IDENTIFICATION OF ENDOGENOUSLY BIOTINYLATED PROTEINS IN MAMMALIAN SPERMATOZOA

A thesis submitted to the
Kent State University Honors College
in partial fulfillment of the requirements
for General Honors

by
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May, 2011
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ACKNOWLEDGEMENTS

I would like to thank my defense committee: Dr. John Johnson, Dr. Robin Joynes, Prof. Gianna Commito and Dr. S. Vijayaraghavan, the Honors College of Kent State University for providing this opportunity, and National Institutes of Health for funding this project. I would especially like to thank my advisor, Dr. S. Vijayaraghavan for guiding me through this project and for being a supporting mentor through my undergraduate studies and beyond. I would also like to thank my brother Tanmoy Das Lala, Shandilya Ramdas, Kurtis Eisermann and all the members of Dr. Vijay’s lab for their support, help and encouragement.
CHAPTER I

INTRODUCTION

A protein’s biological activity can be modified by non-protein co-factors. Co-factors bind to or are covalently linked to an enzyme to assist in biochemical transformations. An enzyme and a co-factor together form an enzymatically active conjugated protein called the holoenzyme. Co-factors can be organic (coenzymes) or inorganic (metals). They can also be classified according to their ability to bind to enzymes. Loosely-bound cofactors are termed coenzymes and tightly-bound cofactors are termed prosthetic groups. The primary difference between a prosthetic group and coenzyme is; the prosthetic group remains attached to the apoenzyme while undergoing oxidation and reduction while coenzymes may undergo reduction while attached to one apoenzyme, and then migrate to another apoenzyme where it can be oxidized. NAD, NADP and CoA are examples of coenzymes whereas hemes, flavins and biotin are prosthetic groups.

Biotin is a cofactor responsible for carbon dioxide transfer in several carboxylase enzymes. It is covalently attached to the active sites of the metabolic carboxylases. Using biotin cofactor as a mobile carboxyl carrier these metabolic enzymes generally capture CO2 from bicarbonate ion and catalyze transfer of this carboxylate to organic acids to form various cellular metabolites[10] .

Biotin protein ligase (BPL), also known as holocarboxylase synthetase (EC 6.3.4.15), is the enzyme that enables covalent attachment of biotin to the carboxylases.
Post-translationally, biotin forms an amide linkage with specific lysine residue of newly synthesized carboxylases with the help of BPL[10]. The image below shows steps of the biotin protein ligase reaction.


Fig 1. Steps of the biotin protein ligase reaction.
**Role of biotin in carboxyl group transfer.**

Biotin is the only prosthetic group that facilitates the transfer of a carboxyl group. The role of biotin is to act as a mobile carboxyl group carrier, transporting the carboxyl group from the site of the carboxyl donor to the carboxyl group acceptor enzyme. Common carboxyl group donors are HCO$_3^-$, oxaloacetate, or methylmalonyl CoA, and carboxyl group accepter enzymes are pyruvate, acetyl CoA, propionyl CoA.

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**Fig 2. General mechanism of biotin function in carboxyl group transfer.** One molecule of ATP is consumed in the carboxyl group transfer reaction.
**Biotin-dependent carboxylases.**

The four biotin-dependent carboxylases in mammals are acetyl-CoA carboxylase (E.C. 6.4.1.2), pyruvate carboxylase (E.C. 6.4.1.1), propionyl-CoA carboxylase, (E.C. 6.4.1.3), and b-methylcrotonyl-CoA carboxylase (E.C. 6.4.1.4). Acetyl-CoA carboxylase (ACC) is found mainly in the cytosol while pyruvate carboxylase (PC), propionyl-CoA carboxylase (PCC) and methylcrotonyl-CoA carboxylase (MCC) are present in the mitochondria [6] (Figure 1).

