Metabolic capacity of high performance fishes

Tuna

Mackerel

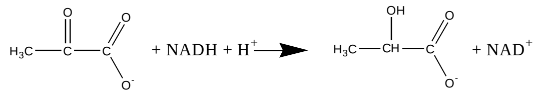
INTRODUCTION

Different species of fishes vary in their capacity for aerobic and anaerobic exercise. Fishes in the family Scombridae, such as tunas, bonitos, and mackerels, are considered to be “high-performance” fishes based on their ability to swim efficiently over long distances and to accelerate rapidly. Tunas are exceptional among the Scombrids because they are regional heterotherms. The red (slow oxidative) muscle fibers used for steady state swimming in tunas is positioned in the core of the body and surrounded by thick layers of white (fast glycolytic) muscle. This morphological adaptation, along with cardiovascular adjustments, allows tunas to retain metabolic heat generated while swimming and maintain elevated temperatures in the swimming muscles. High, stable temperatures in the swimming muscles contribute to high rates of flux through aerobic metabolic pathways and an increased aerobic capacity, traits that are important for endurance swimming.

Tunas may also achieve high rates of flux through anaerobic pathways. Tunas are voracious marine predators, and show an exceptional capacity for burst speed and rapid acceleration. White muscle comprises 46.5- 55.2% of body mass in a tuna (!), and the white muscle fibers have a high glycogen content and exceptionally high activity of key glycolytic enzymes. High rates of anaerobic metabolism during burst activity result in a large amount of lactate build-up, but tunas can rapidly metabolize lactate during post-exercise recovery due in part to their high capacity for aerobic metabolism.

For this lab, we will assess the capacity for anaerobic metabolism and burst speed in two species of Scombrid fishes, the yellowfin tuna and the strictly ectothermic Atlantic mackerel. To do this, we will compare the activity of lactate dehydrogenase (LDH) in white muscle tissue from these two species. LDH is of special importance in anaerobic glycolysis, as it catalyzes the reaction that reduces pyruvic acid to lactic acid and simultaneously oxidizes NADH to NAD+. Regeneration of the NAD+ electron acceptor is a necessary step for continued ATP production by the glycolytic pathway. By measuring the maximal activity of LDH, we can assess the organism’s capacity for anaerobic glycolysis.

Reaction catalyzed by LDH



pyruvate lactate

OBJECTIVES

- To learn how to carry out an assay of enzymatic activity
- To compare the activity of LDH in two species of Scombrid fishes
- To relate differences in LDH activity to the animal's capacity for anaerobic metabolism and burst swimming.

EQUIPMENT AND SUPPLIES

Solutions
Phosphate buffer
NADH solution
Pyruvate solution

***keep all solutions on ice !!**

Tissue preparation
tuna tissue sample
mackerel tissue sample
gloves
ice (in cooler)
marking pen
2 Petri dishes
razor blades
weighing paper
balance
calculator
6 test tubes
blue pipetter
5 ml pipette
beaker of ice water
tissue homogenizer
Centrifuge
2 plastic bulb pipettes

Enzyme assay
Spectrophotometer
Cuvettes
Parafilm
Stopwatch
Vortex
blue pipetter
1 ml pipette
Eppendorf pipetter & 2 tips

PROCEDURES PART I – TISSUE PREPARATION

Before running an enzyme assay, the muscle tissue must be broken down to release the enzymes from the cells. These enzymes must then be diluted to suitable strengths for running the assays.

***NOTE**: Be sure to properly label all test tubes to avoid cross-contamination of samples! Also, keep all tissue samples, homogenates, and solutions on ice, except when centrifuging.*

1) Obtain mackerel and tuna tissue samples from the T.A. Place the samples in separate LABELED Petri dishes. It is important that you keep the samples separate from each other at all times during the experiments, as mixing tissues could alter your results.

2) Place the Petri dishes containing muscle samples on ice, and use razor blades to mince each tissue sample. This step will make homogenization more effective.

3) Place weighing paper on the electronic balance, zero the balance, then weigh out ~0.5 g of the minced mackerel muscle sample. Place the minced muscle in the test tube *on ice* and add 9 volumes of phosphate buffer (assume 1 g of muscle = 1 ml). Use the blue pipetter and 5 ml pipette to transfer phosphate buffer (keep this pipette, as you will use it to transfer phosphate buffer again). Repeat this step for the tuna sample. You now have a 1:10 dilution of tissue:buffer for each sample (i.e. 10% homogenates)

4) Place the test tube containing the mackerel sample and buffer in a beaker of ice water (do not let ice water get into your sample!). Homogenize the sample for 30 – 60 seconds (or until fully homogenized) at medium to high speed. Place test tube with homogenate back on ice. Wash the homogenizer blade by running it for several seconds in a beaker of water. If this does not remove all the extra tissue from the blade, use the brush and water bottle to fully clean blade. *It is important to fully clean blade between samples so that you do not cross-contaminate samples*. Repeat this step for the tuna sample.

5) Make counterbalance tubes for both the mackerel and tuna samples for the centrifugation step. Fill an empty test tube with water until it has the same volume as the tube containing the sample. The counterbalance tubes will be placed on the opposite side of the centrifuge rotor from their respective samples to prevent uneven distribution of weight on the rotor.

6) Centrifuge samples at 6000 rpm for 20 minutes. While samples are in the centrifuge you can prepare the spectrophotometer for enzyme assays and measure out NADH samples in cuvettes so that they can equilibrate with room temperature (next section).

7) Remove sample tubes and use a plastic bulb pipette to carefully transfer 0.5 ml of the supernatant (clear fluid) to a new (labeled!) test tube and place on ice immediately. Use a separate plastic pipette for the mackerel and tuna samples to avoid cross-contamination.

