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STUDIES OF ENDOGENOUS BIOSYNTHESIS OF LIPOIC ACID COFACTOR IN HUMANS: EXPRESSION, PURIFICATION, AND CHARACTERIZATION OF LIPT1 AND LIPT2 ENZYMES

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ABSTRACT

Studies of Endogenous Biosynthesis of Lipoic Acid Cofactor in Humans: Expression, Purification and Characterization of LIPT1 and LIPT2 Enzymes

Lipoic acid is a cyclic disulfide that plays a role in intermediate metabolism. It exists predominantly in the form of its conjugate base, lipoate, under physiological conditions. In humans, lipoate is essential for five redox reactions, four 2-oxoacid dehydrogenases and the glycine cleavage system. In this study, two enzymes, LIPT1 and LIPT2, were investigated. LIPT2 transfers octanoic acid from ACP to GCSH and LIPT1 transfers lipoic acid from GCSH to DLST. GCSH catalyzes the degradation of glycine and mutations in the gene are linked to hyperglycemia. Mutations in DLST have been linked to the development of Alzheimer's in elderly populations. DLST is also an essential part of the Kreb's cycle, therefore mutations in the gene can lead to issues converting ADP to ATP. The primary goal of the study was to overexpress and purify the genes involved in the human lipoic acid biosynthesis pathway so that the activity of LIPT1 and LIPT2 could be observed *in vitro*. In order to characterize the activity of LIPT1 and LIPT2, both genes along with DLST, AaS, ACPS1, Ulp1, and ACP were cloned into dwpSUMO and pET28a plasmids using DH5a *E. coli* cells. Afterwards, the plasmids were transformed into BL21-DE3 *E. coli* cells, overexpressed, and purified. All of the genes of interest were successfully cloned into the dwpSUMO and pET28a plasmids and transformed into BL21-DE3 *E. coli* cells. AaS and Ulp1 were successfully overexpressed and purified. Preliminary results displayed separation between the standards for holo-ACP and acyl-ACP. However, the separation between the peaks corresponding to the holo- GCSH and lipoyl- GCSH was inadequate. Therefore, the HPLC method requires additional alterations before assays can be

conducted. Future directions include overexpressing DLST, ACPS1, and ACP as well as monitoring the lipoic acid transfers by LIPT1 and LIPT2 via HPLC analysis.

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Chapter 1

Significance of Lipoic Acid

Lipoic Acid is a cyclic disulfide that plays a role in intermediate metabolism. It exists predominantly in the form of its conjugate base, lipoate, under physiological conditions. In humans, lipoate is essential for five redox reactions, four 2-oxoacid dehydrogenases and the glycine cleavage system. All these redox reactions are involved in energy and amino acid metabolism. Lipoic acid also is an important antioxidant.[1]

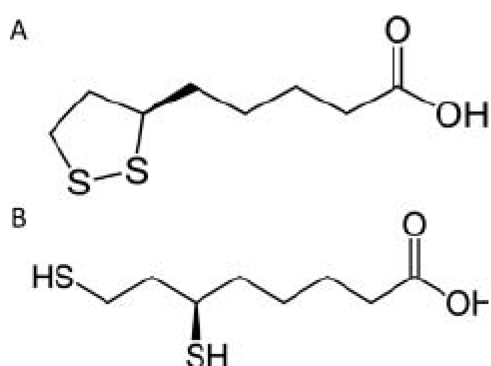

Figure 1.1 Image of lipoate (A) and lipoic acid (B).

There are two forms of lipoic acid, the R and S enantiomers. Only the R enantiomer exists in nature and it is used in aerobic respiration[2].

Biological Sources and Synthesis

Lipoic acid is present in several foods, namely kidneys, hearts, livers, spinach, broccoli, and yeast extracts. Unfortunately, lipoic acid is typically bound and therefore not available to the consumer and the amount of lipoic acid in dietary sources is typically relatively low[3]. Since diet is not a reliable source of lipoic acid, it is mainly obtained via endogenous biosynthesis.

In humans, lipoic acid can be synthesized by the body in the mitochondria. Lipoate synthesis in the mitochondria is a hijack of the fatty acid synthesis pathway, everything is the same until the octanoyl-acyl carrier protein after there are three lipoate specific steps. First, octanoic acid is transferred to glycine cleavage H protein (GCSH) by lipoyl-transferase 2 (LIPT2). A radical-SAM enzyme called LIAS then synthesizes lipoate while attached to GCSH. LIAS removes one of the sulfurs from its 4-iron 4-sulfur cluster and attaches it onto octanoic acid therefore generating lipoic acid[4].

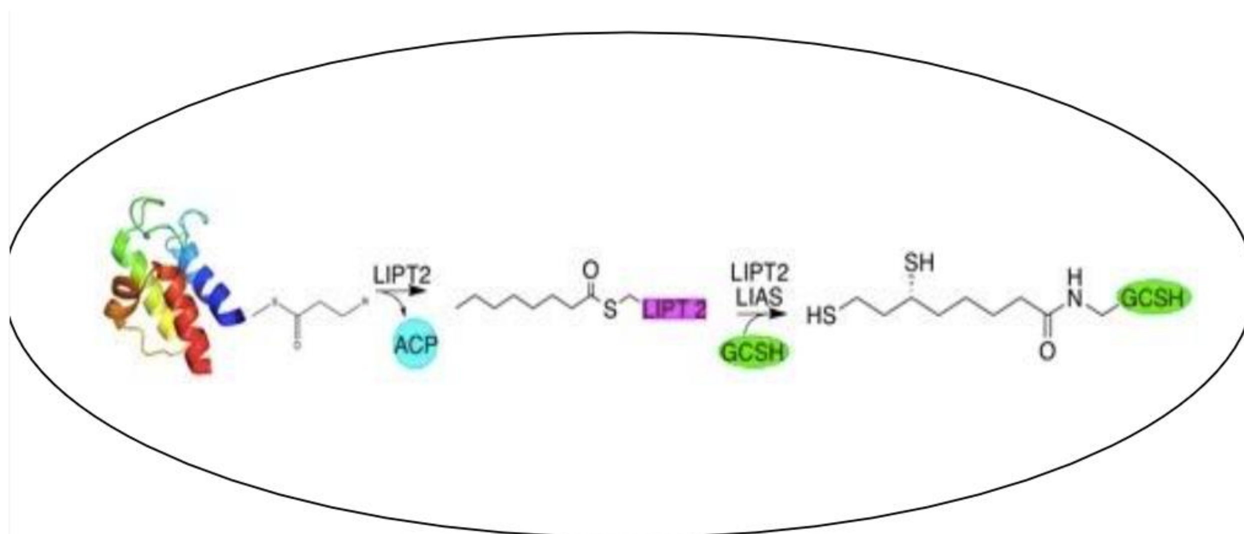

Figure 1.2. Image of the synthesis of lipoic acid by LIAS and transfer from acyl-carrier protein (ACP) to Glycine Cleavage System H-protein (GCSH) by LIPT2.

Lipoic acid is then transferred to dihydrolipoamide S-Succinyltransferase (DLST) by lipoyl-transferase 1 (LIPT1)[4].

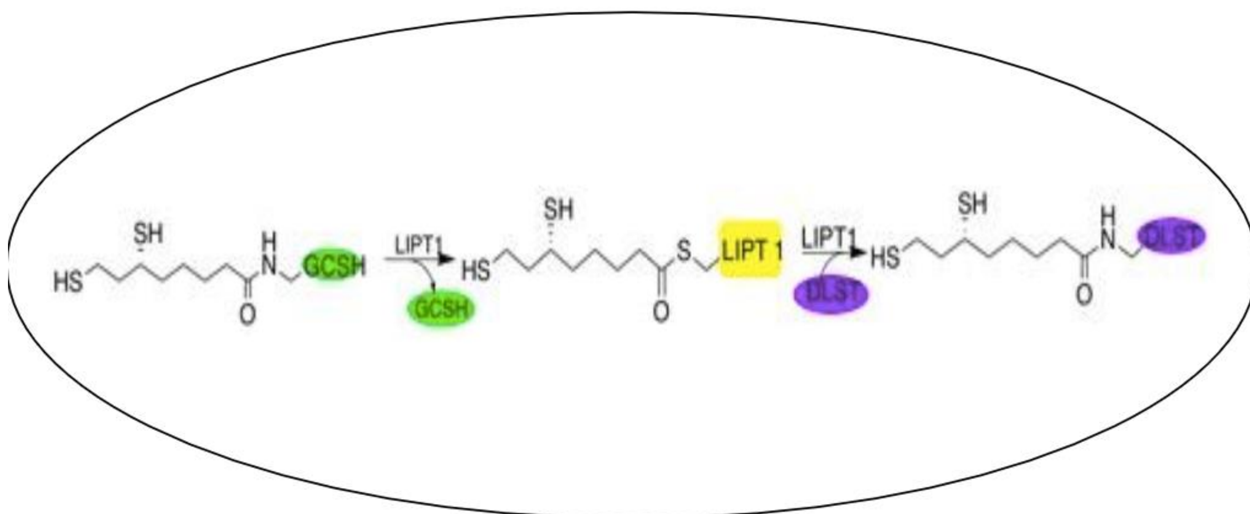

Figure 1.3. Image of transfer of lipoic acid from GCSH to DLST by LIPT2.

