THE EFFECTS OF HUMAN SERUM ALBUMIN MUTATIONS ON
PHYSIOLOGICALLY IMPORTANT FATTY ACID TRANSPORT

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DEDICATION

To my late father Walter C. Tuei, my mother Annrose Tuei, siblings Vincent, Ken, Beatrice, Gladys and Winnie and my brother-in-law Laban for their endless love and support.
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ABSTRACT

Human Serum Albumin (HSA) is the most abundant plasma protein that transports a variety of drugs and endogenous compounds. HSA is the primary transporter for delivering free fatty acid (FFA) to tissues and possesses at least eleven binding sites for this ligand. Although most FFAs are bound to albumin, a small fraction dissociates from the protein and exists in monomeric form within the aqueous phase, referred to as the unbound free fatty acid (uFFA). The interaction of FFA and HSA serves to buffer the level of FFA in serum and therefore regulates the rate at which FFA is transported to appropriate target cells. Since a close relationship of elevated FFA levels to the incidence of Type 2 Diabetes (T2D) have been shown, the HSA/FFA interactions might therefore indicate HSA’s role in the pathogenesis of diabetes by modulating fatty acid availability.

Most hydrophobic ligands that bind to HSA such as FFAs bind to one or two distinct high affinity binding pockets or sites in subdomains IIA and IIIA of HSA. In this study, site-directed mutagenesis and a novel protein expression system called Pichia pastoris system were used to synthesize recombinant HSA proteins with specific mutations on key amino acid residues that are involved in FFA binding on these domains. Binding affinities of recombinant HSA and mutant proteins for long chain FAs (palmitate and oleate) were determined by using a fluorescent probe composed of Acrylodan-Derivatized Intestinal Fatty Acid Binding protein (ADIFAB) FFA quantification method. The modified FFA binding affinity induced by mutations in FA binding sites of HSA may provide the rationale background for further studies associated with cellular HSA/FFA interactions and HSA polymorphism.
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CHAPTER 1
INTRODUCTION

Background

Human Serum Albumin

HSA is a principal component of blood and is the most abundant protein of the plasma (3.5 – 5.0 g/dl) [1]. It is also a prevalent protein in the extracellular fluids, e.g., cerebrospinal fluid. It is synthesized in the liver and has a half-life of 19 days. Its plasma levels are determined by a combination of genetic regulation and dietary factors. The primary role of HSA is to transport fatty acids, but its versatile binding capacities and high concentration mean that it can assume a number of additional features. The protein contributes 80% to the colloidal osmotic blood pressure. A decrease in the osmotic pressure causes an increase in albumin synthesis and vice versa [2]. It is also the protein chiefly responsible for the maintenance of blood pH [3].

HSA is a 66.5-kDa single chain, non-glycosylated polypeptide that organizes to form a heart shaped protein having approximately 67% helix nut no beta sheet [4-6]. The primary sequence of all albumins is characterized by the arrangement of disulphide double loops that repeat in a triplet fashion. A total of 17 disulphide bridges, which are exclusively intrasubdomain and conserved across species, contributes towards HSA’s impressive thermostability [1, 3]. The protein is organized into three homologous domains (I-III), and each domain has two subdomains (A and B) that possesses common structural elements (Figure 1) [3, 4]. The first half of the HSA heart shaped molecule
Figure 1. X-ray crystallographic structure of unliganded HSA
contains subdomains IIA/B and subdomain IIA, the second half contains subdomain IIB and subdomains IIIA/B [5]. Upon examination of the sequence it is speculated that the three homologous domains of albumin arose as a result of a gene duplication event [7]. HSA is not uniformly charged along its length; at pH 7 the charge is -9, -8, and +2 for domains I, II and III respectively [8]. The net negative charge of -15 at neutral pH helps to keep HSA extremely soluble in circulation, which also contributes to its stability.

The amino acid sequence of albumin contains high quantities of cysteine residues and charged amino acids and it contains also low quantities of glycine, methionine, and a single tryptophan (Trp-214). Out of the 35-cysteinyl residues, 34 form the 17 stabilizing disulfide bridges. However, HSA is a heterogeneous mixture of mercaptalbumin (HMA) and nonmercaptalbumin (HNA) [9]. In HMA, the cysteine residue, at position 34, has a free SH group [10] that may be involved in the binding of the biological regulator and neuramodulator, nitric oxide implicating HSA’s role as its reservoir [11, 12]. In HNA, the residue is not free but forms a mixed disulfide with cysteine [10] or glutathione or undergoes oxidation [13] during circulation in the body.

HSA is a very remarkable protein for its binding capabilities to numerous exogenous and endogenous ligands implying that the protein can serve as an almost universal transport and depot protein in circulation[1, 14]. Apart from transporting FAs, it also serves as a transport vehicle for several endogenous compounds such as hemin, bilirubin and tryptophan all of which bind with high affinity [1, 3]. Most of the naturally occurring ligands are hydrophobic and poorly soluble in aqueous medium and thus binding of these ligands to HSA facilitates their transport in blood. By binding to
poisonous metabolites such as bilirubin, it functions as a detoxifying agent; this
detoxification has reached a particular degree of specialization in the Chinese cobra
whose serum albumin prevents the snake from succumbing to its own venom [15].

