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TRANSANALYSIS OF HUMAN SERUM ALBUMIN FUNCTION BASED ON EVOLUTIONARILY AND EXPERIMENTALLY UNIQUE AMINO ACIDS

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ABSTRACT

Human serum albumin (HSA) is a predominant blood protein with binding affinity to many endogenous and exogenous compounds. Following an overview of this protein, the additional capacity for heme binding is analyzed as both an evolutionarily retained and experimentally inducible function. An inclusive analysis of HSA has examined all facets of HSA's current structure, potential variations of this structure that may be induced experimentally, and the potential for evolutionary selection regarding beneficial variance. Initial background information was analyzed including structure and function, the role of HSA within the body, and medical applications, including a proposal for protein modification to facilitate the new application of heme binding and oxygen carrying ability. Once these variations were reviewed, 10 different species (mammalian, reptilian, and avian) were examined for conserved or new heme binding functionality within five relevant heme binding regions. Analysis at these regions, positions 138, 142, 146, 161, and 190 showed relative conservation, but the potential for heme binding as an accommodation for oxidative stress appears negligible within these species. Following this evaluation, procedure and methodology was outlined for the generation of mutant HSA plasmid and possible routes of generating mutant HSA protein with heme binding capability.

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Chapter 1 Introduction and Background Information

General overview of HSA structure and Function

Human serum albumin (HSA) is one of the most prevalent blood proteins, and is commonly introduced as "the most abundant protein in plasma".[1] A monomeric protein, HSA has multiple domains which are used for a variety of functions and binding interactions. These domains give HSA an affinity for a wide variety of ligands, both substances within the body (usually fatty acids, hormones, metal ions etc.), and foreign compounds such as drugs and pharmaceuticals.[1] This remarkable affinity is used in a litany of inherent bodily processes, and has linked HSA to: observed antioxidant processes, a sizeable effect on pharmacokinetics, and the ability to be used in medical applications. On top of this native ability, the genetics and amino acid sequence of HSA have been carefully examined, leading to the potential for a variety of induced functions within the protein. Some such functions includes oxygen and carbon dioxide binding abilities (obtained through various amino acid point mutations) and use as a stable nanoparticle in gene therapy and delivery. Through experimental modification, HSA may be altered to expand functionality, allowing heme binding and the potential for effective gene therapy.[2] These properties will be examined using current literature, and the information gleaned from these reviews will be used in an attempt to propose a new use for human serum albumin.

HSA is an all-α chain nonglycosylated protein which contains 3 domains (I, II, and II).[3] Each domain has helical subdomains A and B which are connected by random protein coils.[3] The binding capabilities of HSA lie within these domains as well as in clefts and polypeptide

linkages which connect domains and subdomains. One of the prominent binding regions are the seven areas of fatty acid equivalent binding cites denoted as FA1-FA7.[3] The FA1 binding site specifically has the ability to bind a heme-Fe(III) complex, which is stabilized in the connecting loop between subdomains IA and IB.[3] The manipulation of amino acids in this area is what inevitably allows for heme binding capability, a property which will be discussed in more detail in the coming chapters.

Overview of HSA's relation to normal bodily function and inherent uses

One of HSA's more useful properties within the body, which has been recently observed, is its role as an antioxidant. Taverna et al., 2013 examine HSA's structure in relation to this property.[4] Generally, it seems that a stable structure and multiple methionine and cysteine residues (only one of which, Cys-34, exists as a free residue) allows the binding of multiple ligands to HSA as well as the possession of free radical trapping properties.[4] One of the most important binding properties which is inherent in the first few amino acids of the N-terminus is the high affinity for metal ions such as copper and iron. It has been shown that these ions (such as Cu(II) and (FeII)) are commonly involved in such reactions as the Fenton reaction and Haber-Weiss reaction if a free radical scavenger such as HSA does not bind them. If unbound, metal ions such as Cu(II) and Fe(II) are components of these two reactions and generate volatile reactive oxygen species (ROS) in association with hydrogen peroxide.[4] The authors also observed free radical trapping properties inherent in HSA's structure. It turns out the Cys-34

