BIO 122 Section 063, Group 06

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Effects of Oxaloacetate on Succinate Dehydrogenase Activity

Introduction:

Eukaryotic cells go through a process called cellular respiration, a process by which one molecule of glucose is converted into roughly thirty molecules of ATP. The main three steps in cellular respiration are glycolysis, the Krebs cycle and the electron transport chain. This experiment involves the Krebs Cycle as that is where SDH is used. SDH maintains a dual role in both the Krebs Cycle and the Electron Transport Chain. The Krebs Cycle, also called Citric Acid Cycle, is a process discovered by Hans Adolf Krebs in 1953. SDH is an enzyme that catalyzes the oxidation of succinate to fumerate while reducing ubiquinone to ubiquinol (Raven et al, 2010). An assay is done to observe the interaction between SDH and Oxaloacetate. It is hypothesized that Oxaloacetate is a competitive inhibitor and will therefore impede SDH activity.

SDH maintains a dual role in both the Krebs Cycle and the Electron Transport Chain. The Krebs Cycle follows the breakdown of citric acid, or citrate, in an enzyme assisted metabolic process. In the matrix of the mitochondria, a 2-carbon acetyl group of acetyl CoA combines with a 4-carbon molecule called oxaloacetate. Once these combine the reaction is catalyzed by citrate synthase it forms citric acid and the Krebs cycle begins to produce the ATP. Oxaloacetate remains dormant for the rest cycle until the end of the Krebs cycle when it begins again. In the final two reactions of the Krebs cycle, a water molecule is added to fumarate, forming malate. Malate is then oxidized, yielding a 4-carbon molecule of oxaloacetate. The final unoxidized product of the entire cycle is oxaloacetate, which can accept another acetyl group to start the cycle again.

In the eighth step of the Krebs Cycle, SDH is an enzyme that catalyzes the oxidation of succinate to fumerate. The enzyme SDH is composed of four subunits which take part of both the Krebs Cycle and the Electron Transport chain. As an integral part of the Krebs cycle, SDH oxidizes, $C_4H_6O_4$, to fumarate, $C_4H_4O_4$, by removing two hydrogen atoms. These hydrogen atoms then reduce cofactor FAD+ to $FADH_2$. The $FADH_2$ are then transferred to the extracellular matrix by the enzyme Ubiquinone. After ubiquinone carries the 2 hydrogen atoms, the hydrogen gradient forms which is then used to drive ATP Synthase to form ATP from ADP and a free phosphate group. (Raven et al, 2010). Most enzymes are regulated by several factors such as allosteric regulators, cofactors, competitive regulators, pH and temperature. For this experiment, nearly all of these factors were controlled. The optimum conditions to obtain 100% SDH enzyme activity included a pH of 7.5 at the temperature of 0^oC. (Ackrell, 1977). An assay was done to observe the interaction between SDH and Oxaloacetate. It is predicted that Oxaloacetate is a competitive inhibitor. Competitive inhibitors bind to the active site of an enzyme and prevent the substrate from attaching. Since Oxaloacetate has a similar shape to succinate, it will impede SDH enzyme activity. Oxaloacetate is a competitive inhibitor to succinate due to its similar structure. Both Oxaloacetate and succinate are a four carbon compounds with two carboxyl groups attached to each end. The difference between the two is the number of oxygen molecules attached. Succinate has two oxygen molecules double bonded to its carbons while oxaloacetate has three. (Karpusas et al., 1990).

In this experiment, several reactants were used to perform specific functions throughout the lab to maintain control and obtain positive data. The reactants used included Sodium Azide, 2-6-

dichlorophenolindophenol (DCPIP), Potassium phosphate, Buffered sucrose, Succinate, and Oxaloacetate. To utilize the application of artificial electron acceptors like DCPIP, the standard pathway of electron transport chain through the mitochondrion must be stopped. One way to stop this is through the use of sodium azide or potassium cyanide. In this experiment, Sodium azide is used. It was also mentioned that a balanced pH of 7.5 is vital to obtain the full scope of enzyme activity for SDH. For this reason, Potassium phosphate buffer is used since it is a great buffer. Buffered sucrose is utilized at the beginning of the experiment to supply the liver cells, more specifically the mitochondria in the liver cells, with a constant supply of glucose while being broken down and centrifuged. Succinate was the dependent variable in this experiment. The amount of succinate produced by the reactions helps determine the level of SDH enzyme activity in mitochondria, regardless of the inhibitors, oxaloacetate, presence. Oxaloacetate acts as an competitive inhibitor for SDH enzyme activity in this experiment. Its presence helped indicate the contrast between mitochondrial fractions with oxaloacetate and those without oxaloacetate – to determine whether oxaloacetate was truly a competitive inhibitor. Another reactant DCPIP or 2,6-dichlorophenolindophenol is a useful artificial electron acceptor which displays a decrease in absorption at 600nm upon reduction and therefore can be used to determine the rate of the enzyme catalyzed reaction. DCPIP appears blue in its oxidized form and transparent in its reduced form. Its loss of color is monitored at a wavelength of 600 nm. (Nissen, 2005).