Generation of LIPT2 substrate

Unfortunately, the substrate for LIPT2, octanoic-ACP, is not commercially available so to study the lipoic acid biosynthesis pathway the substrate must be synthesized first. Apo-ACP is the unmodified form of acyl carrier protein and is the precursor to holo-ACP. The enzyme holo-ACP synthase (ACPS1) catalyzes the reaction that creates holo ACP from apo ACP. Apo-ACP contains a serine, specifically serine 6, which attaches to phosphopantetheine to generate holo-ACP. Then Acyl ACP Synthetase (AaS) adds a carboxylate group to holo- ACP thus forming a thioester bond and generating Acyl-ACP the substrate for LIPT2.[5]

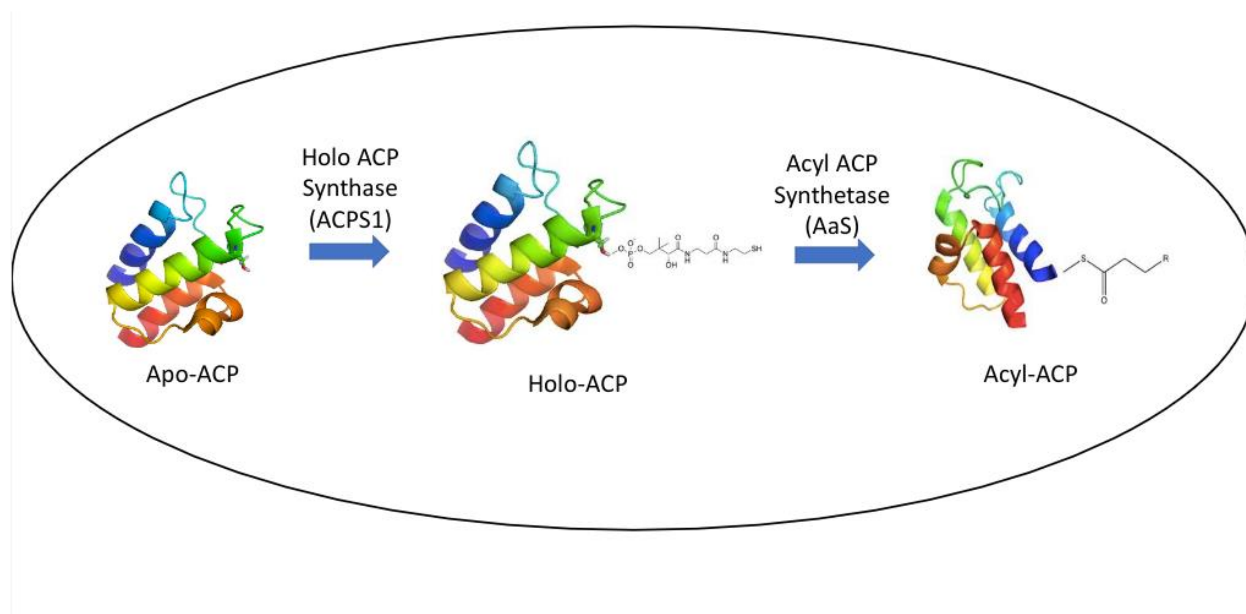

Figure 1.4. Holo As pCP synthase (ACPS1) attaches phosphopantetheine to serine 6 on apo-ACP thus forming holo-ACP. Then acyl-ACP synthetase (AaS) attaches a carboxylate group to holo-ACP which creates a thioester bond and generates acyl-ACP, the substrate for LIPT2.

Lipoic Acid Mutations

As previously discussed, lipoic acid is an essential cofactor in intermediate metabolism and as an antioxidant. It is also involved in enzyme systems associated with the Kreb's or Citric Acid cycle which is involved in energy metabolism. Therefore, mutations in genes that code for enzymes involved in the lipoic acid biosynthesis pathway can cause issues with energy metabolism. Enzymes such as GCSH are also linked to other pathways and mutations in these genes can have several effects outside of energy metabolism. GCSH is linked to the glycine cleavage system which is involved in the breakdown of glycine. Therefore, deficiencies in GCSH can be linked to hyperglycemia. Mutations in LIAS, the radical-SAM enzyme that generates lipoic acid from octanoic acid, results in a variant form of nonketotic hyperglycemia with early onset convulsions.[6]

Project Objectives

The overall objectives of this research were to overexpress, purify, and characterize LIPT1 and LIPT2 enzymes. A literature review was also performed to further understand the physiological importance of LIPT1 and the role that lipoic acid research plays in drug development. To accomplish these goals, there were 3 aims.

Aim 1: Synthesize all enzymes and proteins needed to obtain acyl-ACP.

Aim 2: Overexpress and purify all enzymes in the human lipoic acid biosynthesis pathway.

Aim 3: Observe transfers via HPLC analysis.

Aim 4: Perform a literature review on the effects of LIPT1 mutations and lipoic acid related drug development.

Materials and Methods

Materials- All genes were synthesized at GeneArt. Gene Ulp1 cloned in pET28a was gifted to the Booker lab by Dr. Craig Cameron. Restriction enzymes (NdeI and XhoI) and cloning materials were obtained from New England Biolabs (Ipswich, MA). Plasmid Miniprep kits were purchased from Macherey Nagel (Dueren, Germany). Kanamycin, isopropyl β-ᴅ-thiogalactopyranoside (IPTG), Tris (2- carboxyethyl) phosphine (TCEP-HCl), dithiothreitol (DTT), and ampicillin were obtained from Gold Biotechnology (St. Louis, MO). DNAse I and β-mercaptoethanol (BME) were purchased from Sigma Co. (St. Louis, MO). Ni-NTA resin column for protein purification was purchased from Qiagin. HEPES (sodium salt) and potassium chloride (KCl) were purchased from Dot Scientific (Burton, MI). Imidazole and lysozyme were

obtained from Alfa Aesar (Haverhill, MA). Bradford Protein Assay Reagent was from Amresco (Fountain Parkway Solon, OH). Bovine Serum Albumin (BSA) used for the Bradford Protein Assay standard was from Thermo Fisher Scientific (Waltham, MA). All chemicals and reagents were of the highest grade possible.

General Procedures- All genes were cloned into pET28a and dwp-SUMO plasmids. The plasmids containing the gene of interest were then transformed into chemically competent DH5α-BL21DE3 cells. Three colonies were selected and grown overnight prior to extracting plasmid DNA using the plasmid miniprep protocol (Macherery Nagel). The isolated DNA was then submitted for sequencing to confirm that the gene of interest was successfully isolated. After sequencing, three colonies each containing the gene of interest were selected for an expression study in order to select the ideal colony for protein overexpression.

Overexpression and Purification of Homo Sapien DLST, LIPT2, ULP1, Lipoamidase, ACPS1, AaSH, and LIPT1- A single colony was used to incubate a 300 mL starter culture of *Escherichia coli* BL21-DE3 cells containing a pet28a plasmid with the gene of interest (*H. sapien* DLST-pet28a, *H. sapien* LIPT2-pet28a, *H. sapien* LIPT1-pet28a, *H. sapien* ULP1-pet28a, *H. sapien* Lipoamidase-pet28a, *H. sapien* ACPS1-pet28a, or *H. sapien* AaSH-pet28a). The overexpression was performed in 12 L (4 x 3 L) of M9 minimal Media and LB Media, preincubated at 37 degrees celsius. The cells were grown at 37 degrees celsius and shaken at 180 rpm. At an OD_{600} of 0.6 the cells were induced with IPTG at a concentration of 0.2 mM and the temperature was dropped to 18 degrees celsius. The following day, the cells were harvested via centrifugation at 6,000 xg for 12 minutes and the cell paste was frozen with liquid nitrogen then stored at -80

degrees celsius. Samples were collected prior to induction and during harvesting for SDS PAGE gel.