The biotin-dependent carboxylases play a crucial role in cell metabolism. ACC controls fatty acid synthesis in the cell cytosol by providing the substrate malonyl-CoA [1] (*Figure 1*). ACC may also play an important role in biotin storage [1]. PC is a key enzyme in gluconeogenesis [3] and provides a tricarboxylic acid cycle intermediate [1]. PCC catalyzes an essential step in the metabolism of amino acids such as isoleucine and methionine, odd-chain fatty acids, and breakdown products of dietary carbohydrates [1]. MCC carboxylase catalyzes an essential step in leucine metabolism.
**Cytosol and mitochondria**

\[
\begin{align*}
\text{H}_3\text{C} - \text{C} - \text{SCoA} & \xrightarrow{\text{ACC}} \text{HOOC} - \text{H}_2\text{C} - \text{C} - \text{SCoA} \\
\text{Acetyl-CoA} & \xrightarrow{\text{Biotin}} \text{Malonyl-CoA} \\
\text{H}_3\text{C} - \text{C} - \text{COO}^- & \xrightarrow{\text{PC}} \text{OOC} - \text{H}_2\text{C} - \text{C} - \text{COO}^- \\
\text{Pyruvate} & \xrightarrow{\text{Biotin}} \text{Oxaloacetate} \\
\text{H}_3\text{C} - \text{CH}_2 - \text{C} - \text{SCoA} & \xrightarrow{\text{PCC}} \text{HOOC} - \text{H}_3\text{C} - \text{C} - \text{SCoA} \\
\text{Propionyl-CoA} & \xrightarrow{\text{Biotin}} \text{Methylmalonyl-CoA} \\
\text{Biotin Deficient} & \rightarrow \text{3-Hydroxypropionic acid & 2-Methylcitric acid} \\
\text{H}_3\text{C} = \text{CH} - \text{C} - \text{SCoA} & \xrightarrow{\text{MCC}} \text{HOOC} - \text{CH}_2 - \text{C} = \text{CH} - \text{C} - \text{SCoA} \\
\text{β-Methylcrotonyl-CoA} & \xrightarrow{\text{Biotin Deficient}} \text{β-Methylglutaconyl-CoA} \\
\text{Biotin Deficient} & \rightarrow \text{3-Hydroxyisovaleric acid & 3-Methylcrotonyl glycine}
\end{align*}
\]


**Figure 3:** Biotin-dependent carboxylases in mammals. ACC—acetyl-CoA carboxylase; PC—pyruvate carboxylase; PCC—propionyl-CoA carboxylase; MCC—β-methylcrotonyl-CoA carboxylase.
**Biotin-dependent carboxylases in mammalian tissues.**

Endogenous biotin has been identified immunohistochemically in rat liver[7], human pancreas[6], rat kidney, rodent lung [7], human salivary glands[6], human skeletal muscle [7], mouse Bergmann glial cell[6] and rat adipose tissues [7]. The localization pattern of endogenous biotin has been consistent in all tissues. PC has been localized predominantly in mitochondria of the rat liver. Its significance there includes regulation of flow of carbons between the mitochondria and cytosol during metabolism in mammals. Mitochondrial PC also indicate its role in the regulation of metabolites for gluconeogenesis and lipogenesis[7]. The presence of biotin dependent proteins in the Bergmann glial cells but not neurons suggest these proteins play an important role in metabolism of Bergmann glial cells in relation to the Purkinje cells.[6]

**Role of biotin in spermatogenesis.**

In mammals, spermatogenesis is dependent primarily upon testosterone. It produced by Leydig cells and acts on seminiferous tubules to drive spermatogenesis. Previous studies [5] have shown that biotin deficiency affects spermatogenesis, causing delayed spermatogenesis, a decreased number of spermatozoa, reduced testicular and serum concentration of testosterone and a sloughing of seminiferous tubule epithelium. Biotin improves testosterone levels, probably because it participates in the synthesis of local testicular factors like luteinizing hormone and follicle stimulating hormone, which are required in addition to testosterone for the normal interaction among Leydig, Sertoli and peritubular cells[5]. Addition of biotin or biotin with testosterone, to biotin-deficient
rats, restored normal spermatogenesis. This finding suggests that biotin influenced formation of local testicular factors are required in addition to testosterone and follicle-stimulating hormone for the normal interaction among Leydig, Sertoli and peritubular cells. The study also shows the requirement of biotin for the synthesis of testicular proteins. [4]

**Role of biotinylated proteins in sperm metabolism**

There is controversy about the pathways that operate for energy production in spermatozoa. Glycolysis seems to be the obvious pathway [9]. But if glycolysis is essential how are sperm motile in a glucose free media? Whether gluconeogenesis takes place in the spermatozoa is still unknown and controversial. Some researchers believe that glycogen is lost during the spermatocyte stage of spermatogenesis [9] and that gluconeogenesis does not take place in the spermatozoa. However recent studies show the presence of glycogen in spermatozoa and that gluconeogenesis also occurs in spermatozoa. The evidence for glycogen together with measurable activities of glycogen synthetase and glycogen phosphorylase has been shown in dog, ram, boar and horse sperm [9].