This experiment was performed with the goal of finding evidence that supports or refutes the hypothesis. This experiment was also implemented to develop an understanding of enzymatic activity and the Krebs cycle.

Hypothesis:

The reagent oxaloacetate will inhibit succinate dehydrogenase activity because it acts as a completive inhibitor; binding to the active site and making SDH incapable to bind to its true substrate, succinate.

Results:

1.1 Bradford Assay:

i. Table of Bradford assay data spectrophotometer absorbance readings at 595 nm from various samples of protein concentrations (mg/mL).

Conc (mg/mL)	Absorbance (AU) @ 595 nm
0	0.000
0.05	0.038
0.1	0.095
0.2	0.189
0.4	0.330
0.7	0.612
1	0.833

ii.

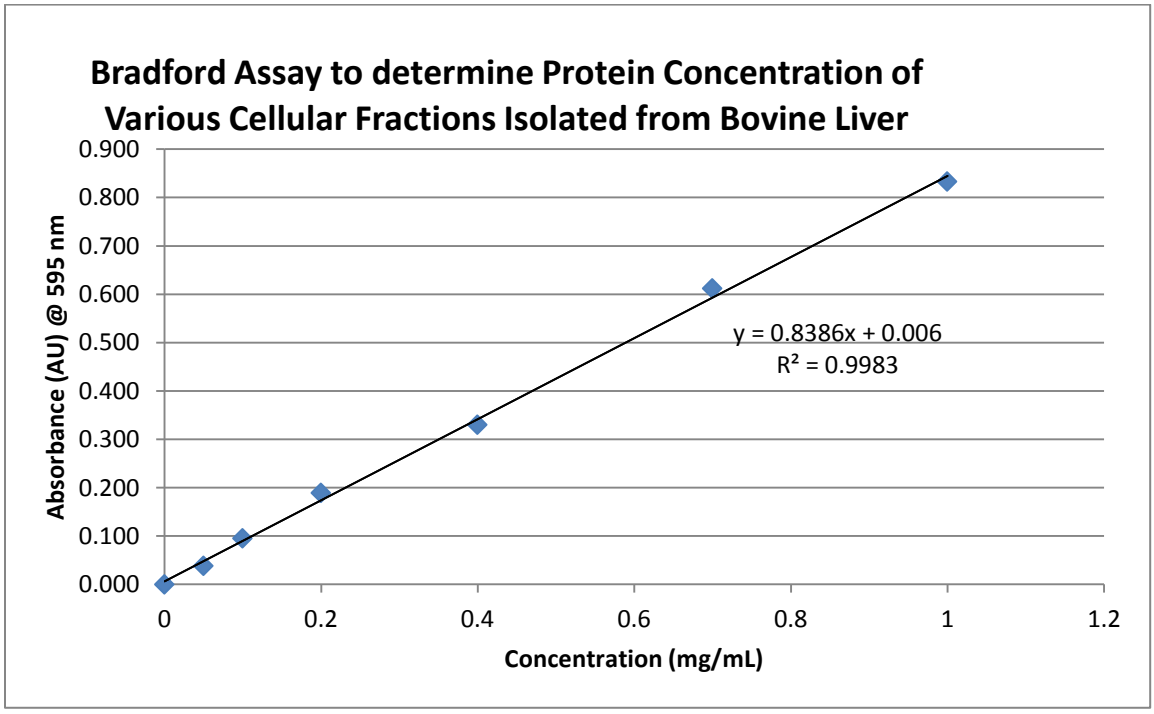


Fig.1 Bradford assay standard curve: linear relationship between concentration (mg/mL) versus absorbance (AU) to determine protein concentrations of crude and mitrochondrial fractions isolated from bovine liver.

iii. Calculations of protein concentrations of mitochondrial and crude extracts

Bradford Assay Standard Curve Equation; y=0.8386x+0.006
Algebraically, the standard curve can be re arranged to solve for sample concentration as a variable x using measured absorbance as variable y.