residue exists in a reduced form of HSA (HSA-SH) observed roughly two-thirds of the time, and possesses a unique binding ability to ROS's as well as reactive nitrogen species. **Figure 1** shows the oxidation and thiolation of HSA-SH under oxidative and nitrosative stress respectively, leading to the binding and neutralization of reactive oxygen and nitrogen species.[4] HSA also has the ability to scavenge and stabilize species such as hydrogen peroxide, peroxynitrite, superoxide, and hypochlorous acid.[4] Ultimately, it seems that the cysteine and methionine residues which are so prevalent in the structure are chiefly responsible for this redox functionality, as the sulfur groups in these residues are prone to being oxidized. The authors of this article go so far as to state that the function of these residues account for roughly 40-80% of antioxidant activity within HSA, and HSA itself is responsible for around 70% of the antioxidative properties within blood serum.[4] Since it is known that these reactive species are responsible for a myriad of problems within the body, HSA's inherent ability to neutralize these particles is certainly an important feature.

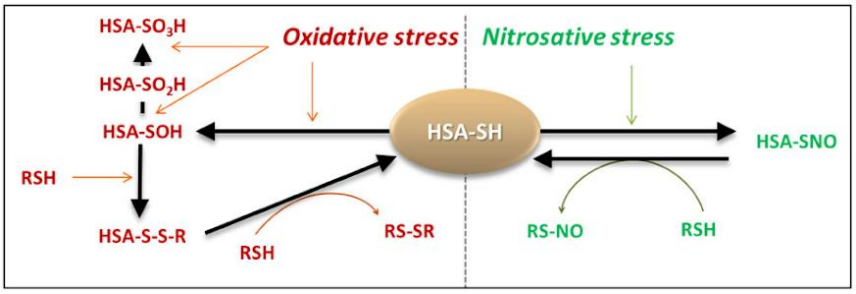


Figure 1 The binding of reactive nitrogen and oxygen species to HSA[4]

Another interesting aspect of HSA that may be examined is its effect on pharmacokinetics. Due to its binding affinity for foreign compounds (commonly at the Cys-34 residue), the use of HSA as a transport protein within the blood plays a role in most bodily drug interactions. Namely, the dendrimer binding abilities associated with drug processing as tested by Froehlich et al., 2009.[5] Dendrimers are synthetic macromolecules that are highly branched and each branch terminates in reactive species. The structure of the poly(amidoamine) dendrimer is depicted in **Figure 2**, illustrating the abundance of terminal reactive species. These reactive molecules are usually associated with various drugs and their delivery with poly(ethylene glycol) modifications to limit toxicity. Dendrimer interaction with HSA was of interest to the authors in order to determine the effect HSA binding has on these drug delivery molecules. The authors examined the effect of specific dendrimers named poly(amidoamine) (PAMAM-G4), poly(ethylene glycol) poly(amidoamine) (m-PEG-PAMAM-G3), and the m-PEG-PAMAM-G3 isomer, m-PEG-PAMAM-G4, when combined with aqueous HSA.[5] Using methods such as absorption, fluorescence, and infrared spectroscopy, authors observed that HSA did complex with the various dendrimers, thus neutralizing their drug binding activity and delivery ability. This binding and dendrimer neutralization also altered the structure of HSA. HSA showed the same denaturation when bound to each of the four tested dendrimers, displaying a decrease in alpha

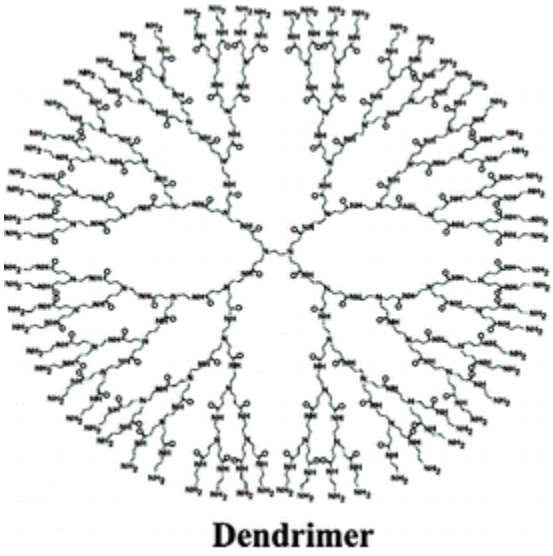


Figure 2 The circular reactive structure of a poly(amidoamine) dendrimer.[5]

helix character and an increase in the prevalence of turns and random coils.[5] This study provides empirical evidence for the reactive involvement HSA has with various synthetic particles, and ways in which their path and/or structure may be altered.