Gluconeogenesis begins in the mitochondria with the formation of oxaloacetate through carboxylation of pyruvate. This reaction which increases the carbon chain length from three to four is catalyzed by pyruvate carboxylase. This enzyme is stimulated by high levels of acetyl-CoA. Oxaloacetate is reduced to malate using NADH, a step required for its transport out of the mitochondria. Malate is then oxidized back to oxaloacetate using NAD$^+$ in the cytoplasm. The remaining steps of gluconeogenesis
occur in the cytoplasm. Oxaloacetate is first decarboxylated and then phosphorylated to form phosphoenolpyruvate by the key enzyme phosphoenolpyruvate carboxykinase (PEPCK). This is an important rate limiting step in gluconeogenesis. The next steps in the reaction are the same as those in glycolysis occurring in reverse. The main reactions of gluconeogenesis is shown in figure 4. Demonstration of the presence of acetyl-CoA carboxylase, pyruvate carboxylase (biotinylated proteins) and PEPCK, which are key enzymes in gluconeogenesis, would strengthen the argument that gluconeogenesis occurs in sperm.

http://themedicalbiochemistrypage.org/gluconeogenesis.html

**Fig 4.** Relevant reactions of gluconeogenesis are depicted here.
CHAPTER II

AIMS

Although the importance of biotin and thereby the importance of biotinylated proteins has been reported for spermatogenesis, there have been virtually no reports on the presence or function of biotinylated proteins in spermatozoa. The purpose of this study was to identify and localize endogenous biotin-containing proteins in mammalian spermatozoa. Since biotin dependent proteins play an important role in regulating metabolism in other tissues, another aim was to understand the function of biotinylated proteins in sperm metabolism.

Are biotinylated proteins present in sperm?

Due to the functional importance of biotin-dependent carboxylases in other tissues[5][6][7], I expected that biotinylated proteins should be present in the sperm. Western blotting analysis of sperm samples with Avidin-HRP should detect biotinylated proteins at positions corresponding to molecular weights of previously characterized biotin-dependent carboxylases.

Are the biotinylated carboxylases present in sperm from other species?

Studies have shown the presence of not only BPL gene across species but the biotin dependent enzymes are ubiquitous in nature[11]. We hypothesized that the biotinylated carboxylases should be present in sperm from several species because their unique role in metabolism.
What are biotinylated proteins?

Biotinylation of proteins is a rare modification and only five biotinylated proteins are found in different organisms [10]. Hence any biotinylated protein if found in sperm should correspond to one of these previously identified biotinylated proteins with their characteristic molecular weights. Thus correspondence of the bands in western blots detected by specific anti-bodies for the carboxylases and the bands detected by Avidin-HRP probed blots should identify these proteins. Isolating biotinylated proteins using streptavidin bead pull down method followed by western blotting should further confirm the identity of these proteins in sperm extracts.

Where are the biotinylated proteins localized in the sperm?

Previous studies have shown localization of ACC in cell cytosol and PCB and PCCA are present in the mitochondria[6] so we hypothesized that majority of the biotinylated proteins should be present in the sperm mitochondria which is located in the mid-piece of the sperm. Immunocytochemistry should show staining primarily in the mid-piece. When head and mid-piece-tail are separated, the expected proteins should be detected only in the mid-piece-tail extracts because the mitochondria in present in the mid-piece.

Are the biotinylated proteins involved in the gluconeogenesis pathway?