Table 1.2 Sample concentrations for crude and mitochondrial fractions based on the Bradford assay standard curve equation multiplied by its dilution factor

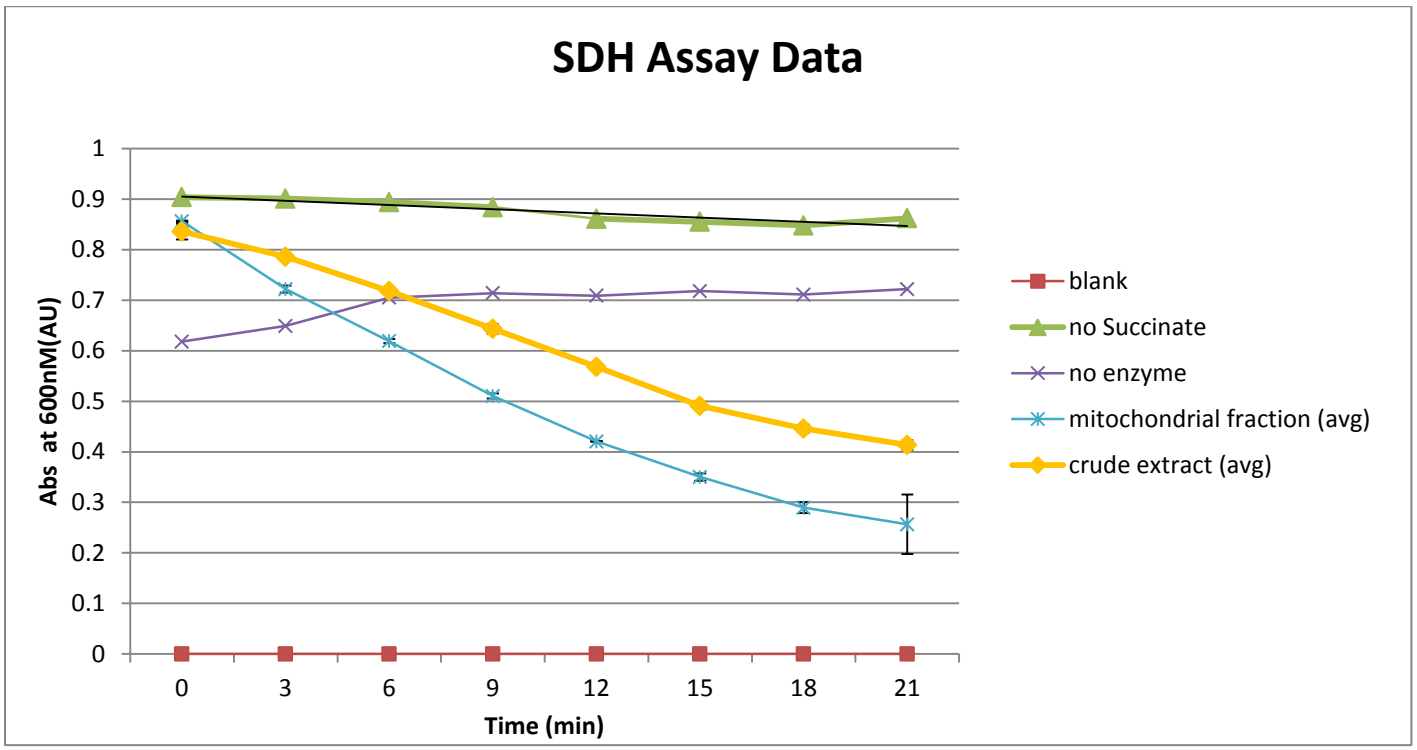
Dilution FACTOR	Sample	Absorbance (AU)	Sample Conc (mg/mL)
20	Mitochondria	1.119	26.54186532
50	Crude	0.433	25.45506547