In addition to antioxidant properties and an influence in drug activity, HSA inherently bears many biomedical applications. Fanali et al., 2012 outline a variety of these applications, usually pertaining to the medical use of HSA infusions and their therapeutic effects.[1] Most of these infusions may be observed in cases of fluid resuscitation to combat ailments such as hemorrhagic shock, surgical blood loss, and hypovolemia to name a few.[1] Due to HSA's prevalence in the blood, it is known that HSA is responsible for 80% of the osmotic pressure within blood vessels, thus making HSA transfusions a cure for edema in starving patients.[1] It is also logical that HSA may function as an effective blood plasma substitute in patients that require regular blood transfusions. In addition to these functions, it has been speculated that HSA reacts with macromolecules and solutes in the subendothelium and interstitial matrix to increase or decrease capillary cell membrane permeability.[1] In addition to using HSA to potentially regulate permeability, human serum albumin may have some therapeutic anticoagulant and antithrombotic functions, binding nitrous oxide and preventing it from its role in platelet aggregation.[1] This prevention of platelet aggregation may lead to the use of HSA as a mild blood thinner in some unique cases.[1]

In addition to the various uses of HSA in medical infusions, HSA may also be used to combat certain diseases. Intravenous HSA is common, for example, in treatment of various chronic liver diseases. Cirrhosis, in particular, often requires HSA treatment, which decreases renal impairment and mortality in these cases. The authors indicate that treatment is usually used to negate the negative effects of paracentesis, or bodily draining, and its association with

hemodynamic destabilization.[1] Human serum albumin is also used in treatment for various nutrient deficiencies and hypoalbuminemia. Due to HSA's prevalence, it may be commonly broken down (leading to the HSA deficiency disorder hypoalbuminemia) under poor nutrient conditions within the body. The authors mention an observed association between hypoalbuminemia and morbidity in hospitalized patients, elderly within the community, and patients prior to surgery.[1] The prevalence of HSA within the body clearly alludes to its numerous properties of functionality within the body, and it should come as no surprise that a variety of these functions may be utilized for medical benefit.

New HSA functionality through mutation and modification

Now that some inherent properties of HSA have been examined, the inducible properties of this Swiss army knife-like protein may be addressed in detail. One trait of HSA mentioned earlier (which is impressive even in wild-type protein) is a remarkable binding ability; despite an impressive range of affinity normally, HSA may be mutated to stabilize the binding of heme groups. Komatsu et al., 2004 attempt this mutation, modifying subdomain IB to allow effective oxygen carrying ability. More specifically, the addition of a proximal histidine residue to subdomain IB will prevent the oxidation of a ferrous HSA-hemin complex, stabilize said complex, and grant it oxygen carrying abilities comparable to that of hemoglobin.[2] The addition of this amino acid as well as a variety of other point mutations were carried out using a site-directed mutagenesis kit, and the existence of the HSA-heme complex was tested using techniques such as SDS-page and infrared spectroscopy.[2] Ultimately, the authors did confirm the presence of this stable complex after HSA mutation, and hemoprotein like affinity for oxygen

and carbon dioxide was also observed (despite being low relative to the affinity of hemoglobin). The implications of this ability are quite sizeable, and may be used in future gene therapy to utilize HSA as a blood protein with even more functionality, hypothetically that of a myoglobin or hemoglobin protein.