The protein has long attracted the attention of the pharmaceutical industry
because of its ability to bind a wide range of drug molecules and alter their
pharmaceutical properties [16]. Binding of drugs to albumin alters the pattern and volume
of distribution, lowers rate of clearance and increases the half-life of the drug [17, 18].
Most of these compounds bind to one of two principal binding sites commonly referred to
as drug sites I and II located within specialized cavities in subdomain IIA and IIIA [4-6,
19]. Site I, mainly binds hydrophobic heterocyclic molecules with centrally located
negative charge (e.g., warfarin, phenylbutazone); drug site II binds aromatic carboxylic
acids with a negative charge at one end of the molecule distal from the hydrophobic
structure (e.g., diazepam, ibuprofen) [1, 3, 20]. It is well known that a pH-dependent
conformational change occurs in HSA around physiological pH [1]. This conformational
change is called neutral to base or N-B transition. At pH 6 almost all the protein is in the
N conformation, whereas at pH 9, the predominant form is B. This N- transitions affect
the binding of some ligands especially site I ligands [21, 22]. Although most ligands for
albumin are hydrophobic anions, heavy metals are also known to bind to the protein [1, 3,
23, 24]. This multiplicity of binding sites on HSA makes it difficult to assess interactions
whether competitive or cooperative between different ligands bound to the protein even
though it is key for our understanding of the role of HSA in vivo.
Role of HSA in FFA Transport

HSA is well known for its ability to bind many fatty acids in different chain length for delivery to and from tissues according to metabolic demands. HSA can bind up to 7 molecules of fatty acids with moderate binding affinities ($K_d$) ranges from $10^{-8}$ to $10^{-6} \text{M}^{-1}$ (Figure 2) [1, 3, 5]. The fatty acids circulate in plasma at a total concentration just under $1 \text{mM}$ but $0.1\%$ of them are really free fatty acids, unbound FFA in the sense of being free in the plasma. The solubility of an ordinary fatty acid such as monomeric palmitate at pH 7.4 is less than $0.1 \text{nM}$. Over $99.9\%$ of the total FFAs are transported by HSA. Under normal physiological conditions, between $0.1$ and $0.2$ mol of FA are bound to albumin but the molar ratio of FA/HSA can rise to $6:1$ or greater in the peripheral vasculature during fasting or extreme exercise [25, 26] or under pathological conditions such as diabetes, liver and cardiovascular disease [1, 27]. It can also be affected by other factors as glycation and competition with toxic metabolites and drugs.

The interaction of FA and HSA serves to buffer the level of FFA and therefore regulates the rate at which FFA is transported to appropriate target cells. Despite the fact that FFA also bind to other blood components, serum levels of FFA are determined principally by FFA binding to albumin because the buffering capacity of albumin greatly exceeds that of any other blood components. To gauge serum FFA levels, therefore, it is important to consider the binding equilibrium between FFA and albumin. Direct membrane interactions of FA-albumin complexes have been implicated for the cellular uptake of FAs [28-30]. However, it is been generally accepted that a major part of fatty acid uptake across blood capillaries is achieved through the spontaneous dissociation of
fatty acids from the albumin into the aqueous phase of the intravascular compartment followed by fatty acid transfer to the apical surfaces of the endothelial plasma membrane by simple diffusion. Therefore, the concentration of HSA in the plasma should play key roles, which determine the real free fraction of FFA, unbound FFA. Changes in HSA/fatty acid binding interaction will modify the fraction of unbound FFA in plasma, thereby resulting in changed availability of unbound FFAs to cells. It is important to understand the specific interactions of fatty acids with certain amino acid on binding sites of HSA. Recent x-ray crystallographic studies have shown that HSA has seven distinct fatty acid binding sites located in three major domains [3, 5, 6]. However, the HSA structure determined by x-ray crystallography provided the location of each fatty acid binding site with great resolution up to 2.5 Å but fail to provide information regarding which fatty acid binding site is principal fatty acid binding site out of seven possible binding sites. Since only one or two fatty acids are usually associated with HSA under normal physiological condition, it is important to determine the primary and secondary fatty acid binding sites of HSA.

**Relationship between FFA and T2D**

T2D is characterized by insulin resistance in liver and muscle and impaired insulin secretion [31]. Studies on the pathophysiology of T2DM showed that both genetic and other acquired factors are involved in the development of insulin resistance of certain cells including adipocytes, muscle cells, and pancreatic β-cells [3, 5, 6, 31, 32]. Out of many acquired factors, which induce insulin resistance, obesity is the one of the major contributing factor to the onset of T2DM. Over 80% of patients with T2DM in the
Figure 2. X-ray crystallographic structure of HSA bound to palmitate
U.S. are overweight and these patients exhibited day long elevated FFA level in the plasma, which does not respond to insulin secretion after meal [31]. Many studies have shown that insulin resistant adipocytes lead to elevated plasma FFA levels due to the failure of insulin to adequately suppress lipolysis and chronically elevated FFA concentrations cause the stimulation of gluconeogenesis, induction of hepatic/muscle insulin resistance, and impairment of insulin secretion by pancreatic $\beta$-cells [31, 33]. Insulin is a potent inhibitor of hormone sensitive lipase, thereby restricting the release of FFA from adipocytes by inhibiting lipolysis. In T2DM patients the ability of insulin to lower FFA concentration in plasma is dysfunctional and prolonged exposure to the elevated FFA in plasma is believed to be the main cause of insulin resistance of muscle and liver cells. Although association between high FFA levels and the incidence of T2DM is well documented in publications, the mechanism of FFA effects on onset of T2DM is not clearly understood. Many studies on the effects of high FFA level mainly focused on FFA effects on adipocytes and muscle cells and these studies also indicated that the elevated FFA level is an important cause of $\beta$ cell destruction, disabling insulin secretion from pancreas after ingesting meal [31, 33-37].