1.2 First SDH Assay (week 3)

i. Table of First SDH assay with mitochondrial and crude extracts

Absorbance Readings	**600 nm**	1st Run	100 µL Extract				
	0	3	6	9	12	15	18	21
Blank	0	0	0	0	0	0	0	0
No Succinate Control	0.904	0.901	0.894	0.884	0.861	0.855	0.848	0.862
No Enzyme Control	0.618	0.649	0.705	0.714	0.709	0.718	0.711	0.722
Mito extract (1)	0.857	0.717	0.616	0.507	0.421	0.345	0.297	0.298
Mito extract (2)	0.855	0.727	0.622	0.514	0.42	0.355	0.282	0.215
Crude extract (1)	0.841	0.783	0.718	0.635	0.562	0.483	0.437	0.403
Crude extract (2)	0.848	0.794	0.725	0.653	0.574	0.498	0.453	0.421
Crude extract (3)	0.818	0.78	0.711	0.642	0.568	0.491	0.447	0.416
Avg Mitochondrial	0.856	0.722	0.619	0.5105	0.4205	0.35	0.2895	0.2565
sd Mitochondrial	0.00141	0.007071068	0.0042426	0.00495	0.00071	0.00707	0.01061	0.05869
Avg Crude	0.83567	0.785666667	0.718	0.64333	0.568	0.49067	0.44567	0.41333
sd Crude	0.0157	0.007371115	0.007	0.00907	0.006	0.00751	0.00808	0.00929

ii. Graph of First SDH Assay with mitochondrial and crude extracts



iii. Calculations of Specific Activities for crude and mitochondrial fractions

Mitochondrial Fraction Specific Activity	Crude Fraction Specific Activity
$\ln\frac{1.732}{1.1125} = 0.443$	$ln\frac{1.762}{1.023} = 0.544$
$\frac{0.443}{12}\min = 0.037\ min^{-1}$	$\frac{0.544}{12} min = 0.045 min^{-1}$
$\frac{0.037 min^{-1}}{0.1\ {min^{-1}}/{units}} = 0.37\ units$	$\frac{0.045 min^{-1}}{0.1\ {min^{-1}}/{units}}$
$\frac{0.37 units}{0.1\ mL} = 3.7\ \frac{units}{mL}$	$\frac{0.45\ units}{0.1\ mL} = 4.5\ \frac{units}{mL}$
$\frac{3.7\ {units}/{mL}}{26.5\ {mg}/{mL}} = 0.1396 \frac{units}{mL}$	$\frac{4.5\ {units}/{mL}}{25.5\ {mg}/{mL}} = 0.1765\ \frac{units}{mL}$

BIO 122 Section 063, Group 06

May 1, 2013

Results for First SDH Assay with mitochondrial and crude extracts

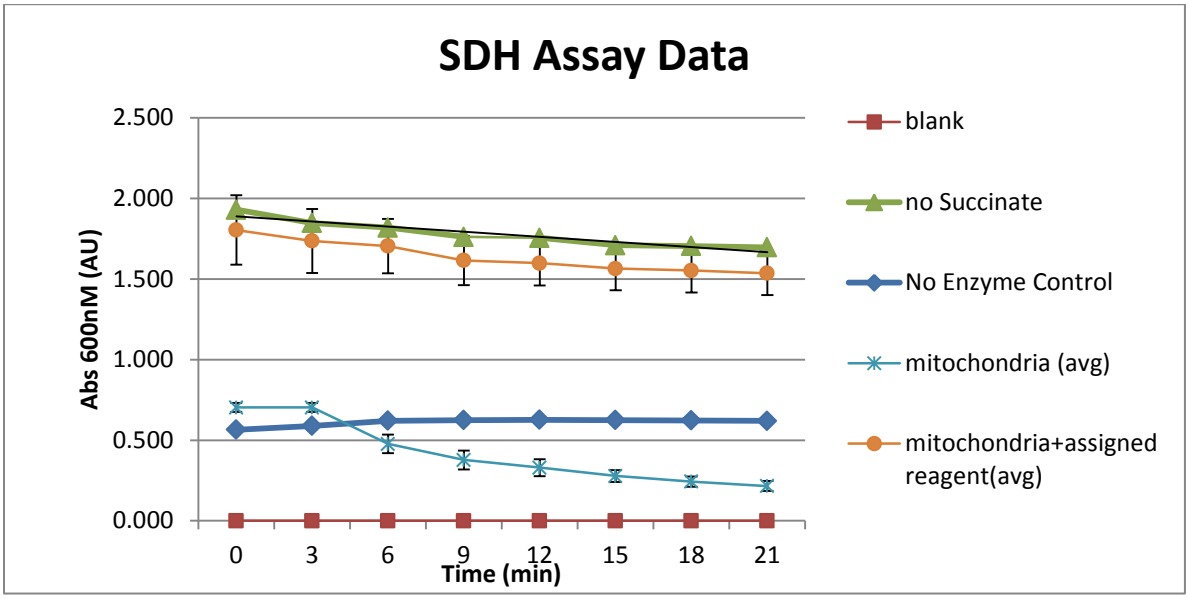
- Mitochondrial fraction has a calculated specific activity of 0.1396 units/ml
- Crude fraction has calculated specific activity of 0.1765 units/ml

