2nd European Union Science Olympiad

in Groningen, Netherlands

TASK **A**

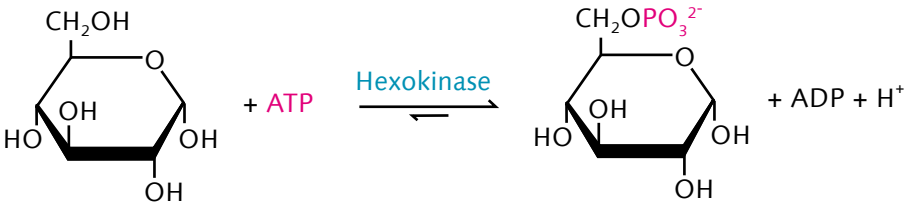
Task A

Investigation of Hexokinase Activity

Introduction

Enzymes are proteins; large molecules that catalyse the thousands of reactions and processes that occur in our bodies. They are biocatalysts; they allow these reactions and processes to proceed at appreciable rates at our body temperature, a relatively low temperature chemically speaking. The enzyme used in this experiment, hexokinase, is an example of one such biocatalyst.

Hexokinase is the enzyme that phosphorylates glucose to glucose 6-phosphate:

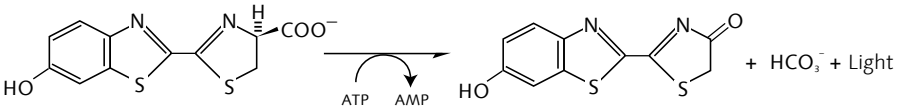


Glucose

Glucose 6-phosphate (G-6P)

This reaction constitutes the first step of the process of glycolysis, the metabolic pathway that breaks down glucose and helps to release energy. In the pancreas hexokinase acts as a receptor or sensor to monitor blood glucose concentrations. After a meal, blood glucose rises and glucokinase is activated. This signals the release of insulin from the pancreatic beta cells. The circulating insulin can then regulate the uptake of glucose into muscle and convert glucose into adipose tissue.

The activity of hexokinase can be measured indirectly by measuring the concentration of ATP through a chemiluminescence reaction. The chemiluminescence assay takes advantage of the light producing reaction of the enzyme luciferase.

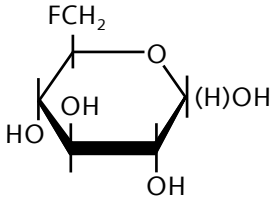


Photirus luciferin

oxidized Photirus luciferin

The chemiluminescence assay is first performed using known quantities of ATP to produce a standard curve. The standard curve is then used to quantitate the amount of ATP used in hexokinase catalyzed reaction. Upon completion of the reactions, the specific activity of hexokinase can be determined under various reaction conditions.

Each team will perform experiments to measure the activity of hexokinase under various conditions (i.e. different substrate concentrations, different hexokinase concentrations, with and without a hexokinase inhibitor). Each team will be supplied with the following materials:



Inhibitor

Material and equipment

- 1 M potassium phosphate buffer containing 200 mM $MgSO_4$ – pH 7.6
- 100 mM ATP/Mg^{2+}
- 100 mM glucose
- 2.650 µg/ml (500 units/ml) hexokinase
- 100 mM inhibitor = 6-fluoro-6-deoxy-D-glucose (see picture above)
- water (deionized/distilled)
- solution pH 12
- luciferase solution
- 96 well microtiter plate
- micro-volume "pipetmen"

General equipment:

- microtiter plate reader (with chemilumenescence feature)
- shaking device

Experimental procedure

Preparing Standard (Calibration) ATP Curve

1. Fill a number of wells of a microtiter plate with 50 µL of solution pH 12.

2. You will have to prepare standard (calibration) ATP solutions in 1 mL eppendorf tubes. The ATP concentrations have to include 0 and 6 mM and a number of concentrations in between. ***Calculate the blanks and fill in the table before you start pipetting!***

As you will be using these standard solution to construct your standard curve from which you will calculate your final results (Hexokinase activity) it is imperative that you work ***extremely accurately***!

Conc ATP standard (mM)	100 mM ATP/ Mg^{2+}stock. (µL)	1M KPi. buffer with 200 mM $MgSO^4$ pH 7.6 (µL)	100 mM Glucose (µL)	H_2O (µL)
0	0	50	10	940
		50	10	
		…	…	
6		50	10	880

3. Take 100 µL of each standard and place it in a microtiter well. Mix for 5 minutes using a shaking device.

4. Add 50 µL of the luciferase solution to each well to initiate the luciferase chemiluminescence reaction. Mix the titer plate as described above for 5 minutes.

5. The plate is wrapped in aluminiumfoil and incubated this way in the dark for 10 minutes. After this time take the microtiter plate (with aluminium foil on) to one of the assistants they will measure the chemoluminescence by using the microtiter plate reader.

6. Plot the chemiluminescence versus ATP standard concentration to get your standard curve.

Hexokinase assay:

1. Fill the wells of a microtiter plate with 50 µL of solution pH 12. For each experiment you will need to fill 8 wells (as you will be taking 8 samples at different time points). So for 3 experiments + 1 control you will need to fill 32 wells. Enjoy this opportunity to hone your pippetting skills, and remember: accuracy is paramount!

2. Prepare 1 mL samples (in eppendorf tubes) as decribed in the pipette scheme below. Prepare the samples (in eppendorf tubes) up to (but not including) the addition of H.K. (Hexokinase). Mix well.

Stock Solutions	**Control** *No hexokinase No inhibitor*	**Experiment 1** *5.3 µg HK (1 unit) no inhibitor*	**Experiment 2** *10.6 µg HK (2 units) no inhibitor*	**Experiment 3** *5.3 µg HK (1 unit) inhibitor*
1M KPi. 200 mM $MgSO^4$ pH 7.6	50 µl	50 µl	50 µl	50 µl
100 mM ATP/Mg^{2+}	50 µl	50 µl	50 µl	50 µl
100 mM glucose	10 µl	10 µl	10 µl	10 µl
Water	890 µl	888 µl	886 µl	788 µl
100 mM inhibitor	No addition	No Addition	No Addition	100 µl
Mix – add hexokinase to start reaction				
2.650 µg/ml (500 units/ml) hexokinase	No addition	2 µl	4 µl	2 µl
Caution	–	After adding Hexokinase take immediately sample 1 (0 minutes) and start the stopwatch.		
Mix well				
Final volume	1000 µl	1000 µl	1000 µl	1000 µl

3. Start the stopwatch and take 100 µL samples at 0, 0.5, 1.5, 2, 4, 5, 8 and 10 minutes of the 'control' sample. Place the samples in the microtiter wells containing the solution pH 12. Remember which sample you put in which well!

7. Repeat step 3 for the other experiments; only add Hexokinase as described in the above pipette scheme and then start recording the time and taking samples.

8. Mix the microtiter plates for 5 minutes using the shaking device.

9. Add 50 µL of the luciferase solution to each well to initiate the luciferase chemiluminescence reaction. Mix the titer plate as described above in step 5 for 5 minutes.

10. The plate is wrapped in aluminiumfoil and incubated this way in the dark for 10 minutes. After this time take the microtiter plate (with aluminium foil on) to one of the assistants. They will measure the chemoluminescence by using the microtiter plate reader.

11. Make a plot on the supplied graph paper of the chemoluminescence versus the time for each experiment. You may plot the results for the 3 experiments + control on the same graph (remember: clarity).