Emerging evidence has suggested that long-chain acyl-CoA (LC-CoA) may be involved in the lipotoxicity of $\beta$ cell that occur after prolonged exposure of FFAs [38, 39]. In association with malonyl CoA resulting from glycolysis, it links fuel metabolism to the secretion of insulin. Cytosolic LC-CoA is the activated intracellular form of FFAs, and is the precursor for triglycerides, diacylglycerol and phospholipids [40]. Its transport into the mitochondria is blocked by carnitine palmitoyl- transferase 1 (CPT 1), which is inhibited
by a rise in malonyl CoA levels. The formation of malonyl CoA is catalyzed by acetyl-CoA carboxylase. LC-CoA induces a rapid, but slowly reversible $K^+_\text{ATP}$ channel opening [41], which may cause defects in insulin secretion (Figure 3). To elucidate the effects of FFA levels on insulin secretion, a study of the effects of unbound FFAs on pancreatic insulinoma cells in the presence of various concentrations of HSA can be informative since unbound FFAs are responsible for the lipotoxic action against pancreatic $\beta$ cells in the future.
Figure 4. Fatty acid metabolism in pancreatic beta cell
Cytosolic long chain acyl CoA (LC-CoA) increase from the metabolism of glucose and either exogenous or endogenously generated free fatty acids (FFA). HSL hormone sensitive lipase, TG triglyceride, ACS acetyl CoA synthetase, CL citrate lyase, ACC acetyl CoA carboxylase, OAA oxaloacetate, CPT-1 carnitine palmitoyl transferase-1
Overview of Research

General Accomplishments

The work presented in this thesis represents the first results of the direct measurements of the FFA concentration for the physiologically relevant long chain fatty acids in equilibrium with recombinant HSA and its mutants. Previously, FFA levels of long chain FA have not been measured directly in serum, in equilibrium or cellular studies. Values have, however been estimated using FA-albumin association constant determined in a series of measurements of the distribution of FA between a water-albumin and heptane phase [42]. Recently, the direct FFA measurements has been made possible by a method using a fluorescent probe composed of a fatty acid binding protein from rat intestine (I-FABP) derivatized with the fluorescent molecule acrylodan (ADIFAB) [43]. Using site-directed mutagenesis and a novel yeast expression system, recombinant HSA proteins with specific mutations of key amino acid residues involved in fatty acid binding were expressed. This FA-HSA equilibrium studies provides specific structural information on their mechanisms of interactions from the FA binding affinities and FFA values generated.

Appropriateness of Using a Protein Expression System

Although HSA has been the subject of a great many physical and biochemical studies for several decades, a clear understanding of the molecular basis of several of its specific binding interactions is only now beginning to emerge. Previous studies have used various experimental techniques to elucidate the specific structural HSA-ligand interactions [1, 3]. These studies, which include chemical modifications of HSA, ligand
analog studies, etc., have been largely unsuccessful in providing structural and mechanistic information because of the limitations of the techniques. Since many ligands display non-specific binding and there is evidence of allosteric interactions between subdomains IIA, IIIA, IB and IIB [44, 45], many researchers have attempted to circumvent this problem by producing proteolytic fragments to study ligand binding [46-50]. However, these types of studies raise serious concerns about the integrity of the fragments produced and the validity of conclusions drawn from binding experiments involving these fragments. In addition, due to the difficulty in creating HSA fragments, many of these studies were done on a variety of albumin species [51] and thus the information generated does not have direct relationship with HSA.

A protein expression system enables the production of HSA with no chemical or enzymatic modification. The protein will not be exposed to any extraneous condition, and thus its folded structure will likely be in its native conformation. Large quantities of the protein can be produced for a variety of studies and these studies will have direct implications to naturally occurring HSA. The in vitro and in vivo properties of recombinant HSA from Pichia pastoris have been well studied and there is close structural and functional similarity to HSA [52]. With recent developments in molecular biology, the techniques of site-directed mutagenesis and recombinant protein expression are widely being applied to biological problems [6, 20, 53-60].
By using site-directed mutagenesis in this novel yeast protein expression system to produce recombinant HSA proteins with specific mutations of key amino acid residues, which are involved in fatty acid binding, can provide specific structural information on the mechanisms of HSA/FFA interactions, i.e., which amino acid residues interact with the ligand’s functional groups to provide the ligand binding free energy.

Significance of Work

Many different genetic HSA variants have been identified electrophoretically through population genetics survey and routine clinical analysis. According to a population genetics survey, the cumulative frequency of albumin variants in most populations is only \( \sim 1 \) in 3000 and most of these variant HSA carriers are heterozygotes and have nearly equal amounts of wild type and mutant albumin in the plasma [61]. Therefore, it is very difficult to assess the effects of certain mutation that happened in HSA on metabolisms of particular interests. To date less than 70 different naturally occurring HSAs have been identified and confirmed by amino acid and DNA sequencing. Although several albumin mutations are found in diverse populations, many mutant albumin species are specific to certain ethnic groups. Some of HSA variants are extremely rare whereas other variants such as albumins Naskapi and Yanomama, are polymorphic, having an allele frequency \( \geq 1\% \) in certain American tribes [62]. Since 1971 the Italian Committee for Standardization of Electrophoretic Laboratory Methods (CISMEEL) has conducted an extensive genetic survey for albumin variants in serum with the participation of clinical laboratories throughout the nation [61]. They found 634 cases of inherited albumin variants in unrelated individuals, which include several
homozygote HSA variants. CISMEL also identified about 100 cases of transient albumin variant induced by pancreatic disease or penicillin therapy and 4 cases of analbuminemias. These finding indicates the prevalence of mutant forms of albumin to certain ethnic groups and thus provides justifications for needs of the HSA polymorphism studies in certain ethnic groups such as Native Hawaiian and pacific islanders. However, it is very hard to carry out genetic survey on certain population since most HSA variants exist as heterozygotes, making it fishing expedition style study. By using recombinant HSA protein and its variants, it will make it possible to produce homogeneous albumin sample and thereby identify target mutations, which are responsible for pathogenesis of certain disease such as T2DM.

