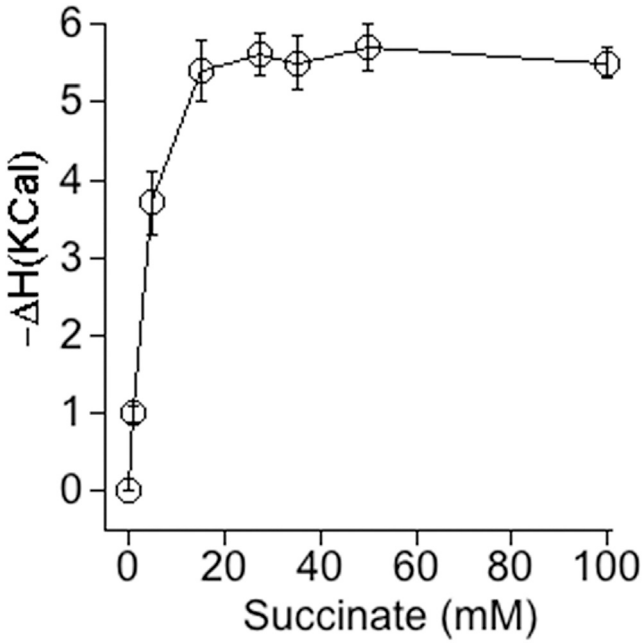

Figure 1. Isothermal titration calorimetry data interpretation with raw data obtained assessing mitochondrial ETC complex stability

Titration of succinate was continued until the enthalpy (ΔH) remained unchanged signifying full saturation of the enzyme. When enthalpy is expressed visually (as shown), the amount of succinate required to reach maximum saturation can be determined and utilized in later steps throughout the procedure. Software built into the calorimeter (MicroCal) was used to calculate ΔH. Data from Bose et al.[1]

Note: G = free energy; T = temperature; S = entropy; H = enthalpy. Enthalpy calculation will yield either a positive or negative value. Negative values signify stability of the assessed reaction while positive values correspond to increased reactivity and less stability. Data calculation was performed via software that was preinstalled on calorimeter system from MicroCal, but calculations may be done manually yielding the same results.

Note: Once ΔH begins to plateau, V_{max} has been reached signifying full enzymatic saturation and consumption of available substrates within mitochondria. At this point, the complex has reached peak stabilization in association with enzymatic saturation and succinate concentration. See troubleshooting 2.

Optional: If desired, repeat beginning at step 2 above with mitoplast substituted for mitochondria sample as a negative constant.

Measurement of mitochondrial complex activity via RIA and conjugate calorimetry

Timing: 8–10 h

This section includes steps of combined calorimetry and steroidogenic activity assay. It was unknown which substrates and cofactors were essential in the stabilization of ETC complex II and its role in regulating steroid metabolism by SCC protein folding. By determining the enthalpy in various combinations of known substrates/cofactors including succinate, mitochondria, mitoplast, and ATP, the possibility to conclude which compounds aid in ETC complex II stabilization became attainable. The difference in required substrates and cofactors for various samples may vary allowing for substitution for compounds of interest. The process of determining which substrates and/or cofactors are necessary for the stabilization of ETC complex II are explained as an optional step below. The enzymatic