The cell pellet was resuspended in 150 mL of lysis buffer (50 mM HEPES pH 7.5, 200 mM KCl, 10 mM Imidazole, and 4 mM TCEP. HCl). Lysozyme at a concentration of 1 mg/mL, DNAse at a concentration of 0.1 mg/mL, and protease inhibitor were added to the cell paste solution. Once thawed, the cell paste solution was sonicated for a total of 7 minutes at 35 seconds/ sonication burst at a setting of 7 and at 2-minute intervals. Following sonication, the solution was centrifuged at 50,000 xg for 1 hour. The supernatant was collected and loaded onto a Ni-NTA resin column that was equilibrated with the lysis buffer. After loading, the column was washed with about 200 mL of wash buffer (50 mM HEPES pH 7.5, 200 mM KCl, 30 mM Imidazole, 10% glycerol, and 2 mM TCEP. HCl). After washing the protein of interest was eluted with an elution buffer (50 mM HEPES pH 7.5, 200 mM KCl, 300 mM Imidazole, 10% Glycerol, and 4 mMTCEP. HCl). 0.1 mL samples were taken for SDS PAGE gel from the supernatant, pellet, flow through during loading and washing, and the pure eluted protein. The eluted protein was concentrated using an Amicon centrifugal filtration device fitted with a 10 KDa molecular weight cut off membrane to a volume of 1 mL. The protein was then loaded onto a nap 10 gel filtration column and eluted with a gel filtration buffer (100 mM HEPES pH 7.5, 200 mM KCl, 30% glycerol, and 5 mM TCEP. HCl). The concentration of the protein was determined via Bradford Assay before being aliquoted, flash-frozen with liquid nitrogen and stored at -80 degrees celsius. The collected samples during the expression and purification process were analyzed by SDS-PAGE to determine expression, solubility, and purity of the protein of interest.

Results

Each of the proteins involved in the synthesis of acyl-ACP (AaS, ACPS1, and ACP) and the lipoic acid biosynthesis pathway (LIPT1, LIPT2, DLST, GCSH) were successfully cloned and transformed. In addition to being cloned and transformed, DLST, AaS, ACPS1, and GCSH were successfully over-expressed. AaS and GCSH were also successfully purified. Ulp1, a protein that cleaves the SUMO tag on the dwp-SUMO vector, was also successfully cloned, transformed, over-expressed, and purified.

Ulp1

The over-expression of Ulp1/pet28a in BL21-DE3 *E. coli* cells in 12 liters of M9 media resulted in a cell paste yield of 30 grams. SDS-PAGE gel analysis revealed that the protein was successfully over-expressed, and that the protein purification method was effective. Bradford analysis revealed that the concentration was 12 mg/mL, and the final yield was 40 mL or 485 mg of pure protein.

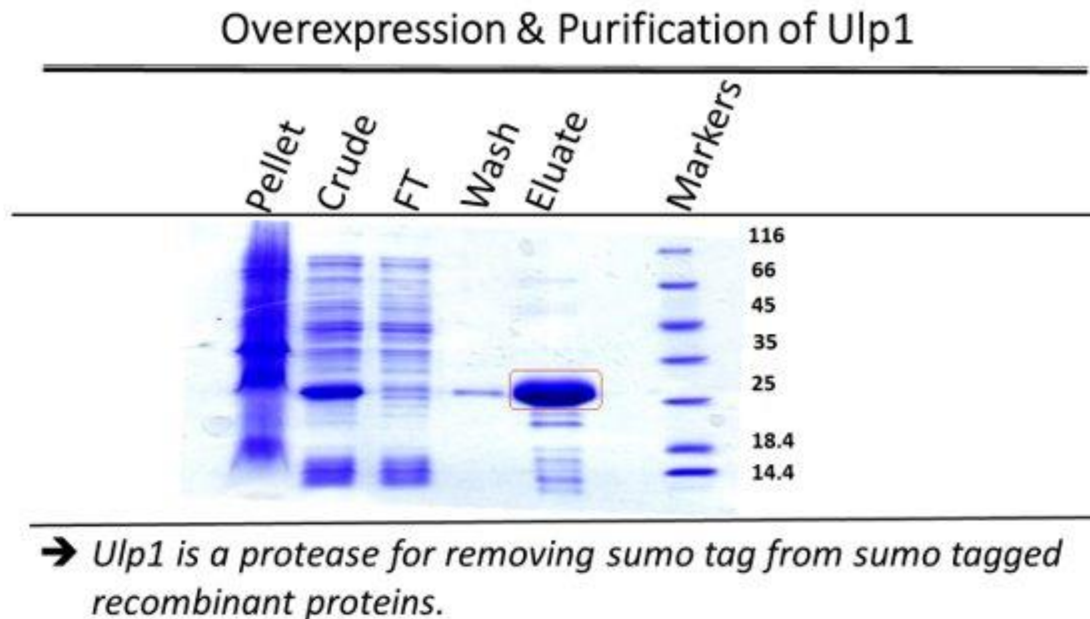

Figure 1.5. SDS-PAGE gel analysis revealed that Ulp1 was successfully overexpressed and purified.

DLST and LIPT2 Protein Expression Study

The over-expression of DLST/pet28a in BL21-DE3 *E. coli* cells in 12 liters of LB media resulted in a cell paste yield of 24 grams. The over-expression of LIPT2/pet28a resulted in a yield of 40 grams of cell paste. SDS PAGE gel revealed that the first attempt to over-express DLST and LIPT2 was unsuccessful. An expression study was performed to identify a colony that was the most effective at expressing DLST and LIPT2. The expression study confirmed that DLST and LIPT2 were successfully over-expressed by both colonies.

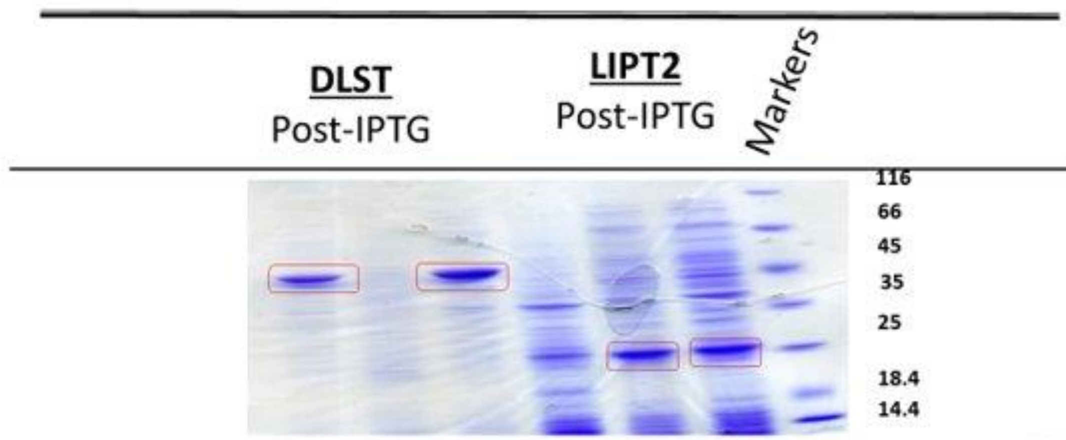

Figure 1.6. SDS-PAGE gel analysis revealed that both DLST and LIPT2 were successfully cloned and overexpressed.

AaS

AaS/pet28a was successfully over-expressed in Bl21-DE3 E. coli in M9 media and resulted in 46 grams of cell paste. SDS-PAGE gel analysis revealed that the protein was also successfully purified. The final protein yield was 222 mg.

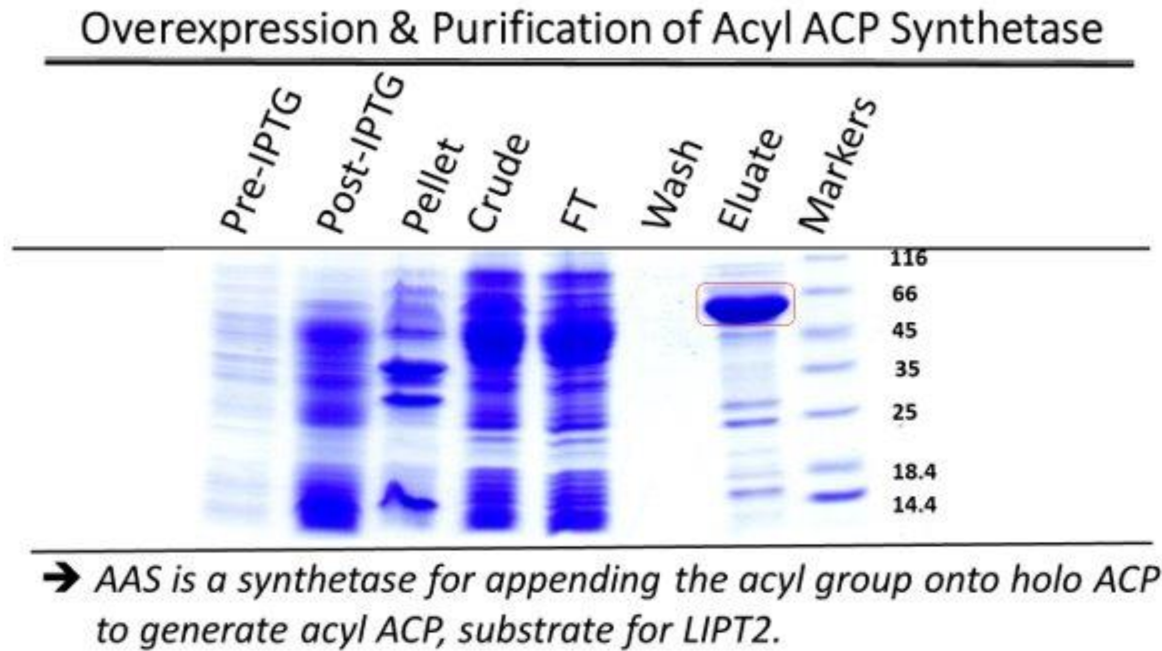

Figure 1.7. SDS-PAGE analysis revealed that AaS was successfully overexpressed and purified.