In this study the information obtained by producing HSA mutants containing amino acid changes introduced into principal fatty acid binding sites will provide valuable data for identifying the specific amino acid responsible for fatty acid binding to HSA. Additionally, the binding equilibrium between FA and HSA mutants will elucidate this effect of HSA mutations on the FA binding affinities and the alteration of FFA fraction. This information could be used to design future in vivo studies on β cell lipotoxicity arising from these possible effects of HSA mutations in altering the FFA concentration, which could establish a physiological relevance to the relationship between plasma FFA levels and HSA polymorphism. Consequently, a basis for HSA polymorphism study that can examine how identifiable genetic differences among certain ethnic groups correspond to health disparities within such populations will be a possible establishment.
CHAPTER 2
MATERIALS AND METHODS

Synthesis and Purification of Recombinant HSA and its Various Mutants

For this study, we synthesized recombinant HSA and the following single and double mutants that were chosen based upon earlier studies of steroids and digoxin binding to HSA mutant proteins [57], NMR [54, 63] and X-ray crystallography [5]. The mutants studied were, R410A/Y411A, W214L/Y411W, R410A, R485L and W214L. Specific mutations were introduced into the HSA-coding region in a plasmid vector containing the entire HSA coding region as described previously [55]. The experimental methods consist of the following steps.

Cloning of HSA Coding Region

With human liver cDNA as template, the entire coding region of the HSA gene, including the native signal sequence, was amplified by polymerase chain reaction using Vent DNA polymerase. The resulting DNA fragment was inserted into the plasmid vector pHIL-D2 using standard cloning techniques. pHIL-D2 is a shuttle vector that can be manipulated by cloning in Escherichia coli and that can also be used to introduce genes into yeast species Pichia pastoris by homologous recombination. Specific mutations were introduced into the HSA coding region using site-directed mutagenesis as described previously [55].

Expression of Recombinant HSA

Each pHIL-D2 expression plasmid contained a methanol-inducible promoter upstream of the HSA coding region. For each expression plasmid, a yeast clone that
contained the expression cassette stably integrated into the yeast chromosomal DNA was isolated. The native HSA signal sequence, which was left on the HSA coding region, directed high-level secretion of mature HSA into the growth medium.

In the initial step, the cells were thawed on ice and thereafter plated out into histidine deficient YPD agar plates (10g yeast extract, 20g peptone, 20 g of agar and 20g of Dextrose (glucose) in 1L of dH2O) and allowed to grow for 24 hours at 30°C. The *Pichia pastoris* lacks the ability to synthesize histidine but the inserted pHIL-D2 vector contains this gene enabling the transformed *Pichia pastoris* to survive on these plates.

Using a single colony, an overnight culture of the cells were grown in 250ml of YPD medium (2.5g yeast extract, 5g peptone and 5g Dextrose in 250ml dH2O) shaking vigorously, 200rpm and at 30°C until an OD$_{600}$ of 1.3-1.5 was achieved.

A scale-up expression was then carried out using 25ml of the overnight culture to inoculate 1.5L histidine free minimal dextrose yeast growth medium (BMGY). The BMGY medium contained 10g yeast extract, 20g peptone, 100ml 1M potassium phosphate buffer pH 6.0, 100ml 10X YNB, 2ml 500x Biotin, 100ml 10X Glycerol dissolved in 1.5L dH2O. Four 2.5L flasks were used in total and were placed in a shaker at 30°C, 200rpm for three days. On the third day the cells were spun down at 5000rpm for 10 minutes at room temperature. To induce expression, the supernatant was decanted and the cell pellet was resuspended in 1/30 of the original culture volume in BMGY induction media. This media has all the other constituents as BMGY media but only methanol is added instead of glycerol. The culture was placed in 4X 2.5L flasks covered
with two layers of sterile cheesecloth and allowed to grow for three days at 30°C with shaking. Every day the cells were actively spiked with methanol at 10% the total volume.

**Verification of DNA Sequence of HSA Clones**

The total genomic DNA from each *P. pastoris* clone used to produce a particular HSA species was isolated using standard techniques. The genomic DNA isolated from each clone was used as template to amplify the entire HSA coding region by polymerase chain reaction. For each clone, the entire HSA coding region was sequenced using the dideoxy chain termination technique, and the translation product corresponding to this sequence matched a previously published cDNA of HSA at all amino acid positions except for the mutation introduced.