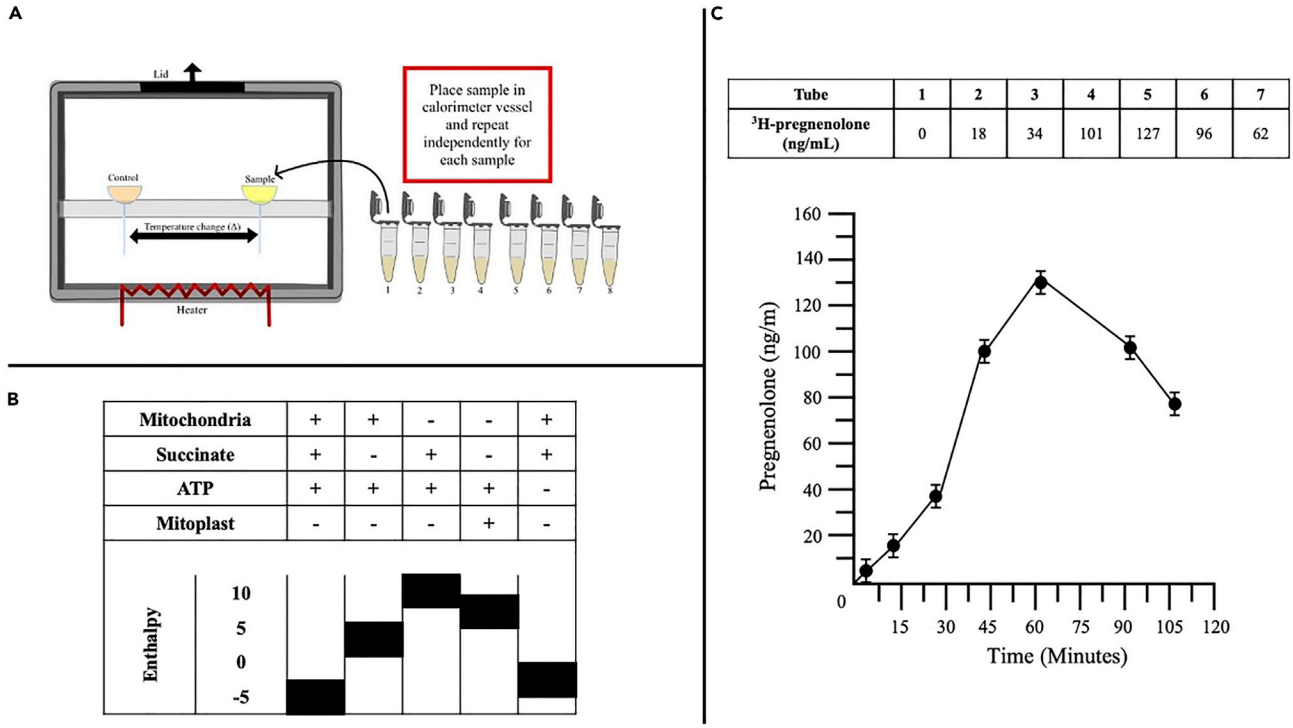

Figure 2. Schematic illustration and example of data for measuring enthalpy and pregnenolone synthesized during ETC complex II formation

Depicted is a technique using a combined calorimetry experiment paralleled to a metabolic activity assay. [Data from Bose et al.[1]].

(A) Illustration provided to emphasize the technique in maintaining corresponding samples for calorimetry and RIA independently. Samples were created in centrifuge tubes for each amount of succinate titrated into the calorimeter for activity measurement via RIA.

(B) To conclude which substrates were required to stabilize ETC complex II, the enthalpy was acquired in the presence and absences of hypothesized substrates/cofactors.

(C) RIA results and relative trend of activity measured. Procedure to perform RIA is available from MP Biomedicals Protocol references. Data is correlated with results obtained from enthalpy measurements to determine the capacity of succinate in ETC SCC-associated complex II steroid metabolism.

substrate assay revealed the essential component in stabilizing the reaction being succinate; however, the question of whether the confirmed substrates regulate enzymatic activity remained. The following steps are designed to measure products of downstream reactions to confirm metabolic capabilities. For this protocol, the conversion of pregnenolone to progesterone, which is an early reaction in steroidogenesis, was measured to confirm intact enzymatic activity (Figure 2).

Note: The mitochondrial protein solution used in this step is the same solution used in steps 1–8 and for all future steps.

9. Prepare calorimeter by adding a fixed 20 μL of mitochondrial sample to isothermal calorimeter.
10. Incubate at 37°C for 1 h.
11. Add 2 μL of 10 mM ATP into calorimeter.
    a. Wait for 30 min following this addition and record enthalpy.
        i. This is the first data point for this portion of the protocol.
12. Titrate each time with 1 μL of 50 mM of succinate into calorimeter.
    a. Wait 2 min after each addition.
    b. Document enthalpy of the reaction.
    c. Continue titration until enthalpy is unchanged.

Note: Amount of added succinate may vary and is dependent on the results acquired from step 8. The amount of substrate added in step 12 will exceed the amount of substrate

required to reach maximum reaction stabilization resembled as plateau phase depicted in Figure 1.