LIPT1 Expression Study

Following a failed attempt to overexpress LIPT1, an expression study was performed to determine which colony and vector was the most effective at overexpressing the protein. There was evidence that all colonies and both vectors expressed LIPT1. However, it appears that not much was produced because while there was a band that corresponded to the molecular weight marker for LIPT1, the band was not much darker than other bands that appeared in the gel.

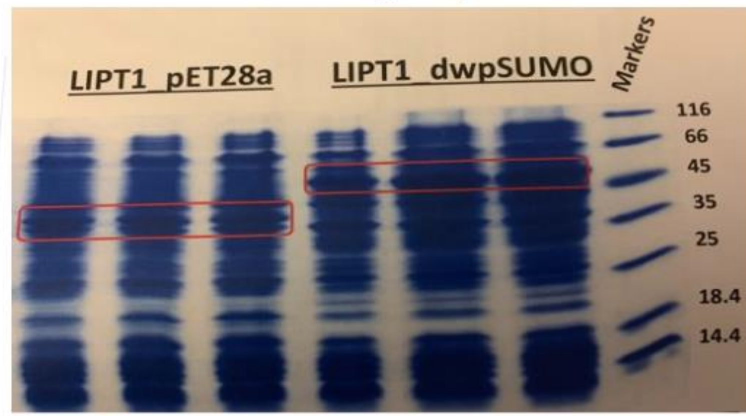

Figure 1.8. SDS-PAGE analysis revealed that LIPT1 was successfully cloned and overexpressed.

Several attempts were made to over-express LIPT1 and LIPT2 and an expression study was performed in order to identify a colony that produced the proteins the most efficiently. However, on a large scale, the bacteria were unable to successfully over-express the proteins. To resolve this issue in the future other bacterial strains and/or plasmid vectors may be utilized.

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Chapter 2

Lipoic Acid Biosynthesis and Malaria Drug Development

Introduction

Causative Agent

Plasmodium is a collective term that refers to a genus of unicellular eukaryotes that are obligate parasites; it is a protozoan and an apicomplexan. Malaria is caused by various organisms belonging to the genus *Plasmodium*. While there are over 100 species of *Plasmodium*, only 5 are known to cause malaria in humans. The species that cause malaria in humans are *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium vivax*, *Plasmodium ovale*, and *Plasmodium knowlesi*. While *Plasmodium* is commonly discussed in regard to human malaria cases, it can cause malaria in various animal species including reptiles, birds, and mammals.[1]

Transmission

It is important to note that malaria is not an infectious disease in traditional terms. It is not like the cold or flu where an individual that has the disease can directly transmit it to another individual. Malaria relies entirely on vectors, specifically female mosquitoes of the genes *Anopheles*, for transmission. While *Plasmodium* can infect various animals, malaria is not considered a zoonotic disease. *Plasmodium knowlesi* can cause a form of malaria referred to as zoonotic malaria. *P. knowlesi* is naturally harbored in macaques in Southeast Asia and they can

spread it to humans. However, there has never been a documented case where a human spread malaria to another animal.[2]

Regionality and Incidence

While malaria is typically associated with Africa, it is a disease that affects over 50% of the world. It is most common in Africa, South Asia, Central and South America, the Caribbean, Southeast Asia, the Middle East, and Oceania. The regions where it is most common are all characterized by warmer climates and access to brackish or freshwater. This is because the female *Anopheles* mosquito requires freshwater to lay her eggs and *Plasmodium* requires a water source in order to remain viable and infectious. *Plasmodium* cannot survive for prolonged periods of time in cold, arid environments. In 2018, there were 228 million cases reported worldwide and 405,000 deaths mainly in African children. Domestically, there are typically about 2,000 cases annually typically in immigrants and travelers returning from regions where malaria is common.[2]

Disease Progression

Incubation Period

Malaria typically has an incubation period of about 7-30 days. However, there is some variation depending on the species that an individual is infected with. *Plasmodium falciparum* and *Plasmodium malariae* are commonly associated with shorter incubation periods. Improper treatment with antimalarial drugs can also delay the appearance of symptoms which can result in a misdiagnosis.[3]

Symptom Development Biology

All symptoms associated with malaria are caused by asexual erythrocytic or blood stage parasites. During the blood stage parasites begin to invade and develop in the erythrocytes or red blood cells. During this stage they start to reproduce and during the process they produce waste products which accumulate in the infected red blood cell. Eventually when the cells lyse the parasites and waste products are emptied into the bloodstream which stimulates the immune response. Macrophages and other immune cells begin to produce cytokines and other soluble factors which results in fever and other associated clinical signs and symptoms. *Plasmodium falciparum* elicits a slightly different immune response than other *Plasmodium* species. Red blood cells infected with *Plasmodium falciparum* adhere to the vascular endothelium of venular blood vessel walls as opposed to freely circulating in the blood. This causes a condition referred to as cerebral malaria which has a high mortality rate and is part of the reason why *Plasmodium falciparum* causes the most aggressive form of malaria.[1]

Forms of Malaria

Uncomplicated Malaria

Uncomplicated or classical malaria is commonly associated with ‘malaria attacks’ which last anywhere from 6-10 hours. The malaria attacks are characterized by 3 distinctive stages, the cold stage, the hot stage, and the sweating stage. The cold stage is characterized by shivering, the hot

stage is characterized by fever, headaches, vomiting, and seizures in young children. Finally, the sweating stage is characterized by sweats, a return to normal body temperature, and fatigue. These malaria attacks can occur every 3rd or 4th day depending on the species of *Plasmodium* that the individual is infected with. *Plasmodium malariae* is associated with attacks every 3rd day and *Plasmodium falciparum, vivax,* and *ovale* are associated with attacks every 4th day. Clinical signs and symptoms associated with classical malaria include fever, chills, sweats, headache, nausea and vomiting, body aches, and general malice.[3]

Severe Malaria

Severe malaria occurs when the infection is complicated by organ failures or abnormalities in blood or metabolism. It typically occurs when the patient has a preexisting condition. Manifestations of severe malaria include cerebral malaria, severe anemia and hemoglobinuria associated with hemolysis, acute respiratory distress syndrome, blood coagulation abnormalities, low blood pressure, acute kidney injury, hyperparasitemia (defined as more than 5% of red blood cells infected), metabolic acidosis, and hypoglycemia. Hypoglycemia occurs most commonly in pregnant women and individuals treated with chloroquine.[3]

Diagnosis and Treatment

Diagnosis

Malaria is most often diagnosed by physically viewing parasites in the blood via microscopy. However, mild anemia, mild decrease in blood platelets or thrombocytopenia, elevation of bilirubin, and elevation of aminotransferases can be indicative of a malaria infection.[4]

Treatment

Treatment of malaria infections is largely dependent upon the severity of the infection, species of malaria parasite, and the part of the world where the infection was acquired. The latter two of the three criterion are used to determine the probability that the organism is resistant to antimalarial drugs. Uncomplicated or classical malaria cases that demonstrate a low probability of antimalarial drug resistance are treated with chloroquine. Uncomplicated cases that demonstrate a higher probability of antimalarial drug resistance are commonly treated with Artemether-lumefantrine (Coartem) or Atovaquone- proguanil (Malarone). For severe cases it is vital that treatment is initiated as soon as possible due to the risk of organ failure. In general, severe malaria cases are treated with intravenous (IV) artesunate.[4]

Life Cycle of *Plasmodium*

Plasmodium has two hosts, the female Anopheles mosquito (the primary host) and the human (the secondary host). During a blood meal the mosquito inoculates sporozoites into the human host. The sporozoites travel to the liver where they infect the liver cells and mature into schizonts. The schizonts rupture and release merozoites into the bloodstream. It is at this stage that there is an important distinction between different *Plasmodium* species. *Plasmodium vivax* and *ovale* can lie dormant and persist in the liver in the form of hypnozoites. *P. vivax* and *ovale*

can then cause a malarial relapse even years after the initial infection. Once the merozoites are in the bloodstream they begin to infect the red blood cells, this marks the beginning of the blood stage. During asexual reproduction, ring stage tropozoites mature into schizonts which rupture and release merozoites. There are some parasites which will have a sexual erythrocytic stage and develop into structures referred to as gametocytes. The female gametocyte is referred to as the macrogamete and the male gametocyte is referred to as the microgamete. During a blood meal the gametocytes are ingested by the mosquito where the travel to the stomach. In the mosquito's stomach the microgametes (male) and macrogametes (female) penetrate and form zygotes. These zygotes become motile and elongated and are referred to as ookinetes. The ookinetes invade the midgut wall of the mosquito where they develop into oocysts. The oocysts grow, rupture and release sporozoites which migrate to the salivary glands. During a blood meal the sporozoites are inoculated into the new human host and the cycle starts over again.[5]