Another inducible function of HSA is described by Look et al., 2015.[6] These authors place focus on an up and coming medical technique known as gene therapy, which effectively counteracts the effect of faulty genes in living systems by introducing a new and "healthy" gene. One of the more prominent issues with this therapy method is the use of effective vehicles for gene delivery within the system. Modified viruses are a common method of delivery due to their simplistic structure and proliferative gene distribution through infection, but these transporters have many drawbacks including high cost to produce and severe immune or inflammatory response.[6] With safe methods of gene delivery limited, the need for a stable and effective transport protein is high, and the authors of this article speculate that HSA may be a solution.

Though nonviral methods of gene delivery do exist (silica nanoparticles, lipoplexes etc.) they are usually cytotoxic or have low efficiency in transfection. HSA, on the other hand, is known to be nontoxic, nonimmunogenic, and biodegradable, making it a practical alternative.[6] To facilitate the use of HSA as a method of gene delivery, the inherent structure must be modified. In the conversion to a nanoparticle (HSA-NP) the negative surface of HSA must be reduced in order to effectively bind negative plasmid DNA. Though the authors performed this cationization, it has been observed that HSA-NP is most effective when specialized for a specific delivery, namely with the binding of a unique ligand. The authors focus on two HSA-NP-peptide complexes incorporating RGD (a short sequence of amino acids

arginine-glycine-aspartate) and Tat (and HIV-1 transactivator of transduction).[6] It seems that both these ligands aid not only in the uptake of various bodily molecules like genetic material, but in cellular penetration as well.[6] **Figure 3** illustrates the structural generation of HSA-NP, the incorporation of plasmid, stabilization, and the structure of the added ligands.[6] After generating the modified HSA nanoparticles (yield was determined gravimetrically) these particles were introduced into HEK293T culture cells as well as human mesenchymal stem cells (hMSC). This proved to test both cellular reception and the stability of HSA-NP, based on its change or undesired effect on the cell.[6] Based on reactions within these cultures (delivering a gene associated with Luciferase which generated glowing cells if the delivery was successful), it

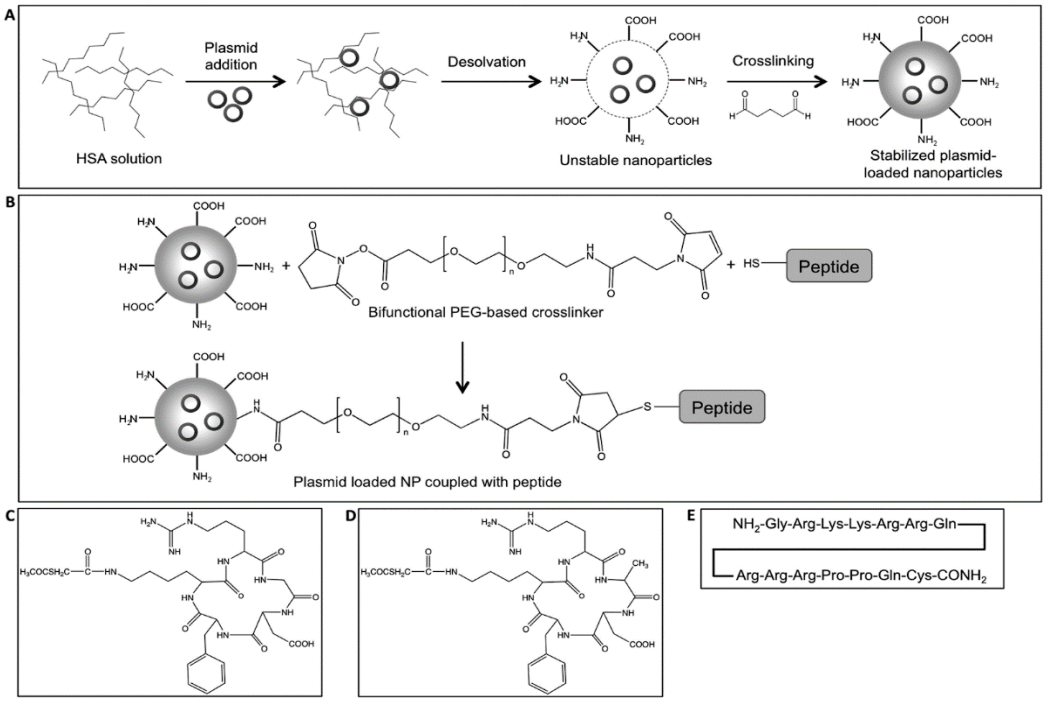


Figure 3 HSA-NP complexed with RGD (C) and Tat (E).[5]