**Purification of Recombinant HSA**

The secreted HSA was isolated from growth medium as follows. The medium was brought to 50% saturation with ammonium sulfate at room temperature. The temperature was then lowered to 4°C, and the pH was adjusted to 4.4, the isoelectric point of HSA. The precipitated protein was collected by centrifugation and resuspended in 10X phosphate-buffered saline. Dialysis was carried out for 48 hours at 4°C against 100 volumes of phosphate-buffered saline (150mM NaCl, 40mM phosphate, pH 7.4). A sample of the precipitant before and after dialysis was run on a 10% sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) for 3 hours using commercial HSA as a standard. The sample for SDS-PAGE was prepared by denaturing the protein at 100°C for 5 minutes in a 1X loading dye containing 50μM dithiothreitol. The bands on the gel were visualized by staining with coomassie brilliant Blue (Sigma chemical) for
one hour. The solution was loaded onto a column of Cibacron blue immobilized on Sepharose 6B (Sigma) [64]. After washing the column with 10 bed volumes of phosphate-buffered saline, HSA was eluted with 3M NaCl. The eluent was dialyzed into phosphate-buffered saline and passed over a column of Lipidex-1000 (Packard instruments) to remove hydrophobic ligands possibly bound to the HSA [65]. The resulting protein migrated as a single band on SDS-PAGE. The final protein concentration was checked using the Bicinchoninic Acid Protein Assay kit (BCA) from Sigma based on Lowry method.

**FFA Fluorescence Measurements**

**ADIFAB Preparation**

ADIFAB was purchased from Molecular Probes as 200μg of lyophilized powder and stored at -20°C. For stability it is best to store ADIFAB at high concentration after reconstitution at 4°C [66]. Thus the 200μg of powder was brought up in 130μl of storage buffer consisting of 50mM TRIS, 1mM EDTA and 0.05% Sodium Azide at pH 8.0. This resulted in a stock of approximately 100μM ADIFAB. This low salt was used only for storage for not more than three months.

**Fatty Acid Preparation**

The sodium salts of the fatty acids, palmitate and oleate (Figure 4) were used to prepare the aqueous solutions. The stock solutions were prepared at a fatty acid concentration of 30mM in deionized water plus 4mM NAOH and 25μM butylated hydroxytoluene (BHT). BHT was dissolved in ethanol at 50mM and the final ethanol concentration in FA–sodium salt solution did not exceed 0.05% of ethanol. ADIFAB has been found to
react with organic solvents such as ethanol reducing the peak at 432nm and thereby
giving appearance of bound fatty acid [43] thus was the need of minimizing the ethanol
concentration in the measurements. 500μM dilutions of these stocks were made in water
plus 4mM NaOH but no additional BHT. Total Fatty acid was determined by WAKO
NEFA C kit and aliquots stored in -20°C for no longer than 3 months.

**FFA Determination in FA/HSA complexes**

Upon excitation at 386 nm, ADIFAB fluorescence at 432 nm in the absence of
fatty acid and at 505nm in the presence of fatty acids. Thus the intensity ratio (R) of
505nm to 432 nm is indicative of the amount of fatty acid present. Measurements of R
values were done with an SLM 8000C fluorometer using the photon counting mode.
ADIFAB was used to determine FFA levels in the presence of palmitate or
oleate/albumin complexes. Wild type, R410AA, W214L/Y411W, R410A, R485L and
W214L recombinant albumin mutant proteins were all treated identically as follows. For
each complex, FFA levels were determined by measuring the fluorescence as follows.
First, the value of the ADIFAB ratio is measured without FA present (R₀) and thus the
fluorescence intensities were measured at 505nm and 432nm in a cuvette with 4μM
albumin in measuring buffer pH 7.4 containing 20mM HEPES, 140mM NaCl, 5mM KCl
and 1mM Na₂HPO₄ (blank intensities). 0.2μM ADIFAB was then added to the cuvette
and again the intensities at 505nm and 432nm were measured. The R₀ value is equal to:

\[
R_0 = \frac{I_{505}^0 - I_{505}^{blank}}{I_{432}^0 - I_{432}^{blank}}
\]
Figure 4. Molecular structures of palmitate and oleate
Thirdly, the appropriate aliquots of FA were added to the cuvette to give total FA concentrations of between 0 and 24 \( \mu \text{M} \). Once again the fluorescence intensities at 505 nm and 432 nm were measured and \( R \) calculated as:

\[
R = \frac{I_{505} - I_{505}^{\text{blank}}}{I_{432} - I_{432}^{\text{blank}}}
\]

The same albumin (4\( \mu \text{M} \)) and ADIFAB (0.2\( \mu \text{M} \)) concentrations were used in each sample and the total volume of samples was 1.5 ml. Just prior to FA addition, the concentrated stocks of FA sodium salts were warmed to temperatures above the FA melting point (62°C for palmitate and 37°C for oleate). FA was then added in small volumes to the sample in the third step described above which was maintained at 37°C and immediately mixed by drawing the solution in and out of the pipette. Between each FA addition the cuvette was allowed to incubate for 10 minutes at 37°C. After the 10 minutes incubation the 432 nm and 505 nm intensities were determined from the three samples all at 37°C. This procedure was repeated for total FA to total albumin ratio (\( \nu \)) values between 0 and 6 and in steps of approximately 1.0. ADIFAB itself has little effect on the FFA concentration in these measurements because of the FA binding capacity of albumin and because the concentration of albumin and total FA are much greater than that of ADIFAB [67].

As consequence, the FFA concentration was determined according to [43] from the 505 nm and 432 nm fluorescence by:

\[
[\text{FFA}] = \frac{K_0 Q (R - R_0)}{(R_{\text{max}} - R)}
\]

(1)

Where \( R \) and \( R_0 \) are the measured ratios of 505 to 432 nm intensities in the presence and or in the absence of FFA with blank intensities subtracted respectively, \( R_{\text{max}} \) is the value
when ADIFAB is saturated, $Q = \frac{I_F(432)}{I_b(432)}$, and $I_F(432)$ and $I_b(432)$ are the ADIFAB intensities with zero and saturating concentrations of FFA respectively. Values of $Q$ and $R_{\text{max}}$ were found to be 19.5 and 11.5 [43] respectively and $K_d$ values of ADIFAB on binding oleate and palmitate are 0.28 $\mu$M and 0.34 $\mu$M respectively according to [66].