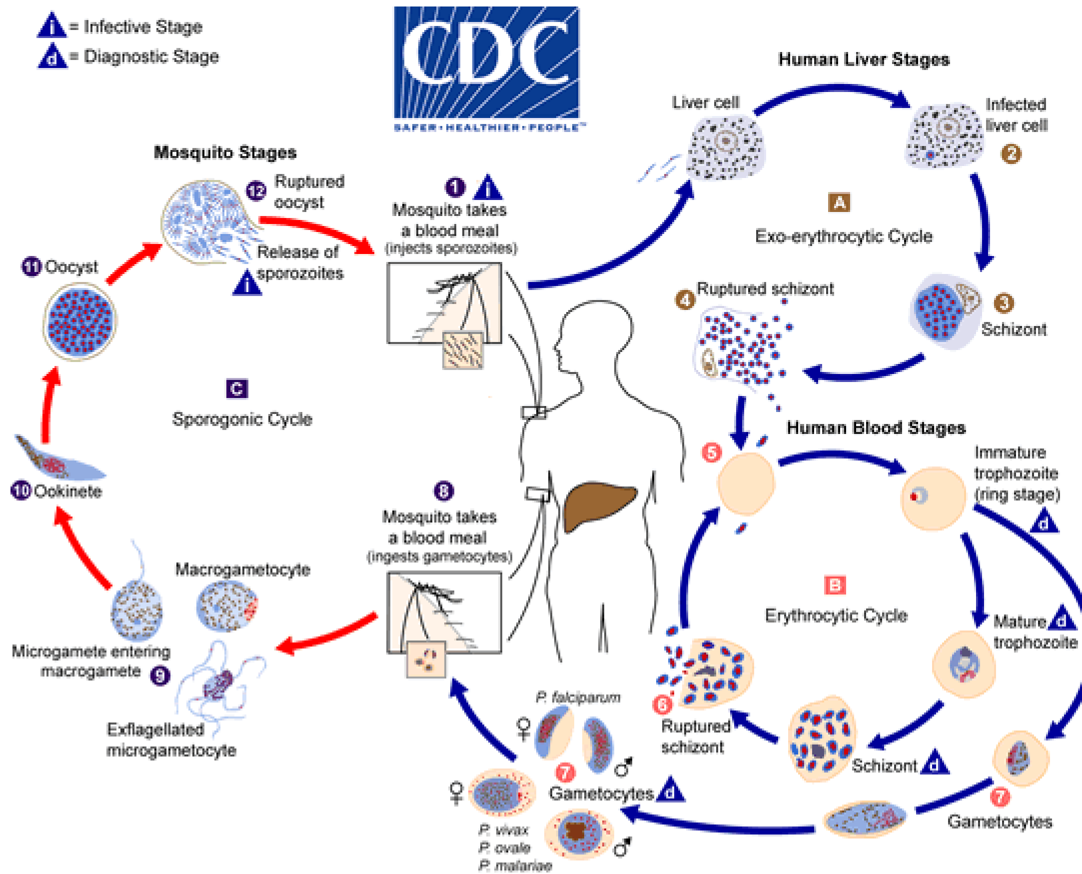

Figure 2.1. The life cycle of *Plasmodium* species, the parasite that causes malaria. The infective stage of *Plasmodium* are the sporozoites which are injected into the host by the mosquito.

Human Factors and Malaria- Genetic Factors

Sickle Cell Trait

There are two main genetic factors which can influence an individual's susceptibility to a malaria infection. Both factors are associated with the red blood cells. The first is the sickle cell trait. Individuals that are heterozygous for abnormal hemoglobin gene HbS are less susceptible to

malaria infections caused by *Plasmodium falciparum* due to the presence of both normal and sickle red blood cells. This trait is most commonly found in Africa and those of African descent.[6]

Duffy Blood Group

The Duffy glycoprotein is a receptor that is present on red blood cells for chemicals secreted during an inflammatory response. The duffy glycoprotein can also act as the receptor for *P. vivax*. Individuals that lack this receptor are referred to as Duffy negative and are protected against infections caused by *P. vivax*. The majority of Africans are Duffy negative. Due to this, most malaria cases in Africa are caused by *P. ovale* as opposed to *P. vivax*.[7]

Acquired Immunity

Individuals that suffer from several malaria attacks can develop partial protective immunity and enter into what is referred to as a semi-immune state. These individuals are still able to be infected and may not develop severe disease[8]. Newborns that are born to mothers that previously experienced malaria attacks are protected during the first few months of life due to the ability of the maternal antibodies to migrate across the placenta. However, these antibodies do decrease with time. Also, pregnant mothers will lose their partial protective immunity over the course of their pregnancy[9].

Antimalarial Drug Resistance and Vaccine Development

Antimalarial drug resistance is an increasing area of concern and is most commonly associated with *P. falciparum* and *P. vivax*. Amongst all treatment methods, chloroquine resistance is the

most common. Unfortunately, due to the complex *Plasmodium* life cycle there is currently no licensed vaccine for malaria prevention. This leaves room for the exploration of new potential drug targets.

One potential area for drug development lies in *Plasmodium*'s dependence upon lipoic acid. *Plasmodium* has two methods for the acquisition of lipoic acid, the lipoic acid salvage pathway and the lipoic acid biosynthesis pathway. Both are suitable drug targets due to the vital role that lipoic acid plays in *Plasmodium* development. Current challenges to lipoic acid targeted drug development lie in the similarity of human and *Plasmodium* lipoic acid biosynthesis pathways and the lack of knowledge surrounding the lipoic acid salvage pathway. [10]

***Plasmodium* Lipoic Acid Biosynthesis and Salvage Pathways**

Plasmodium can acquire lipoic acid via two pathways: the lipoic acid salvage pathway and the endogenous lipoic acid biosynthesis pathway. The lipoic acid salvage pathway relies on two lipoic acid protein ligases, LplA1 and LplA2, both can be found in the mitochondria but LplA2 can also be found in the apicoplast. LplA1 only uses lipoic acid that is salvaged from the host and lipoylates alpha-ketoglutarate dehydrogenase, branched chain alpha-ketoacid dehydrogenase and glycine cleavage system H-protein (GCSH). Little is known regarding the specific function of LplA2. The lipoic acid salvage pathway is essential during the intra-erythrocytic and liver stage development of *Plasmodium* species.

The *Plasmodium* endogenous biosynthesis pathway is housed in the apicoplast and utilizes three proteins, octanoyl- acyl carrier protein, protein N-octanoyltransferase (LipB), and lipoate synthase (LipA), to generate lipoic acid from octanoyl ACP. SImilar to the human lipoic acid biosynthesis pathway, octanoyl ACP is obtained via the type two fatty acid biosynthesis pathway which is also housed in the apicoplast. Lipoic acid serves as the co-factor for the acetyltransferase subunit of pyruvate dehydrogenase, also located in the apicoplast, which produces acetyl-CoA and feeds into the type two fatty acid biosynthesis pathway.[10]

Inhibition of the lipoic acid biosynthesis pathway will not necessarily halt development and cure an individual of the infection. This is because the lipoic acid biosynthesis pathway is not essential for intra-erythrocytic development of *Plasmodium*. However, parasites with deletions in pyruvate dehydrogenase or components of the type two fatty acid biosynthesis pathway displayed developmental arrest during the liver stages. This introduces the possibility of utilizing the lipoic acid biosynthesis pathway as a target for vaccine development.[10]

Lipoic Acid and Malaria Drug Development

The growing concern of antimalarial drug resistance has caused scientists to begin investigating new drug targets. One such target involves the use of lipoic acid. Malaria parasites rely heavily on their apicoplast to maintain its redox balance. One molecule proposed to play a role in apicoplast redox regulation is alpha-lipoic acid which serves as an antioxidant. The proposed role of lipoic acid is based on a connection between redox regulation and pyruvate metabolism in the

apicoplast via the pyruvate dehydrogenase enzyme complex. The pyruvate dehydrogenase complex consists of three enzymes which serve to generate acetyl-CoA for the fatty acid biosynthesis pathway by transferring an acetyl group from pyruvate to coenzyme A. These reactions rely on lipoic acid as a substrate that is reduced and re-oxidized to allow for continual cycles of pyruvate dehydrogenase complex activity.