Detection and identification of biotinylated protein pyruvate carboxylase suggests it plays a role in gluconeogenesis in sperm because it is a key enzyme involved in the synthesis of phosphoenolpyruvate (PEP) from pyruvate during gluconeogenesis, in other
tissues. Detection of PEPCK in the sperm would further strengthen the hypothesis of gluconeogenesis occurrence in the sperm as PEPCK is the enzyme responsible for the rate controlling step for gluconeogenesis.
CHAPTER III
MATERIALS AND METHODS

Sperm Protein Extraction

Testes of mature bulls were obtained from a local slaughter house, Kent, OH. Caudal sperm from bull and other species (mouse, rat and hamster) were collected from the distal caudal epididymis. Sperm were washed in CESD (100 mM NaCl, 40 mM KCl, 20 mM Tris-HCl, pH 7.4), for 5 minutes at 500 x g and then for 5 minutes at 1150 x g. After the second wash they were then resuspended in a homogenization buffer (10 mM Tris, pH 7.2; 1 mM EDTA; 1mM EGTA; 0.1 mM sodium orthovanadate) and incubated for 20 minutes. The suspended sperm was then spun down at 16,000 x g for 20 minutes. The supernatant, which is the soluble protein containing fraction of sperm, was boiled after addition of 6x sample buffer and used for Western Blot analysis.

The pellets of sperm from different species were prepared in four different conditions to test which condition resulted in maximum extraction of the insoluble mitochondrial proteins of interest.

For the first condition, sperm pellet was resuspended in HB+ buffer and sonicated for 6s bursts, three times. The sonicated sample was then centrifuged for 20 minutes at 16,000xg. Second, the sperm pellet was resuspended in HB+ buffer for 30 minutes followed by centrifugation for 20 minutes at 16,000xg. Third, the sperm pellet was
resuspended in 1% SDS for 15 minutes followed by centrifugation for 20 minutes at 16,000x g. Fourth, the sperm pellet was resuspended in RIPA+ buffer 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGT, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM b-glycerophosphate, 1 mM Na₃VO₄, 1 µg/ml leupeptin for 15 minutes followed by centrifugation for 20 minutes at 16,000x g. The supernatants of all four samples were boiled with 6x sample buffer.

The boiled supernatants were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a Bio-Rad Mini Protean electrophoresis chamber. Samples were loaded in 10% polyacrylamide gels and run at 200 volts for an hour.

**Western Blot**

Proteins were electrophoretically transferred from the gel to polyvinylidene fluoride membranes (Millipore Corp.). The membranes were blocked with 5% non-fat dry milk in Tris buffered saline containing Tween (TTBS: 25 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.4). It was blocked with milk because proteins are not present in all parts of the membrane. Proteins in milk bind to all those parts of the membrane to prevent non specific antibody binding. The blots were incubated with the primary antibody in dilutions directed by the manufacturer, over night at 4 degrees celcius. Antibodies were obtained from Novus, Santa Cruz and Bio-RAD. Antibodies were diluted in 5% milk/TTBS solution. Blots were washed 3 times in TTBS for 10 minutes. The blots were then incubated with their corresponding anti-mouse or anti-rabbit secondary antibody conjugate to horse radish peroxidase(Amersham) for one hour at
room temperature. Blots were washed again three times for 10 minutes each. Blots were developed with the use of ECL chemiluminescence kit (Amersham) and Fuji dark box. Table 1 shows all the antibodies used for western Blot.

### Table 1: Antibodies used for Western Blot

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Dilution</th>
<th>Secondary-AB</th>
<th>Detects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avidin-HRP</td>
<td>Bio-Rad</td>
<td>1:1000</td>
<td>N/A</td>
<td>Biotin</td>
</tr>
<tr>
<td>PCB</td>
<td>Santa Cruz Biotechnology, Inc</td>
<td>1:500</td>
<td>Anti-Goat</td>
<td>Pyruvate Carboxylase</td>
</tr>
<tr>
<td>PCCA</td>
<td>Novus</td>
<td>1:500</td>
<td>Anti-mouse</td>
<td>Propinyl coA carboxylase</td>
</tr>
<tr>
<td>PEPCK</td>
<td>Santa Cruz Biotechnology, Inc</td>
<td>1:200</td>
<td>Anti-Goat</td>
<td>Phosphoenolpyruvate carboxykinase</td>
</tr>
</tbody>
</table>