The amount of fatty acid that was bound to ADIFAB probe was next calculated as:

$$\text{ADIFAB}_{\text{bound}} = [\text{ADIFAB}]_{\text{total}} \frac{Q (R - R_o)}{(R_{\text{max}} - R) + Q (R - R_o)}$$  \hspace{3cm} (2)

Finally, the amount of FA bound to albumin, after each titration was calculated as [68]:

$$[\text{FA}]_{\text{bound}} = [\text{FA}]_{\text{total}} - [\text{FFA}] - [\text{ADIFAB}]_{\text{bound}}$$  \hspace{3cm} (3)

### Analysis of Data

All titrations of a particular HSA species with either oleate or palmitate were done three times. The number bound $[\text{FA}]_{\text{bound}} / [\text{HSA}]_{\text{total}}$ fraction were determined at each point along the titration. Each of the three data sets for each $K_d$ determination was fit to the equation shown below by nonlinear regression (least-squares method) using the computer program Prism (Graphpad).

$$\text{Number bound} = \frac{B_{\text{max}} \cdot X}{(K_d + X)}$$  \hspace{3cm} (4)

This binding equation assumes one binding component with binding capacity ($B_{\text{max}}$) and a dissociation constant ($K_d$) value. The variable $X$ represents the FFA concentration. A curve corresponding to the best fit of the data to this one component equation was then generated for the binding of each HSA species to each FA. Since all three of the replicates were done identically, averaging the number bound and FFA concentration at each point on the titration for all the three data sets created an average data set.
CHAPTER 3
RESULTS

Purification of Recombinant HSA and its Various Mutants

After a week of growth and induction, the transformed *Pichia pastoris* were centrifuged and the supernatant was harvested. The HSA protein should be in the supernatant because the inserted gene still contained the native signal sequence for albumin. This would enable the newly synthesized protein product to be inserted into the endoplasmic reticulum and secreted to the external media through the yeast cell’s secretory pathway. Wild type rHSA and the double mutants; W214L/Y411W and R410A/Y411A as well as the single mutants; R410A, R485L and W214L were successfully synthesized. Figure 5. represents SDS-PAGE column showing the HSA bands in the right location size of 67 kDa before and after performing affinity chromatography. The band shows a concentrated fraction of the protein after elution.

HSA Binding Affinities to FFAs

It was found that the ADIFAB’s fluorescence shifted from 432nm to 505nm upon binding of free fatty acids and increased as the total fatty acid concentration was increased in the reaction mixtures. Figure 6 shows the ADIFAB fluorescence spectra pattern when oleate was titrated into the measuring buffer containing wild type rHSA and ADIFAB. Figure 7 shows the binding isotherms of wild type rHSA, W214L/Y411W and R410A/Y411A for palmitate. The curves were generated based on one binding component. Visually, the shapes of the curve for the double mutants are not altered from that of the wild type rHSA. However, the corresponding $K_d$ values for their respective
curves showed a slight increase in W214L/Y411W than R410A/Y411A when compared to the wild type rHSA (Table 1). On the other hand, the single mutants; R410A, W214L and R485L showed altered binding isotherm patterns when compared to the wild type rHSA with R485L being the most notable (Figure 8). Their corresponding Kd values as shown in Table 1 followed the same pattern with R485L having less binding affinity.

The Kd values of rHSA and its mutants for oleate binding followed the same pattern as for palmitate. However, oleate showed higher affinities to all the HSA species when compared to palmitate binding affinities. Fig 9 shows the binding isotherms of wild type rHSA and the double mutants on binding oleate, while Figure 10 depicts the single mutants. Table 2 shows their corresponding Kd values.
Figure 5. An 8% SDS–PAGE showing the wild type rHSA protein before and after performing affinity column chromatography. Column 1 is commercial HSA, column 2 is the dialyzed sample before performing affinity chromatography, column 3 is the wash and column 4 is the eluent.
Figure 6. Fluorescent monitored titration of ADIFAB with wild type rHSA/Oleate mixtures
ADIFAB concentration of 0.2μM in measuring buffer containing 20mM HEPES, 140mM NaCl, 5mM KCl and 1mM Na₂HPO₄, pH 7.4 at 37°C and wild type rHSA is 4μM. Oleate concentration is labeled adjacent to the corresponding data trace. The final oleate concentration was 24μM.
Figure 7. Binding isotherms of wild type rHSA, W214L/Y411W and R410A/Y411A for palmitate
The FFA concentrations were determined with ADIFAB fluorescence ratios measured at pH of 7.4, [ADIFAB] (0.2μM), [HSA] (4.0 μM) and total FA titrated between 0-24μM at 37°C. The curves correspond to the best fit of the data from three experiments to one component binding.
Figure 8. Binding isotherms of R410A, W214L and R485L for palmitate
The FFA concentrations were determined with ADIFAB fluorescence ratios measured at pH of 7.4, [ADIFAB] (0.2μM), [HSA] (4.0 μM) and total FA titrated between 0-24μM at 37°C. The curves correspond to the best fit of the data from three experiments to one component binding.
Table 1. $K_d$ values for palmitate binding to HSA

The average $K_d$ values determined by fluorescence spectroscopy are shown for the binding of each HSA species with palmitate. Values represent ± 1 standard deviation from three experiments to one component binding.