13. Repeat succinate titration in the previous step until enthalpy begins to increase signifying loss of ETC complex stabilization.

Pause point: If the reaction is continually producing a positive value of ΔH, reassess necessary substrate concentration needed to reach stabilization calculated in step 6. This is a good point to explore hypothesized substrates and/or cofactors that may be a limiting factor in complex stability. See troubleshooting 4.

Optional: Test combinations of substrates by removing and/or adding different compounds in steps 11 and 12. Resulting ΔH measurement will signify the enzymatic requirement for stability. This step is not essential if the substrates and cofactors are known for mitochondrial stability and functionality; however, this step provides opportunity to test unknown substrates and their contributions to the enzymatic reaction being investigated.

Note: At this point in the protocol, the goal is to prepare samples for RIA identical to sample conditions observed in steps 9–13. By doing so, it is important to recall the number of titrations performed. This number plus 1 is the number of tubes that must be prepared and labeled. The "plus 1" accounts for the enthalpy of the initial mitochondria protein sample.

14. Label tubes 1 through Xn equivalent to the number of titrations performed in previous step for RIA preparation.

Note: For explanation, if 30 data points were obtained from calorimetry above, tubes should be labeled 1–30.

15. Add 20 μL of mitochondrial sample to each labeled tube.
16. Add 1 μL of 50 mM succinate independently to labeled tubes increasing amount by 1 μL each addition in correspondence to total succinate titrated at each data point.
    a. The first tube labeled tube should not contain succinate and will be correlated to data point "time 0" of the calorimetry data collected. The amount of succinate added to the second labeled tube will correspond to the data point obtained after the first titration of 1 μL of succinate in above calorimetry. In the third labeled tube, 2 μL of succinate is added in correspondence to the third data point in the calorimetry performed and so on.

Note: At this point in the procedure, the RIA samples in labeled tubes must be identical to the amount of succinate added at each data point in calorimetry. Since the sample amount in the calorimeter is fixed, the amount of succinate continues to increase with each data point; however, the succinate in the sample used for RIA will be unchanged.

17. Following the addition of succinate to each independent tube, add 1–2 μL of 100 mM concentration of Trilostane.
    a. Immediately following additions, transfer to a 37°C water bath for 2 h.
        i. The incubation time is independent for each labeled tube and must be maintained precisely.
18. Centrifuge at 3,000 × *g* for 10 min at 4°C–8°C.
19. Collect supernatant from each tube independently for RIA beginning at step 20 below.

Note: If RIA to determine ETC complex activity and metabolic conversion is not performed immediately following step 19, the supernatant must be stored at −86°C and gradually

thawed on ice prior to starting RIA experiment. Trilostane is soluble in Ethanol and stops further conversion.

Direct measurement of progesterone by radioimmunoassay

Timing: 2–3 h

In this procedure, we used radioimmunoassay (RIA) for a quantitative analysis of the enzymatic activity. The antibody used to determine activity may vary depending on the target product downstream for the reaction of interest. When performing RIA for specific compounds, use caution and proper PPE with radioactive components. Protocol for RIA provided by MP Biomedicals and additionally listed in "materials and equipment" section.

Note: All samples must remain on ice throughout completion of metabolic reactions. RIA counting may produce variations in results due to static on scintillation vials thus ensure to allow sufficient time to decrease outliers. RIA procedure may differ depending on target product synthesized. If the radiolabeled product differs, interpretation of the data remains consistent and is discussed in "quantification and statistical analysis" section.

20. To measure the conversion of pregnenolone to progesterone, use the supernatant obtained in step 19 for RIA procedure provided by MP Biomedicals. See troubleshooting 5.

Optional: After the ETC complex activity has been determined by RIA above, thin layer chromatography (TLC) was used to further confirm the conversion studied throughout the protocol. This is an additional confirmatory step and not required. Confirmation of enzymatic products may be performed in ways other than TLC; therefore, we have provided key points for TLC as this is a widely used method in the scientific field. To initiate the conversion of cholesterol to pregnenolone, incubation of isolated mitochondrial protein sample and a significant amount of cholesterol must be done. Essential substrates of the desired metabolic reaction must also be added during the incubation process. Stopping the conversion of pregnenolone to progesterone is accomplished by incubation with Trilostane, which is an inhibitor of 3-βhydroxy steroid dehydrogenase. Following incubation, extraction of steroids/metabolites is performed allowing for the analysis of conversion via TLC.