seemed this system does have transfection efficacy, and exhibits limited destabilization or harmful effects towards the cultured cells.[6] The use of a preexisting body compound such as HSA may be one of the most viable methods of gene delivery, even if slight modifications are

required. If this process may be shown as it has in this study to be safe and effective, the idea of gene therapy in human subjects may be less a notion of science fiction.

Proposal for new HSA functionality based on existing modification or new technique for modification

It is quite clear from the literature reviews that HSA has a multitude of functions, relating mostly to an extensive binding affinity to different compounds within blood plasma. I believe that most of HSA's therapeutic potential lies within the modification and amplification of this affinity. The abundance of methionine and cysteine residues (specifically the inclusion of sulfur) undoubtedly contribute to oxygen and nitrogen reactivity. Though Komatsu et al., 2014 explored a variety of single and double mutations to stabilize heme binding, a variety of other mutations within HSA may be explored to increase this stabilization.[2] Multiple mutants induced concurrently may generate a more stable complex, and therefore increase oxygen carrying capacity. These double mutants may be a variety of combinations, such as an additional histidine and the alteration of isoleucine to the smaller leucine residue in the 142 amino acid position (denoted as I142L). This combination would likely stabilize the heme group more than an additional histidine alone. Other adjustments in the size of residues may also be effective with the histidine alteration, such as the substitution of leucine with the large aromatic tyrosine residue.

The large functional variance of HSA suggests that there may be a large evolutionary divergence among organisms in structure and function of related serum albumin (SA). Wardell et al., 2002 observed a retention of heme binding sites between mammals and amphibians despite

the fact that a large portion of SA was dissimilar.[7] Based on this observation, it is likely that functional variance may be observed throughout a variety of mammalian species such as mice, bears, and those that are under oxidative stress like whales or dolphins. If the prevalence of mutations associated with heme binding can be observed and or mapped, such genetic analysis may give insight into the evolutionary answer for serum albumins variance and its possible function. The comparison of amino acid sequences for functionality will be covered in Chapter 2, and an attempt to experimentally induce functionality will be outlined in Chapter 3.

Chapter 2 Genetic analysis and comparison of serum albumin variants

A large variety of functionality has been addressed in HSA in both *in vitro* and *in vivo* settings, but how has serum albumin (SA) been retained throughout other mammalian species? Has evolution retained useful areas of SA (such as that of heme binding) or denoted new variance to aid with species-specific problems? To answer these questions, sequences of SA (both recorded and predicted which is denoted as **P** in the chart below) have been compared for similarity and functionality. Data from 10 species were collected based on a specific set of criteria: relative similarity with HSA (predominantly mammals), variation in species and living environment, and strains on the species such as the potential for oxidative stress. Species have been summarized in Table 1.

Table 1. Species with SA function and conserved regions.

Animal	**Genus Species**	**Gene**	**Protein Ex #**
Mouse	*Mus musculus*	Albumin, mRNA	NP_033784
Pig	*Sus scrofa*	Albumin, mRNA	NP_001005208
Bottlenose Dolphin	*Tursiops truncatus*	Albumin, mRNA variant X7	XP_019788269
Beluga Whale	*Delphinapterus leucas*	Albumin, mRNA	XM_022599584
Polar Bear	*Ursus maritimus*	Albumin, mRNA variant X3	XP_008691428
Weddell Seal	*Leptonychotes weddellii*	Albumin, mRNA	XP_006729869
Human	*Homo sapiens*	Albumin, mRNA	CAA23754
Chicken	*Gallus gallus*	Albumin, mRNA	NP_990592

Chimney swift	*Chaetura pelagica*	Albumin, mRNA	XP_010001931
Tuatara	*Sphenodon punctatus*	Serum albumin mRNA, partial cds	AAM46104