**Preparation of sperm head and tail fractions**

Sperm heads and tails were obtained by sonication and sucrose gradient centrifugation [12] Bull caudal sperm washed with CESD(100 mM NaCl, 40 mM KCl, 20 mM Tris-HCl, pH 7.4) were resuspended in PBS and sonicated 3 time in bursts of 30s each. This resulted in the detachment of the mid-piece-tail from the head. The sucrose gradient was made by weighing out appropriate quantities of sucrose and dissolving in water to produce 1.80M, 2.05M and 2.20M sucrose solution. 0.5mL of each solution was layered in centrifuge tubes starting with 2.20M solution at the bottom, 2.05M solution in the middle and 1.80M solution on top. 1mL of sonicated sperm solution was gently added on the top of the sucrose gradient. It was then centrifuged for 40minutes at 100,000 x g. Most of the mid-piece-tail fraction was found in the 1.80M
interface and the 2.20M interface contained mostly heads. Purity was assessed by light microscopy. Separated fractions were subsequently resuspended in 1% SDS for 5 minutes to extract the mitochondrial proteins followed by centrifugation at 16000 x g for 20 minutes.

**Immunocytochemistry**

Caudal sperm was washed with phosphate-buffered saline and fixed in 4% formaldehyde. 0.5% triton was added to permeabilize the sperm cells. The sperm cells were spread on a poly-L-lysine coated slide and dried. The slides were washed three times with TTBS for 10 minutes and blocked overnight with 2.5% bovine serum albumin (BSA) and goat serum. The slides were then washed three times for 10 minutes each and incubated with the recommended dilutions of primary antibody in goat serum (GS)/BSA/TTBS for 5 hours at room temperature. Control slides for avidin-HRP were incubated with GS/BSA/TTBS without the antibody. Control slides for PCB and PPCA were incubated with GS/BSA/TTBS with the secondary antibody. Slides were then washed three times with TTBS. Both control and experimental slides were incubated for an hour with corresponding fluorescence conjugated secondary antibodies. After washing the slides, they were mounted and viewed using FluoView 500 fluorescence microscope (Olympus, Center Valley, PA). Table 2 list all the antibodies used in immunocytochemistry.
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Dilution</th>
<th>Secondary AB</th>
<th>Detects</th>
<th>Control Slide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy3-conjugated-Avidin</td>
<td>Rockland Inc.</td>
<td>1:1000</td>
<td>N/A</td>
<td>Biotin</td>
<td>GS/BSA/TTBS/Water</td>
</tr>
<tr>
<td>PCB</td>
<td>Santa Cruz Biotechnology, Inc</td>
<td>1:200</td>
<td>Anti-goat-Fluor</td>
<td>Pyruvate carboxylase</td>
<td>GS/BSA/TTBS/Sec AB</td>
</tr>
<tr>
<td>PCCA</td>
<td>Novus</td>
<td>1:200</td>
<td>Anti-mouse-Cy3</td>
<td>Propionyl-coA carboxylase</td>
<td>GS/BSA/TTBS/Sec AB</td>
</tr>
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**Streptavidin Pull Down**

"Pull-down" is a small-scale affinity purification technique similar to immunoprecipitation (IP), except that the antibody is replaced by some other affinity system. In this case, the affinity system is the well-known and specific biotin-streptavidin interaction.

Sperm extracts were made as described above. The 50µl of streptavidin agarose beads (Thermo Scientific) were washed three times with Tris-buffered Saline (Thermo Scientific). Sperm extracts (1ml) were incubated with the 50µl beads for 3 hours at 4 degrees Celsius with rotation. Control tubes contained streptavidin agarose beads and water. The beads-extracts mix was spun down at 16,000 x g for 5 minutes. The supernatant was saved as flow-through and the beads were washed 3 times with PBS. Beads were then boiled in 100 µl of 1% SDS for 10 minutes at 100 degrees Celsius.
according to Invitrogen guidelines. The boiled sample was centrifuged at 1000 x g for 10 minutes and supernatant was collected for western blotting.
CHAPTER IV

RESULTS

Are biotinylated proteins present in sperm?