EXPECTED OUTCOMES

The experimental sample should reach full saturation demonstrated through a plateau phase in the initial calorimetry providing means for sufficient protocol to determine the quantitative measurement of sample activity. For the experimental procedure this protocol was based on, necessity of succinate in regulating SCC complex was confirmed via permanent knockdown of malate dehydrogenase (MDH) in MA-10 cells with a silencing vector (Bose et al.[1]). Given the results yielded marginal effect on mitochondrial protein expression, we expect that our protocol will provide the means for adequate and reproducible methods to assess the essential role of potential mitochondrial complex cofactors in stabilization and metabolism.

QUANTIFICATION AND STATISTICAL ANALYSIS

The enthalpy of the performed reactions via isothermal titration calorimetry is calculated by heat produced divided by the substrate concentration of the corresponding reaction. Once the calculations are complete, the change in enthalpy distinguished whether the reaction is stable or unstable. A negative ΔH value, being a negative change in reaction temperatures, signifies stability thus suggesting full saturation of the enzyme as expressed throughout the protocol. As the substrate concentration increases within the vessel in the calorimeter, the values recorded will show little change the

closer the reaction approaches maximum enzymatic saturation. The importance in determining the amount of succinate required to reach full saturation provided insight into whether the sample had the capacity of being stable and how much substrate is required to reach highest stability. With the data interpreted from the calorimetry in determining ETC complex stabilization, the substrate concentration sufficient for the sample to reach full saturation can be computed. That amount of substrate will be a constant when configuring the activity assay via RIA.

Using the known amount of sufficient substrate, it is then correlated to the amount of pregnenolone/progesterone produced in a downstream reaction. The knowledge of mitochondrial steroid metabolism is the basis for enzymatic activity measurement. Cholesterol conversion to pregnenolone followed by pregnenolone conversion to progesterone provided means to evaluate ETC Complex II impact on metabolism. By performing RIA to determine the amount of steroid metabolism occurring at each titrated concentration of succinate, a known amount of downstream product was obtained. With the question remaining on whether succinate played a role in ETC SCC-associated complex II regulatory steps in steroid substrate conversion, association with complex stability via isothermal calorimetry bridged the concept of measuring enzymatic product and changes in heat production within the reaction allowing verification and inference into the ETC complex enzymatic capacity related to the amount of substrate required for the metabolism of mitochondrial products. Utilization of enthalpy (ΔH) from calorimetry measurement in parallel with activity measurement demonstrated the formation of complex ability and capacity in steroid metabolism.

Although regulatory capacity of succinate to steroid metabolism in relation to ETC complex II was determine through combined calorimetry and RIA, further confirmation of the regulatory substrate was obtained by performing the above experiment with malate dehydrogenase knockdown (MDH2) in MA-10 cells. Replication of activity measurement protocol yielded no difference in data between mouse Leydig cells and knockdown cell line confirming observed activity represented enzymatic reactions from SCC-associated ETC complex II. With the replicable data obtained, it is feasible to conclude the essence of new compounds in ETC metabolism and capacity to regulate downstream reactions.

LIMITATIONS

Potential limitations may include stunted cell growth and/or contamination in cell culture as well as potential impurities in sample. The mitochondria must be active metabolically and should be tested all the batches prepared at a time mitochondrial preparation by measuring the ATP. Each mitochondrial aliquot should be used once and should not be back and forth freeze thaw cycle.

TROUBLESHOOTING

Problem 1

Variable enthalpy readings of mitochondria protein sample without clear trend towards stabilization in step 6.

Potential solution

- If mitochondrial sample fails to stabilize, ensure viability of equipment, reagents, and sample.
- Use of buffers that have not been degassed prior to use and/or have not been prepared using degassed water can cause a wide variation or artefactual data. Degas water and prefilter through 0.22 μm before use.
- Calorimetric measurements should be done in a vibration and noise free room to eliminate potential background that could obscure data values.