1.2.1 Second SDH Assay of mitochondrial extract with and without reagent (week 4)

1.2.1.1 Table for Second SDH assay data of mitochondrial extract with and without oxaloacetate

Absorbance Readings	**600 nm**	1st Run	100 µL Extract					
	0	3	6	9	12	15	18	21
Blank	0.000	0.000	0	0	0	0	0	0
No Succinate	1.930	1.847	1.818	1.761	1.755	1.709	1.704	1.696
No Enzyme Control	0.565	0.589	0.621	0.625	0.627	0.625	0.623	0.62
Mito extract (1)	0.683	0.540	0.436	0.335	0.293	0.252	0.221	0.194
Mito extract (2)	0.723	0.618	0.518	0.419	0.367	0.304	0.266	0.238
Mito extract with reagent (1)	1.942	1.855	1.75	1.641	1.622	1.573	1.563	1.536
Mito extract with reagent (2)	1.555	1.507	1.518	1.45	1.449	1.426	1.411	1.4
Mito extract with reagent (3)	1.914	1.846	1.846	1.753	1.724	1.694	1.684	1.67
Mito extract (average)	0.703	0.703	0.477	0.377	0.33	0.278	0.2435	0.216
Mito extract (SD)	0.028284	0.028284271	0.057982756	0.059397	0.052326	0.03677	0.03182	0.031113
Mito extract with reagent (average)	1.803667	1.736	1.704666667	1.614667	1.598333	1.564333	1.552667	1.535333
Mito extract with reagent (SD)	0.215806	0.198370865	0.168633725	0.153207	0.139019	0.13421	0.136793	0.135001

1.2.1.2 Graph of Second SDH Assay of mitochondrial extracts with and without oxaloacetate



1.2.1.3 Calculations of Specific Activities of mitochondrial extract with and without reagent

Mitochondrial Extract without Oxaloacetate	Mitochondrial Extract with Oxaloacetate
$ln\frac{0.703}{0.278} = 0.9277$	$ln\frac{1.736}{0.278} = 0.104$
$\frac{0.9277}{12} min = 0.077 min^{-1}$	$\frac{0.104}{12} min = 0.0087 min^{-1}$
$\frac{0.077 min^{-1}}{0.1\, {min^{-1}}/{units}} = 0.77\ units$	$\frac{0.0087 min^{-1}}{0.1\, {min^{-1}}/{units}} = 0.087\ units$
$\frac{0.77 units}{0.1\ mL} 7.7\, {units}/{mL}$	$\frac{0.087\ units}{0.1\ mL} 0.87\, {units}/{mL}$
$\frac{7.7\, {units}/{mL}}{26.5\, {mg}/{mL}} = 0.3412 \frac{units}{mL}$	$\frac{0.87\, {units}/{mL}}{26.5\, {mg}/{mL}} = 0.0308 \frac{units}{mL}$

Results for Second SDH Assay with mitochondrial extracts with and without oxaloacetate

- Mitochondrial fraction without OAA has a calculated specific activity of 0.3412units/ml
- Mitochondrial fraction with OAA has a calculated specific activity of 0.0308 units/ml
- Mitochondrial fraction without OAA has more activity than with OAA

Conclusion:

1. **Role of Bradford assay**
   - Bradford Assay determines protein concentration
   - Standard curve is a linear relationship between measured absorbance values and concentration
   - Protein concentration values used to calculate specific enzyme activity in units/mL
   - Specific enzyme activity of mitochondrial extract with and without oxaloacetate is compared and interpreted.

2. **Blank and negative controls**
   - Blanks have no enzyme activity, is a control variable against other reagents, and is used to calibrate absorbance values
   - Negative controls contain no enzyme activity nor succinate
   - Assays did not behave constantly as expected: no enzyme activity displayed an increasing linear curve indicating some activity
   - No enzyme control is a comparison variable for samples with / without enzyme activity

3. **Differences in specific activities**
   - First SDH Assay, mitochondrial extract (0.1396 units/mL) has greater specific activity than crude extract (0.1765units/mL)
   - Results as expected; more activity in mitochondrial versus crude extract because mitochondrial has more specific enzymes despite lower calculated specific activity due to dilution factor of 20 for mitochondrial : 50 for crude
   - Second SDH Assay, mitochondrial extract without OAA (0.3412 units/ml) versus mitochondrial extract with OAA (0.0308 units/ml)
   - Results as expected; more mitochondrial specific activity without OAA than with OAA supports hypothesis: OAA inhibits succinate dehydrogenase activity and decreases the amount of specific activity

References and Citations

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