In figure 4 Bull caudal sperm extracts were made in various buffers including HB+, 1% SDS and RIPA. The HB+ buffer extracts only soluble proteins whereas 1% SDS and RIPA can extract both soluble and insoluble proteins including mitochondrial proteins. These extracts were subject to gel electrophoresis followed by analysis with Avidin-HRP. Three bands are visible in lanes 3 and 4 at 240kDa, 130kDa, 72kDa showing the presence of biotinylated proteins in the sperm. These molecular weights correspond to three biotin-dependent carboxylases, Acetyl-CoA carboxylase (240kDa), pyruvate carboxylase (130kDa) and propionyl-CoA carboxylase (72kDa) characterized in other tissues. There was no band detected at 61kDa which corresponds to methylcrotonyl-CoA carboxylase, a fourth biotinylated protein usually found in tissues. Lanes 1 and 2 did not contain all three bands because these extracts contained only soluble proteins and whereas most of the biotinylated proteins are insoluble. All three biotinylated carboxylases are likely mitochondrial-insoluble proteins and thus present in the insoluble fractions (Lanes 3 and 4).
Fig 4. Western blot of bull caudal sperm treated with different extraction buffer to optimize extraction conditions, and identify the presence of endogenous biotinylated proteins in the sperm Avidin-HRP(1:1000).

Lane 1: Soluble Fraction extracted with HB+ after sonication of sperm.
Lane 2: Soluble Fraction extracted with HB+, no sonication.
Lane 3: Pellet re-suspended in 1% SDS
Lane 4: Pellet re-suspended in RIPA.
Are the biotinylated carboxylases present in sperm from other species?

Next we examined if sperm from different mammals also contained biotinylated proteins. In figure 5 extracts of sperm from hamster, rat, mouse and bull were made in 1% SDS and the extracts were subjected to SDS-PAGE. Two of the three biotinylated proteins corresponding to Pyruvate Carboxylase(130kDa) and Propinyl-CoA Carboxylase(72 kDa) are seen in sperm from all the species tested. The difference in amounts of proteins that were present could be due to differences in anti-body reactivity between species. The reason the 170 kDa band was not detected is not known.

Fig 5. Western blot of caudal sperm of different species probed with Avidin-HRP(1:1000)
Identities of the biotinylated proteins.

To confirm the identity of the two mitochondrial biotinylated proteins detected by Avidin-HRP we used specific antibodies against these proteins. Six blots from the same gel, were transferred onto a pvdf membranes and cut into 3 strips. In figure 6, the first strip was probed with Avidin-HRP, the second with Pyruvate Carboxylase antibodies and third with Propinyl-CoA Carboxylase antibodies. It can be seen that Pyruvate Carboxylase in Lanes 3 and 4 corresponds to the 130kDa band detected by avidin HRP and Propinyl-CoA Carboxylase corresponds with the 72kDa band detected by avidin-HRP.
Fig 6. Western blot of mouse and bull caudal sperm, identifying the possible proteins with its specific antibodies. Lane 1 and 2 probed with Avidin-HRP (1:1000), Lane 3 and 4 probed with Pyruvate Carboxylase antibody (1:500) and Lanes 5 and 6 probed with Propinyl Coa Carboxylase antibody (1:500). (**Pyruvate Carboxylase, * Propinyl Coa Carboxylase)
To further confirm that the antibodies for pyruvate carboxylase and propinyl CoA carboxylase were specifically binding to the biotinylated proteins detected by avidin HRP we use strepavidin pull down of sperm extracts. Strepavidin pull down protein were analyzed by Western blot (Figure 7). Lanes 1 to 4 were probed with PCCA (1:500) and lanes 5 to 8 were probed with PCB (1:500). Strong bands due to PCCA and PCB in lanes 2 and 6 indicate that most of the biotinylated proteins bound to the beads. Lanes 4 and 8 were control; beads incubated with water. This blot confirms that antibodies detecting proteins at 130kDa and 72kDa are indeed pyruvate carboxylase and propinyl CoA carboxylase respectively.

Fig 7. Proteins from Strepavidin pull-down shows are PCB and PCCA. Proteins isolated by strepavidin pull down as described under methods were analyzed by western blot and probed with PCCA and PCB antibodies.
Where are the biotinylated proteins localized within sperm?