As previously discussed, *Plasmodium* has two methods for acquiring lipoic acid. They can obtain it via the lipoic acid salvage pathway which is where they take lipoic acid from the human host or they can utilize their own lipoic acid biosynthesis pathway. The *Plasmodium* lipoic acid endogenous biosynthesis pathway is housed in the apicoplast and is catalyzed by two enzymes. It should be noted that the *Plasmodium* lipoic acid biosynthesis pathway operates independently of the lipoic acid salvage pathway in the mitochondria. In order to determine whether or not lipoic acid plays an essential role in redox regulation and parasite metabolism, studies were performed on *Plasmodium* parasites with lipB gene deletions. LipB, also known as octanoyl-ACP: protein *N*-octanoyltransferase, is one of two enzymes that catalyze the lipoic acid biosynthesis pathway. LipB serves to transfer the octanoyl group from octanoyl-ACP to the lipoyl-domain of E2. This study revealed that there is a connection between lipoic acid availability and redox regulation. Also, in parasites with LipB gene deletions there were changes in carbon metabolism which suggests a connection between redox regulation in the apicoplast and mitochondrial and cytosolic metabolic pathways. LipB gene deletion also interferes with parasite development in the mosquito, which aligns with the important roles that redox regulation and fatty acid biosynthesis play in the insect stage of the *Plasmodium* life cycle.[11]

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Chapter 3

Lipoic Acid and Malaria Vaccine Development

History of Malaria Vaccine Development

The first step towards an effective malaria vaccination took place in 1973 when there was the first report of human protection from malaria via vaccination. While this was a monumental development, it was a very impractical way of vaccinating large populations due to the fact that the vaccination consisted of the bites of roughly a thousand mosquitoes infected with malaria parasites that had been X irradiated. This vaccine showed that it is possible to confer immunity

against malaria but that more research was required to discover a more practical method of manufacturing.[1]

Pre-erythrocytic Vaccines

Throughout the history of vaccine development scientists have targeted various stages of the *Plasmodium* life cycle. The main life stage that has been targeted by vaccines are the pre-erythrocytic stages or the life stages prior to when the parasite invades the red blood cells. The pre-erythrocytic stages include the sporozoite and liver stages. The sporozoite is the infective stage of *Plasmodium.* During a blood meal, the female Anopheles mosquito will inject anywhere from five to twenty sporozoites which migrate to the hepatocytes, liver cells, within minutes. This marks the beginning of the liver stage. While in the hepatocytes, the sporozoites develop into schizonts. These schizonts eventually rupture and release several thousand merozoites into the hosts' circulatory system where they are either picked up by phagocytes or invade erythrocytes, red blood cells. This marks the beginning of the blood stage. Vaccines that target the pre-erythrocytic stages aim to induce antibodies against sporozoites to prevent the parasites from entering the liver stage. This vaccine also aims to induce infected hepatocyte destruction by cytotoxic T-cells without harming the host. This is an ideal life stage to target because as previously mentioned, the mosquito injects relatively few sporozoites into the host. The liver stage is where the sporozoites can mature and replicate into large numbers that overwhelm the hosts' immune system. The most promising candidate for this type of vaccine is RTS, S, a recombinant protein vaccine. While RTS, S does confer clear immunity to *Plasmodium falciparum*, the immunity does not last long and would therefore require periodic booster shots.[2]

Blood Stage Vaccines

Blood stage vaccines specifically target the life stages where the parasites have invaded the erythrocytes and are rupturing them to release more merozoites into the bloodstream. There are two classes of blood stage vaccines, anti-invasion and anti-complication. Anti-invasion vaccines focus on preventing the invasion of red blood cells by merozoites which in turn prevents malaria disease development. It should be noted that all clinical signs of malaria are related to the blood stage. Development of such vaccines is complicated due to limitations of available animal models and lack of a human challenge model. Most anti invasion vaccines focus on Merozoite surface protein-1 (MSP-1). However, the discovery of parallel invasion pathways and the ability of MSP-1 to block the activity of malaria-protective antibodies complicate the development of such a vaccine. Anti-complication vaccines aim to prevent the development of severe malaria caused by *Plasmodium falciparum*. *Plasmodium falciparum* has a protein called erythrocyte membrane protein-1 (PfEMP-1) which gives it the ability to adhere to vascular endothelium cells. This adherence protein is what gives *Plasmodium falciparum* the ability to cause severe malaria. However, this protein is variable and a high rate of antigenic variation which complicates vaccine development.[3]

Sexual Stage Vaccines

Sexual stage vaccines target the gametocytes and fertilization. When the schizonts rupture and release merozoites, some of the merozoites mature into gametocytes which are ingested by the mosquito during a blood meal. The 'male' gametocyte is referred to as a microgamete whereas the 'female' is referred to as the macrogamete. Sexual reproduction then occurs in the mosquito

midgut and the resulting sporozoites migrate to the salivary glands where they can be injected into the host. These vaccines induce production of antibodies to gametocyte antigens which prevents fertilization in the mosquito midgut. When the mosquito ingests the blood meal, it also ingests antibodies which prevent fertilization. While these vaccines have proven to be effective, they receive little funding due to the lack of a market in developed countries. Since the vaccine targets sexual reproduction, which occurs in the mosquito, it is not effective in preventing vaccinated individuals from developing disease. However, it is useful in protecting communities from infection and could prove to be useful in controlling outbreaks when used in conjunction with other prevention measures.[4]

Potential Solutions

Due to the various complications associated with developing vaccines that specifically target various *Plasmodium* life stages, scientists have recently begun to explore the possibility of utilizing genetically engineered parasites for vaccines. The whole parasite vaccines are based on the sporozoite form of the parasite and target the pre-erythrocytic life stages. Animal models have shown that such vaccines induce neutralizing antibody responses against sporozoites and cytotoxic T cells which destroy infected hepatocytes. Immunization with live whole parasites capable of reproduction affords the opportunity for superior immunity and protection when compared to live sporozoites that are incapable of replication. Such vaccines have proven to be safe, feasible, and efficacious. They also present the opportunity to include immunomodulatory elements such as antigens from other *Plasmodium* species and antigens for pathogens coendemic with malaria. While they appear to be the way of the future in terms of vaccine development they

still come with complications, namely in the manufacturing process. Large scale production of live sporozoite vaccines along with preservation, storage and delivery, and public perception are all considerable obstacles.[5]

Lipoic Acid and Malaria Vaccine Development

As previously discussed, due to the role that the lipoic acid biosynthesis pathway plays in liver stage development of *Plasmodium* it is a potential target for vaccine development. The liver stages are preferential targets because compared to the intra-erythrocytic stages there are fewer parasites, and the infected host is asymptomatic. Vaccines are also believed to be more effective when they target the pre-erythrocytic and liver stages because at these stages the parasites do not exhibit significant antigenic variation and polymorphisms that cause the vaccine to fail against heterologous parasite strains.

The use of live attenuated parasites is a promising candidate for malaria vaccines due to their ability to confer immunity and protection against disease. One potential method of generating such vaccines involves genetically engineering malaria parasites with gene knockouts in genes that encode pyruvate dehydrogenase or components of the type two fatty acid biosynthesis pathway. Both pyruvate dehydrogenase and the type two fatty acid biosynthesis pathway are connected to the *Plasmodium* endogenous biosynthesis pathway which is required for *Plasmodium* liver stage development in the human host. Deletion of these genes will result in a parasite that is able to elicit an immune response but unable to further develop and cause disease thus making it an ideal organism for immunizations.[6]

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Chapter 4

Lactic Acidosis and LIPT1 Mutations

Introduction

Lactate is produced by most body tissues, but the majority is produced in the muscles. Under normal physiological conditions, it is rapidly cleared by the liver with some clearance by the kidneys. During aerobic respiration, pyruvate is produced via glycolysis and enters Krebs cycle, this process bypasses lactate production. During anaerobic conditions when oxygen is scarce, lactate, the end product of glycolysis, enters the Cori cycle as the substrate for gluconeogenesis. Lactic Acidosis occurs when anaerobic respiration causes a buildup of lactic acid in the tissues and/or the liver and kidneys fail to clear it efficiently. This can be caused by decreased oxygen delivery or a defect in mitochondrial oxygen utilization. The buildup of lactic acid causes an imbalance in blood pH, which is typically slightly alkaline. If left untreated the condition can be fatal.[1]

Types of Lactic Acid and Lactic Acidosis

There are two forms of lactic acid, L and D-lactate. Most lactic acidosis is caused by a buildup of L-lactate, the predominant form of lactic acid produced by humans. There are three main types of lactic acidosis, type A, type B, and D-lactic acidosis. Type A lactic acidosis is caused by tissue hypoperfusion and is the most serious form. In Type A acidosis, lactic acid is overproduced by ischemic tissue as a byproduct of anaerobic metabolism during an oxygen deficit. Type B lactic acidosis is caused by an impairment of cellular functioning. It is commonly

linked to systemic and congenital conditions, cancer, and certain drugs. D-lactic acidosis is caused by a buildup of D-lactate. It is caused by the systemic absorption of D-lactate, a product of bacterial carbohydrate metabolism.[2]