<table>
<thead>
<tr>
<th>HSA</th>
<th>PALMITATE</th>
<th>$K_d$(nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WILD TYPE</td>
<td>42.67 ± 6.78</td>
<td></td>
</tr>
<tr>
<td>W14L/Y411W</td>
<td>33.16 ± 3.42</td>
<td></td>
</tr>
<tr>
<td>R410A/Y411A</td>
<td>37.94 ± 5.83</td>
<td></td>
</tr>
<tr>
<td>R410A</td>
<td>54.58 ± 16.12</td>
<td></td>
</tr>
<tr>
<td>W214L</td>
<td>66.19 ± 16.78</td>
<td></td>
</tr>
<tr>
<td>R485L</td>
<td>99.43 ± 17.60</td>
<td></td>
</tr>
</tbody>
</table>
Figure 9. Binding isotherms of wild type rHSA, W214L/Y411W and R410A/Y411A for oleate
The FFA concentrations were determined with ADIFAB fluorescence ratios measured at pH of 7.4, [ADIFAB] (0.2 μM), [HSA] (4.0 μM) and total FA titrated between 0-24 μM at 37°C. The curves correspond to the best fit of the data from three experiments to one component binding.
Figure 10. Binding isotherms of R410A, W214L and R485L for oleate
The FFA concentrations were determined with ADIFAB fluorescence ratios measured at pH of 7.4, [ADIFAB] (0.2 μM), [HSA] (4.0 μM) and total FA titrated between 0-24 μM at 37°C. The curves correspond to the best fit of the data from three experiments to one component binding.
Table 2. $K_d$ values for oleate binding to HSA

The average $K_d$ values determined by fluorescence spectroscopy are shown for the binding of each HSA species with oleate. Values represent $\pm 1$ standard deviation from three experiments to one component binding.

<table>
<thead>
<tr>
<th>HSA</th>
<th>OLEATE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_d$ (nM)</td>
</tr>
<tr>
<td>WILD TYPE</td>
<td>32.94 ± 3.99</td>
</tr>
<tr>
<td>W14L/Y411W</td>
<td>27.94 ± 3.02</td>
</tr>
<tr>
<td>R410A/Y411A</td>
<td>32.50 ± 3.03</td>
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<tr>
<td>R410A</td>
<td>37.90 ± 2.30</td>
</tr>
<tr>
<td>W214L</td>
<td>40.39 ± 3.12</td>
</tr>
<tr>
<td>R485L</td>
<td>62.31 ± 11.58</td>
</tr>
</tbody>
</table>
CHAPTER 4
DISCUSSION AND CONCLUSION

Since previous studies have showed that the IIIA subdomain of HSA is the one of the two major ligand binding sites and could load up to two fatty acids [5, 58], we hypothesized that this subdomain of HSA is the principal FA binding site and any mutation introduced into the site of HSA could result in changed FA binding to HSA. For instance, X-ray structural studies on FFA binding to HSA clearly showed that arginine at position 410, tyrosine 411 and arginine 485 of subdomain IIIA are in direct contact with the carboxylate groups of bound fatty acids. Additionally, recent studies using X-ray crystallography and NMR spectroscopy has shown that the fatty acid binding sites 4 and 5 within domain III represent two of the three high affinity fatty acid binding sites on HSA [63]. In the mutagenesis studies, we therefore substituted these amino acids with other amino acids of different properties. For example, hydrophobic alanine and leucine were used to substitute for the basic amino acid arginine at position 410, aromatic hydrophobic tryptophan was used to substitute for aromatic polar amino acid tyrosine at 411 and hydrophobic leucine was substituted for basic amino acid arginine at 485. Tryptophan at position 214 in subdomain IIA was also changed to leucine since it has been shown to be the key amino acid involved in various HSA/ligand binding interactions [3].
The subdomains IIIA and IIA binding sites for fatty acids have asymmetric distribution of amino acid residues leading to a hydrophobic surface on one side and a basic or positively charged surface on the other. The region is thus primarily an elongated sock-shaped pocket wherein the foot region is mainly hydrophobic and the leg is primarily hydrophilic [3].

HSA mutants with the double mutations namely R410A/Y411A and W214L/Y411W exhibited slightly increased binding affinities to both oleate and palmitate when compared to wild type recombinant HSA. On the other hand, the single substitutions R410A, R485L and W214L decreased the binding affinities with R485L most notable. Specifically, these results suggest that the double substitutions involving the changes of the polar amino acids arginine and tyrosine at position 410 and 411 with alanine and tryptophan respectively, enhanced binding and thus indicating that hydrophobicity is the major ligand binding force for the FFA binding to HSA. This alteration in binding supports the X-ray crystallographic data that showed that R410A and Y411A are the key amino acids in the binding pocket of subdomain IIIA of HSA. Interestingly, the single mutants R410A and W214L showed a decrease in affinity implying that replacing arginine and tryptophan with the smaller hydrophobic amino acids alanine and leucine was not favorable for fatty acid binding. It is reasonable to speculate that R410 is engaged in hydrogen bonding with FFA carboxylic group.
Another single mutant, R485L showed significant decrease in binding affinity for palmitate and oleate. Substituting R485 with leucine is unfavorable for fatty acid binding implying again that arginine at position 410 is involved in hydrogen bonding with FFA carboxylic group.