The supernatant contains soluble enzymes, such as LDH. The pellet contains cytoskeletal material, oraganelles, and cell membranes, which you don't want!

8) Use blue pipetter and 5ml pipette to add 4.5 ml phosphate buffer to the supernatant in each test tube. You have now further diluted your original homogenate to a 1:100 (tissue:buffer) dilution (i.e. 1% homogenate). There is a lot of LDH in fish muscle! In order to analyze activity as accurately as possible we need to dilute our samples. The 1:100 dilution is the sample that you will use for the assay. Be sure to keep it on ice!

PROCEDURES PART II – ENZYME ASSAY

You will use a spectrophotometric assay to measure LDH activity in the mackerel and tuna samples. Enzyme activity is a measure of the maximal rate of a reaction in the presence of saturating concentrations of substrate. Activity is measured in units of umols•min^{-1}•g wet weight tissue^{-1}. This is commonly referred to as Units•g^{-1}. Although enzyme activity is not a direct measure of enzyme concentration in the tissue, if the concentration of an enzyme is doubled, activity will also double. Therefore, we can compare activity of an enzyme in different tissues and assess how the enzyme's concentration is adjusted to reflect the metabolic needs of the cell.

The spectrophotometer measures the absorbance of light at different wavelengths. NADH absorbs light at 340 nm, whereas NAD does not. For our assay, we will place all of the ingredients necessary for the reaction catalyzed by LDH to proceed in the cuvette and then record the change in absorbance as NADH disappears. Remember that LDH catalyzes a reaction in which NADH is oxidized to form NAD+ as pyruvic acid is converted to lactic acid (see equation in Introduction). The rate of change in absorbance therefore provides an indication of the rate at which the reaction catalyzed by LDH is proceeding (i.e. LDH activity)

NOTE: Before placing cuvettes in the spectrophotometer, be sure to wipe the side of the cuvette clean using a Kimwipe. Also, make sure you place the cuvette in the cuvette holder such that the CLEAR sides (not the frosted sides) are facing into the spectrophotometer beam.

1) Confirm that the wavelength on the spectrophotometer is set to 340 nm

2) Set the display mode to Transmittance by pressing the mode control key.

3) Place black box in the cuvette holder and close lid. If display does not read 0.00% T, press the 0%T button.

4) Fill a clean square cuvette with phosphate buffer and place in the cuvette holder. If display does not read 100% T, press 0A/100%T button. Set display mode to Absorbance by pressing the mode control key. If the display does not read 0.00 A, press the 0A/100%T button again.

5) Use a plastic bulb pipette to add 3 ml of NADH solution to two cuvettes. Place the cuvettes in a shallow bowl of water (taking care not to let water get in the cuvettes!) so that they equilibrate with room temperature more quickly.

6) Make sure that you have a stopwatch, pencil, and paper handy. When you are ready to run a sample do the following:

NOTE: SPEED IS ESSENTIAL!!!
ALSO: Only prepare one cuvette for analysis at a time. It doesn't matter whether you do the tuna or mackerel sample first.

- use blue pipetter and 1 ml pipette to add 0.2 ml (200ul) of pyruvate solution to the cuvette

- use the Eppendorf pipetter and tip to add 0.02 ml (20ul) of homogenate – put Parafilm over the top of cuvette, invert once (to mix all ingredients in the cuvette), and then place cuvette *immediately* in the spectrophotometer and start the stopwatch

- record your first Absorbance reading immediately and then every 10 seconds for the next 2 minutes, or until the absorbance change slows considerably. Record for *at least* 1 minute.

- Remove the cuvette and rinse once with alcohol and twice with dH_2O

- Repeat these steps for your second tissue sample.

ANALYSIS

1) To calculate enzyme activity we must first determine the slope (m) of the line relating Absorbance (A) to time. Generate a graph and plot Absorbance (Y) vs. time (X). Note on your graph where the change in Absorbance (A) begins to slow down considerably, and only use data recorded before that point to calculate the slope (A/sec). The slope will be negative because you are measuring NADH disappearance – just convert it to positive.

2) Once we know the slope, we can then use the following equation to determine enzyme activity:

Activity ($Units \bullet g^{-1}$) = [(m*60)/ ε] * [(total assay vol)/(vol of homogenate in assay)] * dilution factor

m = slope (this is initially in units of A/sec – we multiply by 60 to convert to A/min)
ε = 6.22 (absorption coefficient for NADH)
total assay volume = 3.220 ml (total volume in cuvette)
vol of homogenate = 0.020 ml (volume of homogenate in cuvette)
dilution factor = 100 (our final homogenate dilution was 1:100 tissue:buffer)

For example, if your slope was 0.0060 A/sec:

LDH activity = [(0.0060*60)/6.22] * [3.220/0.020] * 100 = *931 Units•g*$^{-1}$

For each tissue sample (tuna and mackerel), please provide a printout of graphs relating absorbance to time. You may make calculations of enzyme activity on the graph printout, but please be sure that your writing is legible! Hand in the graphs and calculations along with answers to the questions in the following section.

QUESTIONS

1) Are there differences in the LDH activity in white muscle from tuna and from mackerel? If so, how might you explain these differences? What would these differences tell you about the capacity for anaerobic metabolism and burst activity for these two species of fish?

2) What are the benefits of having high anaerobic capacity? What are the benefits of having a high aerobic capacity?

3) Explain why it is beneficial for species with high anaerobic capacity to also have a high aerobic capacity.

REFERENCES

Dickson, K.A. 1996. Locomotor muscle of high-performance fishes: What do comparisons of tunas with ectothermic taxa reveal? Comp. Biochem. Physiol. 113A (1): 39-49.