Disease Etiology and Pathogenesis

Etiology

Lactic Acidosis has several causes but is most commonly caused by tissue hypoperfusion or the inadequate delivery of vital oxygen and nutrients to body tissues. Another cause of lactic acidosis is also septic shock. Septic shock is linked to macrocirculatory dysfunction causing arterial hypotension, microcirculatory dysfunction, and decreased oxygen and nutrient extraction by peripheral tissues. This can lead to an increase in lactic acid buildup. In septic patients elevated lactate is associated with mortality independent of shock. Cardiogenic, obstructive, and hemorrhagic shock can also lead to elevated lactate. The elevation is related to increased tissue lactate production and is unrelated to the liver's ability to clear lactic acid. Cardiac arrest can cause ischemia due to lack of blood flow and associated inflammation can cause an initial rise in lactate. Amongst patients experiencing traumatic injuries hypoperfusion is common which can lead to increased lactate production. Certain forms of seizures can also cause a rapid increase in lactate production. However, it is typically cleared quickly by the liver after the seizure is resolved. Excessive muscle activity can cause increases in lactate production due to anaerobic metabolism. Betaagonists used in asthma treatment are linked to lactic acidosis cases due to excessive adrenergic stimulation. Regional ischemia, burns and smoke inhalation, and diabetic ketoacidosis also have links to lactic acidosis. Thiamine deficiencies can cause lactic acidosis

due to their role as cofactors for cellular enzymes which are essential for aerobic metabolism. In its absence anaerobic metabolism dominates. Malignancy, specifically rapidly progressive leukemia or lymphoma with liver involvement is also linked to increased lactate production. This is likely due to an overexpression of glycolytic enzymes, mitochondrial dysfunction, impaired hepatic clearance, and malnutrition leading to thiamine deficiency. Liver dysfunction can lead to lactic acidosis due to the liver's role as the primary organ for lactate clearance. Numerous pharmacologic agents and toxins can lead to increased lactate production and put users at risk of lactic acidosis. *Metformin, a* biguanide used for the management of diabetes mellitus, is one such pharmaceutical that is linked to the increased risk of elevated lactate. It has the ability to inhibit gluconeogenesis and cause mitochondrial impairment. However, it remains controversial as to whether or not it alone can cause lactic acidosis. Commonly lactic acidosis caused by *Metformin* is seen in patients that have lactate accumulation due to kidney failure, liver failure, or overdose. In the event of renal failure, it is commonly corrected by using hemodialysis to correct the acidosis and remove *Metformin.* Alcohol abuse is also linked to lactic acidosis due to the increased risk for other causes of elevated lactate.[1]

Risk Factors: LIPT1 Mutations

As previously discussed, the lipoic acid biosynthesis pathway is associated with the pyruvate dehydrogenase complex. Lipoic acid covalently attaches to the pyruvate dehydrogenase complex E2 subunit and is functionally required. Defects in genes encoding components of the lipoic acid biosynthesis pathway can affect the pyruvate dehydrogenase complex as well as other systems that are reliant on lipoic acid as a co-factor. Pyruvate dehydrogenase complex deficiencies are

associated with a wide range of diseases, most notably fatal infantile lactic acidosis and Leigh disease. [3]

In one case a newborn presented with moderate metabolic acidosis. The infant had a venous pH of 7.16 and an arterial blood gas of pH 7.14. The infant's condition declined rapidly, and it experienced respiratory distress and shock. Ultimately the infant died at 1.5 hours of life. Necropsies and genetic testing revealed that the infant had LIPT1 gene mutations. Case 2 was the sibling of infant 1 which presented with similar symptoms, progressive respiratory distress. Capillary blood gas revealed that the infant had severe metabolic acidosis. Infant 2 had a capillary blood gas pH of 6.80. Chemistry revealed that the infant had a blood lactate concentration of 16 mmol/L/. Lactic acidosis is typically defined by lactate> 5 mmol/L and pH<7.35. The infant ultimately died at 2 hours of life, but the parents consented to whole exome sequencing (WES). WES revealed that infant 2 also was compound heterozygous for 2 nonsense mutations in LIPT1.[4]

Diagnostics, Treatment, and Prevention

Diagnostics

The diagnosis of lactic acidosis is based on a fasting blood test which assesses the lactate levels in the blood. Lactic acidosis is diagnosed when the individual has a blood pH of less than 7.35 and a blood lactate concentration greater than 5-6 mM/L. An oxygen flux test can also be used. This method is commonly used in the diagnosis of occult tissue hypoperfusion in patients w/ low-grade elevations in lactate levels.[4]

Therapeutic Approaches

Treatment of lactic acidosis involves first addressing the primary cause and providing supportive care. Traditionally, intravenous bicarbonate was used to buffer pH changes. However, this method is widely discouraged due to the potential adverse effects on cardiac function. Newer methods include peritoneal dialysis and hemodialysis in which large amounts of alkali can be provided without causing hypernatremia or hypervolemia associated with bicarbonate infusion. Peritoneal dialysis with bicarbonate-based dialysate is the ideal means of delivering bicarbonate. Methylene blue is thought to increase lactate metabolism by altering the cellular oxidative state; however, there is little efficacy associated with this method. Sodium nitroprusside has been used as a method to alleviate regional hypoperfusion. Recently, dichloroacetate has been explored as a potential treatment due to its ability to activate pyruvate dehydrogenase and enhance lactate metabolism.[5]

Preventative Strategies

Lactic acidosis caused by exercise is easily treated by drinking more water and initiated an exercise routine gradually. Lactic acidosis caused by medications and toxins can be avoided by prescribing alternative pharmaceuticals, especially to individuals experiencing liver or renal failure.[6]

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ACADEMIC VITA

ILANA MOSLEY

Relevant Coursework

Genetics

Elementary Biochemistry and Lab 1

Immunology

Cell Biology

Organic Chemistry 1,2, and lab

Animal Science and Nutrition

Lab Animal Medicine

Comparative Vertebrate Anatomy

Microbiology and Lab

Principles of Animal Disease Control

Education

The Pennsylvania State University- University Park, Pennsylvania 2021

Major: Veterinary and Biomedical Sciences

Graduation Date: Spring 2021

Research Experiences

UT Health GRADSURP; Houston, Texas- COVID-19

This past summer I was accepted into the UT Health GRADSURP Program in the Texas Medical Center to do research in the lab of Dr. Rebecca Berdeaux. Unfortunately the program was cancelled due to COVID-19.

Booker Lab, Dr. Squire Booker; University Park, Pennsylvania- 2017- present

As a member of the Booker lab, my responsibility is to aid Dr. Booker with his research by studying the human lipoic acid biosynthesis pathway.

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Abstract:

Studies of Endogenous Biosynthesis of Lipoic Acid Cofactor in Humans: Expression, Purification and Characterization of LIPT1 and LIPT2 Enzymes

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Lipoic acid is a cyclic disulfide that plays a role in intermediate metabolism. It exists predominantly in the form of its conjugate base, lipoate, under physiological conditions. In humans, lipoate is essential for five redox reactions, four 2-oxoacid dehydrogenases and the glycine cleavage system. In this study, two enzymes, LIPT1 and LIPT2, were investigated. LIPT2 transfers octanoic acid from ACP to GCSH and LIPT1 transfers lipoic acid from GCSH to DLST. GCSH catalyzes the degradation of glycine and mutations in the gene are linked to hyperglycemia. Mutations in DLST have been linked to the development of Alzheimer's in elderly populations. DLST is also an essential part of the Kreb's cycle, therefore mutations in the gene can lead to issues converting ADP to ATP. The primary goal of the study was to overexpress and purify the genes involved in the human lipoic acid biosynthesis pathway so that the activity of LIPT1 and LIPT2 could be observed in vitro. In order to characterize the activity of LIPT1 and LIPT2, both genes along with DLST, AaS, ACPS1, Ulp1, and ACP were cloned into dwpSUMO and pET28a plasmids using DH5a E. coli cells. Afterwards, the plasmids were transformed into BL21-DE3 E. coli cells, overexpressed, and purified. All of the genes of interest were successfully cloned into the dwpSUMO and pET28a plasmids and transformed into BL21-DE3 E. coli cells. AaS and Ulp1 were successfully overexpressed and purified. Preliminary results displayed separation between the standards for holo-ACP and acyl-ACP. However, the separation between the peaks corresponding to the holo- GCSH and lipoyl- GCSH was inadequate. Therefore, the HPLC method requires additional alterations before assays can be conducted. Future directions include overexpressing DLST, ACPS1, and ACP as well as monitoring the lipoic acid transfers by LIPT1 and LIPT2 via HPLC analysis.

Brindley Lab, Dr. Brindley; The George Washington University, Washington, D.C.- June 2018- August 2018

While in the Brindley lab, I researched schistosomes and the role that they play in the development of Squamous Cell Carcinoma of the bladder. While in the Brindley Lab I was exposed to aseptic techniques, tissue culture, and animal research using hamsters, mice, and snails. The research that I conducted in the Brindley Lab was published in Nature and is titled "Differential responses of epithelial cells from urinary and biliary tract to eggs of Schistosoma haematobium and S. mansoni".