Our findings on the effects of HSA’s mutation on FFA binding provide a possible explanation on the role of HSA in affecting the secretion of insulin from pancreatic β-cells by modulating the exposure of these cells to FFAs. Therefore, a study of serum albumin’s effects on FFA stimulated insulin secretion from cultured pancreatic β-cells will validate the findings of this work. Since HSA is the only FFA carrier molecule in human serum and that many studies have shown the close relationship between elevated FFA levels and the incidence of T2D, HSA may play a role in modulating the effects of FFA in the pathogenesis of T2D. The concentration of the unbound FFAs available to the cells will be determined to a large extent by the affinity of FFAs to albumin. Since the level of FFAs will determine the degree of B-cell lipotoxicity, any mutations in the fatty acid binding sites of HSA could influence the effect by modifying FFA binding affinity. Specifically, we may project from these findings that the double mutants might potentially bind fatty acids with a much higher affinity and thus depriving fatty acids available for pancreatic β-cells uptake and subsequently leading to decreased lipotoxicity as compared to the single mutants with lowered binding affinities.

In summary, site-directed mutagenesis and the Pichia pastoris system provide a valuable tool to generate recombinant HSA and its mutants for protein functional studies. HSA ligand binding interactions can be modified by single nucleotide polymorphism and
rationale for a possible HSA polymorphism study. Amino acid changes on key FFA binding sites of HSA's subdomain IIA and IIIA altered palmitate and oleate binding affinities. This study might contribute to the understanding of HSA's mutational effects on FFA stimulated insulin secretion from cultured pancreatic β-cells as well as its role in the pathogenesis of T2D.
APPENDIX
OVERVIEW OF SITE DIRECTED MUTAGENESIS AND PROTEIN EXPRESSION SYSTEM

Summary

The purpose of this appendix is to provide a visual overview of the technology used to produce recombinant HSA mutants described in chapter 2. The information is presented in a series of figures, which describes the steps involved that would help elucidate the molecular biology and yeast expression system to non-experts in this field. Figure 11 shows the plasmid vector used to mutate the HSA coding region and to introduce the mutated coding region into the yeast species *Pichia pastoris* by homologous recombination. The functions of each gene in the vector are described in the figure legend. Figure 12 illustrates the basic site directed mutagenesis procedures used to introduce desirable mutations in the HSA coding region using various restriction enzymes and ligases. Figure 13 describes the procedures for introducing the linearized expression vector containing mutations into yeast genome by electroporation and homologous recombination. Figure 14 shows the method used to select the best yeast clones that have integrated the expression cassette in the *Pichia pastoris* alcohol oxidase gene (AOX) and to identify those clones secreting recombinant HSA.
Figure 11. pHiL-D2 HSA expression vector

The promoter and terminator sequences from the *Pichia pastoris* alcohol oxidase gene are labeled as promoter and terminator, respectively. The HSA coding region, which was, amplified from liver cDNA using PCR and inserted into pHiL-D2 to construct the above vector is labeled as HSA gene. A DNA sequence that allows the plasmid to replicate in *E. coli* autonomously of chromosomal DNA is labeled Ori. A gene that confers resistance to the antibiotic ampicillin and allows for screening of *E. coli* cells that have the plasmid is labeled Amp. A gene which allows a *Pichia pastoris* strain unable to synthesize histidine to make histidine when the gene is integrated in the yeast chromosomal DNA, is labeled HIS4. 5’ AOX and 3’ AOX refer to regions that are homologous to the 5’ and 3’ ends of the alcohol oxidase gene and provide targets for homologous recombination between the expression cassette and the *Pichia pastoris* alcohol oxidase gene (AOX).
pHiL-D2/HSA

10.2kb
Figure 12. Site directed mutagenesis of the HSA coding region

The diagram shows how an oligonucleotide containing a single base pair mismatch with the wild type HSA coding region is used to introduce a stop codon into the HSA coding region.
Linearize plasmid → Introduced into *Pichia pastoris*
Figure 13. Introduction of the HSA expression cassette into *Pichia pastoris*

The diagram shows the mechanism of homologous recombination between the HSA expression cassette and the alcohol oxidase gene (AOX) of *Pichia pastoris*.

The linearized vector DNA with Amp and Ori removed is introduced into *Pichia pastoris* by electroporation.
PHIL-D2/HSA 10.2 kb

Not I Enzyme digest

By Electroporation

Transform DNA into *Pichia pastoris* strain lacking HIS4 gene

(DOUBLE HOMOLOGOUS RECOMBINATION)

Select for transformants on histidine deficient plates—those colonies that have taken up the HIS4 gene will survive
Figure 14. Identification of *Pichia pastoris* clones expressing HSA

Many *Pichia pastoris* clones will have taken up the HIS4 gene without integrating the rest of the expression cassette in the *Pichia pastoris* genome. Those clones in which homologous recombination has occurred specifically at the alcohol oxidase gene (AOX) are most likely to contain the entire expression cassette. Integrating into AOX destroys the AOX gene resulting in a slow growth on methanol phenotype as the AOX gene product is needed for metabolism of methanol.
1. Select colonies from the -HIS plate and patch colony onto a -HIS, +GLY and a -HIS, +MEOH plate. Colonies which grow slowly on methanol no longer contain the AOX gene indicating homologous recombination into the AOX gene.

2. Select colonies shown in red which are unable to grow on methanol plate and grow for three days in media containing glycerol as the carbon source.

3. Pellet the cells and remove the media.

4. To induce expression, resuspend pellet in media containing methanol as the carbon source to induce the expression of HSA gene incorporated into pichia pastoris genome.

5. After two to four days of induction collect supernatant and analyze by SDS-PAGE. Of the colonies expressing HSA scale up using the clone showing the highest expression level.
LITERATURE CITED