To determine if the insoluble proteins detected are localized to the sperm mitochondria, we purified head-tail preparations. This was done by sonicating sperm and separating the fractions with glucose density gradient a method that can isolate head and tail fraction (Figure 8). Since 2 out of the 3 biotinylated proteins detected are normally found in mitochondria in other tissues, we were also able to confirm this in the spermatozoa. Accordingly Pyruvate Carboxylase(130kDa) and Propinyl Coa Carboxylase(72 kDa) were detected in significant amounts in the tail fractions which contain the mitochondria and with little contamination from sperm heads. The faint band at 130kDa in the head fraction is due to a 3% contamination with tails.
Next we used immunocytochemistry to determine localization of biotinylated proteins within the spermatozoa. Formaldehyde-fixed bovine and mouse sperm were treated with Avidin-Cy3 (Fig 9, 10), PCB-DyLight(Fig 11) and PCCA-Cy3 (Fig 12) antibodies. In both mouse and bovine spermatozoa we observed, the majority of the biotinylated proteins are present in the mid-piece-tail region. Biotinylated proteins seem to be absent in the head of the spermatozoa.
Fig 9. Fluorescence cytochemistry showing the localization of the biotinylated protein in the mid-piece with Avidin-Cy3 in Bull Sperm.
Fig 10. Fluorescence cytochemistry showing the localization of the biotinylated protein in the mid-piece with Avidin-Cy3 in Mouse Sperm.

Fig 11. Immunocytochemistry showing the localization of Pyruvate Carboxylase in the mid-piece of mouse sperm. Fixed sperm were developed with antibodies to pyruvate carboxylase as described under methods. Left panel (Fluorescence) right panel (Bright Field)
Fig 12. Immunocytochemistry showing the localization of Propionyl CoA Carboxylase in the mid-piece and principal pieces of mouse sperm. Fixed sperm was reacted with PCCA antibodies followed by secondary antibodies conjugated to Cy3. Left Panel (Fluorescence) Right Panel (Bright field).
Are the biotinylated proteins involved in the gluconeogenesis pathway?

Since we detected pyruvate carboxylase, a key enzyme in gluconeogenesis, we wanted to see if PEPCK, another important enzyme in the pathway, is present in spermatozoa. The PEPCK antibody was able to detect significant amounts of PEPCK in both the cytosol and mitochondria of bull sperm.

Fig 13. Western blot of bull sperm extracts (soluble and insoluble fractions) in different buffers. Lanes on the left of PPM is the soluble fraction and to the right are pellets of insoluble mitochondrial proteins in 1% SDS. The blot was probed with PEPCK antibody (1:200).
CHAPTER V

DISCUSSION

Biotin is essential and plays a key role in cell metabolism [2]. Defects in BPL, the enzyme that conjugates biotin to proteins, lead to Multiple Carboxylase Deficiency (MCD) which leads to loss of functions carried out by biotin carboxylases. Patients’ suffer from seizures, reduced muscle tones and impaired immune system [10]. Previous studies have also shown that biotin regulates the expression of the catabolic carboxylases PCC and MCC at the posttranscriptional level [16]. Biotinylated pyruvate carboxylase is absolutely essential for gluconeogenesis.

The role of gluconeogenesis in sperm has been debated. The only evidence of gluconeogenesis is documented in dog sperm [9]. However, sperm from many species, including human, remain motile in glucose-free media. Another study postulated that mitochondrial energy is possibly used to drive gluconeogenesis and provide glucose for glycolytic energy production in the flagellum [9]. Other studies looking at boar sperm were unable to detect any gluconeogenesis metabolites [9]. The identification of enzymes involved in gluconeogenesis could suggest a possible role for it in sperm energy production.

In this study we have used principles of avidin-biotin technology to detect, identify and localize the endogenously biotinylated proteins in sperm because the affinity of
avidin for biotin is one of the strongest interactions ($K_a=10^{-15} \text{ M}$) known in biology[11]. Although avidin is conjugated to reporter groups like enzymes, fluorescence and radiolabels, its biotin binding affinity stays unaffected [11]. Our results, for the first time unequivocally demonstrated the presence of endogenously biotinylated protein in mammalian spermatozoa.