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Abstract:

Differential responses of epithelial cells from urinary and biliary tract to eggs of Schistosoma haematobium and Schistosoma mansoni

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Chronic Urogenital Schistosomiasis (UGS) and deposition of Schistosoma haematobium eggs, a group 1 carcinogen according to the International Agency for Research on Cancer, can lead to squamous cell carcinoma of the bladder. Physiopathological and molecular mechanisms linking UGS with bladder cancer have yet to be clarified. Previous studies investigated the effects of culturing epithelial cells from two discrete human tissues, HCV29, established from normal urothelium, and H69, established from cholangiocytes, in the presence of S. haematobium or S. mansoni eggs. Growth of HCV29 and H69 cells co-cultured with the schistosome eggs was monitored in real time. Gene expression analysis of pathways involved in oncogenesis, epithelial to mesenchymal transition and apoptosis was undertaken. Schistosome eggs promoted

proliferation of urothelial cells. The enhanced growth induced by S. haematobium eggs was expected given that eggs of S. haematobium reach the lumen of the bladder via urothelium whereas S. mansoni eggs transverse the intestinal wall.

Based on the results of previous studies, it was hypothesized that post exposure to eggs of S. mansoni, cell cycle regulators would be up-regulated and following exposure to S. haematobium proteins involved in cell-cell junctions and growth factors would be down regulated; tumor suppressors were expected to be down regulated post exposure to schistosome eggs. For this study, levels of three proteins known to respond during malignant transformation were investigated: BAX, MASPIN (SERPIN B5), and p53. Following co-culture of HCV29 cells with eggs of S. haematobium or S. mansoni for 2 hours, more BAX was induced in HCV29 cells by exposure to S. mansoni eggs (p= 0.9996) but not to S. haematobium eggs or the control HCV29 cells (p>0.9999). At 24 hours, more BAX was detected in cells exposed to S. haematobium eggs (p=0.0152); however, there was not a difference between the amount of BAX detected in cells exposed to S. haematobium and S. mansoni eggs (p=0.8904) or cells exposed to S. mansoni and control HCV29 cells (p=0.0887). The amount of BAX (p=0.0025) and p53 (p=0.0018) detected among the 2 hour and 24 hour groups was significantly different, with higher concentrations at 24 hours (p=0.0025). At 2 hours, there was not a difference in the amount of p53 detected among cells exposed to S. haematobium (p=0.9999) or S. mansoni (p>0.9999) and control HCV29 cells. At 24 hours, there was significantly more p53 detected in cells exposed to S. haematobium and control HCV29 cells (p=0.0013) but not between S. mansoni and control HCV29 cells (p=.2115). The concentration of MASPIN in all samples were below the sensitivity level of 110 pg/mL.

The elucidation of molecular mechanisms linking the UGS and the development of bladder cancer may lead to the discovery of new treatments for this neglected tropical disease-related cancer.

Annual Biomedical Research Conference for Minority Students; Indianapolis, Indiana-November 2018, November 2019

During this conference I had the opportunity to do a poster presentation on the research that I performed in the summer of 2018 in the lab of Dr. Paul Brindley and the lab of Dr. Squire Booker. Abstracts are attached above underneath each respective experience.

Publications

Nature; 24 July 2019

Nacif-Pimenta, R., da Silva Orfanó, A., Mosley, I.A. et al. Differential responses of epithelial cells from urinary and biliary tract to eggs of Schistosoma haematobium and S. mansoni. Sci Rep 9, 10731 (2019). https://doi.org/10.1038/s41598-019-46917-y

Extracurriculars

National Organization for the Professional Advancement of Black Chemists and Chemical Engineers: Outreach Coordinator, Dr. Squire Booker; State College, Pennsylvania-August 2020-May 2021

The National Organization for the Professional Advancement of Black Chemists and Chemical Engineers or NOBCChE aims to increase diversity and inclusion in the STEM field by encouraging minority students to pursue careers in the fields of chemistry and chemical engineering. As outreach director I work with the other executive board members to plan events and update our social media pages. I also manage our relationships with other clubs and the greater State College community by planning community service and outreach events.

Introduction to Animal Science (ANSC 201) Teaching Assistant, Mr. Dale Olver; State College, Pennsylvania- January 2020-May 2021

As a teaching assistant for ANSC 201 my responsibility is primarily to guide and encourage the learning and understanding of basic animal science to my students. I achieve this by teaching a hands-on lab section that meets once a week and reinforces topics covered in class such as anatomy and nutrition. In order to gauge the understanding of my students I write and administer lab quizzes and exams throughout the semester.

Susquehanna Service Dogs Puppy Raiser, Dr. Nancy Dreschel; State College, Pennsylvania-September 2019-January 2021

As a Susquehanna Service Dogs Puppy Raiser my responsibility was to socialize my puppy and teach him basic cues and guide him along his path of becoming a service dog. Recently my dog, SSD Boodles, passed his evaluations and is currently in advanced training learning more specific skills to aid his future partner.

Schreyer Honors College Scholar Ambassador, Mrs. Rosanna Mersinger; State College, Pennsylvania-August 2018-May 2021

As a Schreyer Honors College Scholar Ambassador my responsibility is to represent the Honors College and aid in recruitment efforts. I serve on panels and give tours of the college to prospective students and parents in order to give them an idea of what life is like for Penn State students. I also provide information to current students looking to apply for admission to the honors college during their second or third year. I also aid in the coordination of different events hosted by the Honors College for the Penn State community such as Founder's Day and Scholar's Day.

Presidential Leadership Academy, Mrs. Melissa Doberstein; State College, Pennsylvania-August 2018-May 2021

As a member of the Presidential Leadership Academy, I have the opportunity to collaborate with other student leaders and formulate solutions to local and global problems. Through the PLA I have had the opportunity to meet weekly with the president of my university to discuss problems that face the university and give him advise on how the student body views and responds to university policies. I also have the opportunity to draft grant proposals, develop stronger leadership methods, and facilitate discussions regarding controversial topics.

Millennium Scholars Program, Dr. Amy Freeman; State College, Pennsylvania- June 2017-May 2021

The Millennium Scholars Program is a scholarship program that awards full tuition (out-of state students) and full ride (in-state students) to high achieving students pursuing STEM careers. The main objective of MSP is to increase diversity and inclusion in the STEM field by encouraging minority students to engage in undergraduate research and ultimately obtain a PhD. The Millennium Scholars Program has the highest GPA requirement of any organization on campus,

a 3.5 cumulative and semester, and requires graduating seniors to submit a graduate-level research thesis. Through MSP I have also had the opportunity to mentor other minority students pursuing STEM careers.

Ireland Study Abroad, Dr. Tracy Hoover and Dr. Dennis Decoteau; Dublin, Ireland- June 2018

During the two week maymester to Ireland I completed a comparative agriculture study between the United States and Ireland. I also learned more about the Irish Potato Famine and how it impacted both Ireland and the United States.

<u>Awards, Honors, Scholarships</u>

Millennium Scholars Program- University Park, Pennsylvania

The Millennium Scholars Program awards high achieving students pursing a PhD in a STEM field with full tuition (out-of state students) or full ride (in state students) scholarships.

Provost Award- University Park, Pennsylvania

The Provost Award is a highly competitive scholarship awarded to exemplary students. Each recipient's award is reviewed annually based on grade point average.

Schreyer Honors Scholar-University Park, Pennsylvania

During my senior year of high school, I was inducted into the Schreyer Honors College. Through the Honors College I have been able to enhance my undergraduate experience through more intimate classroom settings, study abroad and internship opportunities, and financial support.

Douglas Honors Study Abroad Scholarship- University Park, Pennsylvania and Dublin, Ireland

The Douglas Honors Study Abroad Scholarship is a study abroad scholarship awarded to exemplary students by the College of Agriculture. I was awarded this scholarship to help pay for my study abroad trip to Ireland.

Gerald and Leoda Gummo Ex Fund- University Park, Pennsylvania and Dublin, Ireland

The Gerald and Leoda Gummo Ex Fund is a study abroad scholarship awarded to exemplary students by the College of Agriculture. I was awarded this scholarship to help pay for my study abroad trip to Ireland.

Internship-PA Commonwealth- University Park, Pennsylvania and Dublin, Ireland

The Internship-PA Commonwealth Scholarship is a study abroad scholarship awarded to exemplary students by the College of Agriculture. I was awarded this scholarship to help pay for my study abroad trip to Ireland.

Settlemeyer Fund- University Park, Pennsylvania and Dublin, Ireland

The Settlemyer Fund is a study abroad scholarship awarded to exemplary students by the College of Agriculture. I was awarded this scholarship to help pay for my study abroad trip to Ireland.

Oswald Scholarship- University Park, Pennsylvania

The Oswald Scholarship is awarded to high-achieving veterinary and biomedical sciences students in the College of Agriculture.