- Bubbles should be removed carefully, and blank run must be performed with the buffer each time prior to use to remove free oxygen that may offset data readings.
- Ensure speed of substrate injection is correct. Injection speed being too fast or slow may cause variations in data.
- Consider extending/shortening time intervals between substrate injections in steps 5 and 6. This may normalize data points by allowing equilibration of reaction.
- If sample stabilization continues to fail, potential for mitochondrial sample being metabolically inactive is possible prompting further evaluation. See troubleshooting 2.

Problem 2

Failure of mitochondria protein sample to reach stabilization in steps 1–8.

Potential solution

- To determine mitochondrial viability, the use of thin layer chromatography (TLC) is used to verify the presence of VDAC2 in the sample.
  - VDAC2 presence confirms that the functional mitochondria components were not damaged during the isolation process.
- To correct this potential error, carefully perform stated number of up and down strokes during homogenization from steps 8 and 18 of "Isolation and Purification" section. Particularly when isolating mitochondria from a tissue rather than cell culture, overuse of the Dounce homogenizing process can lead to breaking of the mitochondrial membranes. Errors in homogenization results in the loss of functional ETC complexes that were imbedded in the mitochondrial membranes.
- Cell culture should not undergo mitochondria isolation prior to 4^{th} passage. After removing cell lines from storage, cell function may be slowed and required additional growth before *in vivo* activity levels are reached.
- Persisting problems after sample viability has been confirmed may suggest contamination/inadequacy of buffer and stock solutions. Ensure proper storage parameters have been met and check pH. Titration may be necessary and pH for each solution can be found on "materials and equipment" section.

Problem 3

Lack of pellet formation during isolation in step 24 of "mitochondria sample preparation."

Potential solution

- Ensure adequate cell culture growth before undergoing mitochondria isolation. Efficiency of the protocol is improved when mitochondria protein sample concentration is 1 μg/μL. If cells are not growing enough on single dish to reflect this concentration, combination of up to 3 cell culture petri dishes may be done to increase concentration.
- Measure concentration via Bradford Protein Assay prior to storage.
- Increasing sample concentration may improve stabilization and require less substrate for subsequent steps.

Problem 4

Stabilization of sample in step 13 is not consistent with results from step 8.

Potential solution

- Mitochondria sample used in all steps must be from the same batch. Using different batches will cause inaccurate data and inability to relate calorimetry findings to activity assay. It is essential to keep all samples uniform throughout protocol.

- If sample was not a large enough volume to suffice entire protocol, mitochondria protein sample must be repeated yielding a large batch size.
- Sample must remain on ice when not in use. Inconsistent results or failure to reach stability in steps 9–13 may suggest compromised sample. Repeating these steps with a new aliquot of original stored batch will suffice if uncontaminated.
- If problem is not resolved, refer to troubleshooting 1 and 2 for additional solutions.

Problem 5

Presence of outliers in RIA results following step 20.

Potential solution

- Following completion of protocol provided in RIA MP Biomedical Kit, allow scintillation tubes to settle overnight to reduce static that may be skewing data. Tubes can be run through scintillation counter for a second time which may reflect data with less variations.
- If data is still skewed, ensure that each sample contains viable mitochondria. Steps to confirm are listed following step 20 as an "Optional" step.
- Storage and troubleshooting for RIA MP Biomedical protocol may be helpful in correcting continued problems that are unrelated to isolated mitochondria protein sample.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Himangshu Bose, Department of Biomedical Sciences, Mercer University School of Medicine, 1250 East 66th Street, Savannah, GA 31404. Email: bose_hs@mercer.edu.

Materials availability

All reagents will be available to the user in writing and will be shipped out after approval from the office of the technology licensing (OTL).

Data and code availability

Data and code are embedded into ITC and does not require additional software. Processing of data is done directly from ITC.

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AUTHOR CONTRIBUTIONS

H.S.B. designed and performed experiments. N.E.D. participated in the initial standardization, wrote the manuscript, and prepared schematic presentations. H.S.B. edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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