SDS-PAGE separation of sperm protein extracts probed with avidin-HRP showed three distinct bands representing biotinylated proteins. In mammals, four biotinylated proteins have been identified and their corresponding molecular weights have been determined. These are acetyl-coA carboxylase (170 kDa), pyruvate carboxylase (130kDa) propionyl-coA carboxylase (72kDa) and methylcrotonyl-CoA carboxylase (61kDa). All three of the bands were of molecular weights of previously known proteins; acetyl-coA carboxylase (170 kDa), pyruvate carboxylase (130kDa) and propionyl-coA carboxylase (72kDa). SDS-PAGE followed by immunoblotting with specific antibodies for pyruvate carboxylase and propionyl-CoA carboxylase showed bands at 130kDa and 72kDa respectively. The positions of these bands corresponded to the positions of the bands detected by avidin-HRP. The antibodies detected bands that correlated to the bands seen with avidin conjugated with HRP. To further determine the identity of the bands detected by avidin-HRP and specific anti-bodies, we isolated all the sperm biotinylated proteins using streptavidin pull-down. SDS-PAGE analysis of the proteins pulled down by streptavidin followed by western blot with avidin-HRP and with specific antibodies confirmed the presence of pyruvate carboxylase and propionyl-CoA carboxylase in sperm. In summary, we confirmed that the biotinylated proteins present in sperm are
acetyl-coA carboxylase (170 kDa), pyruvate carboxylase (130kDa) and propionyl-coA carboxylase (72kDa).

In order to determine where the biotinylated proteins are present in sperm extracts were made from head and tail fractions of bull spermatozoa. These extracts were analyzed for the presence of the biotinylated proteins using SDS-PAGE and immunoblotting using specific antibodies. This experiment was performed to provide insight into the localization of these enzymes. Propionyl-CoA carboxylase and pyruvate carboxylase were detected in the tail fraction. This suggests that these enzymes could be present in the mid-piece region of the tail where the mitochondria are present. There was a faint band in the head fraction when probed with anti-pyruvate carboxylase. This was due to contamination of the head-fraction with tail mid-piece fractions.

Determining the localization of a protein helps in elucidating its function. In sperm, proteins involved in metabolism and providing energy are localized primarily in the mitochondria. Immunocytochemistry was used to determine the localization of the identified proteins. Fluorescence was detected in the mid-piece of sperm when probed with specific antibodies and avidin-Cy3. The mid-piece of sperm contains tightly coiled mitochondria and the primary site of energy production. A weak signal was detected in head when probed with anti-pyruvate carboxylase.

Pyruvate carboxylase is involved in converting pyruvate to oxaloacetate in the first step of gluconeogenesis in other tissues. The presence of pyruvate carboxylase supports the theory that gluconeogenesis could take place in sperm. The next logical step
was to detect if any of the other enzymes involved in gluconeogenesis were present in sperm. Using specific antibodies for PEPCK we were able to detect its presence. Since PEPCK plays a role in the rate limiting step of gluconeogenesis we decided to determine its presence. The presence of pyruvate carboxylase, propionyl-coA carboxylase and PEPCK in sperm makes a strong case for a role of gluconeogenesis in sperm.

Future studies could purify and sequence the biotinylated proteins in sperm for irrefutable evidence of their identity. The absence of the fourth biotinylated protein methylcrotonyl-CoA carboxylase could be further confirmed by using specific antibodies in western blot and immunocytochemistry.

The occurrence of gluconeogenesis in bull, mouse and other mammalian sperm could be further tested by NADP\(^+\) NADHP activity assays. The coenzyme NADP and NADPH are essential for gluconeogenesis. Determination of the presence of other key gluconeogenic enzymes such as fructose-1,6 bisphosphatase and aldolase B should provide conclusive evidence for the role of pyruvate carboxylase and for gluconeogenesis occurs in spermatozoa.

In summary my work has conclusively established, for the first time, the presence of biotinylated proteins, acetyl-coA carboxylase (170 kDa), pyruvate carboxylase (130kDa) and propionyl-coA carboxylase (72kDa) in spermatozoa. Further studies will be required to determine the role of these enzymes during sperm maturation and function and contribute to our understanding of male gamete function. These studies will enable
us to better understand metabolic pathways in spermatozoa. It is possible that defects in these pathways could be a cause in male infertility.
References