To begin a cross-species analysis of SA, the multiple sequence tool Clustal Omega (version 1.2.4) (www.ebi.ac.uk/Tools/msa/clustalo/) was used in coordination with the alignment-viewing program Jalview (version 2.10.3) (www.Jalview.org). Within these programs, each protein is compared for properties of conservation, quality, consensus, and occupancy among each amino acid. A coloring functionality allows amino acids with unique properties to be highlighted with various shades according to certain properties like charge, hydrophobicity, size, or propensity to generate a helix. Segments of the alignments from Jalview are included based on the section reviewed and a full Clustal omega alignment can be found in Appendix A. In addition to these functions, Jalview also generates a phylogenetic tree showing relative similarity of proteins based on their sequence. This tree was analyzed along with protein sequence to further illustrate the relationship between SA variants.

Genetic Analysis

The first area of SA that was examined is the common binding region of HSA, subdomain IB. Komatsu et al., 2004 generated mutations within this region to stabilize heme binding, and Wardell et al., 2002 observed conservation in this region coupled with an innate heme binding ability.[2,7] **Figure 4** depicts Subdomain IB along with relevant heme-binding sites. In addition to this, roughly 5 amino acids have been shown by Carter et al., 1993 to serve a key role in heme binding in HSA: Tyr138, Tyr161, Ile142, His146, and Lys190.[8] Positions comparable to these in a local alignment were analyzed throughout each of the other nine species for conservation and potential similarity in properties.

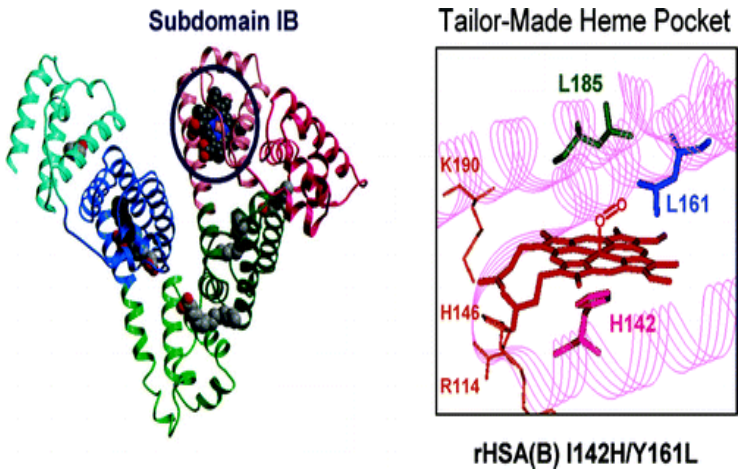


Figure 4 Subdomain IB and heme binding areas.[2]

Due to the nature of the Clustal Omega alignment program, (as it aligned each species based on functionality and amino acid similarity rather than the exact residue number/position) each exact amino acid position is difficult to analyze. To adjust for this, areas near each relevant position in HSA are addressed as a whole, and properties of amino acids in these regions are examined. Areas of hydrophobicity associated with the hydrophobic binding pockets of HSA subdomain IB will be given special focus along with the 5 amino acids outlined by Carter et al. Each species will be addressed in turn for their respective amino acids and the properties of these amino acids.

The first area which will be examined is the hydrophobic adjacent region of tyrosine (Tyr) residue 138 in HSA (**Figure 5**). Interestingly enough, this region is very well conserved throughout all mammalian species. Even the Tuatara possess a Tyr residue despite the fact that it is considerably upstream (likely due to the variation in post translational modification in reptiles compared to mammals). Despite this conservation, each bird species reviewed has a phenylalanine (Phe) residue in the location of interest. Though not identical to Tyr, Phe is comparable in all properties aside from the fact that it is less polar and slightly more hydrophobic. Positions adjacent to this location (before and after respectively) are also well conserved in mammals (all lysine and leucine residues) and show comparable variance in birds (histidine and isoleucine residues) and reptiles (asparagine and isoleucine). Though amino acids in these adjacent positions are not identical, they share the same properties of charge, size, and hydrophobicity, indicating an area of conservation at Tyr138.

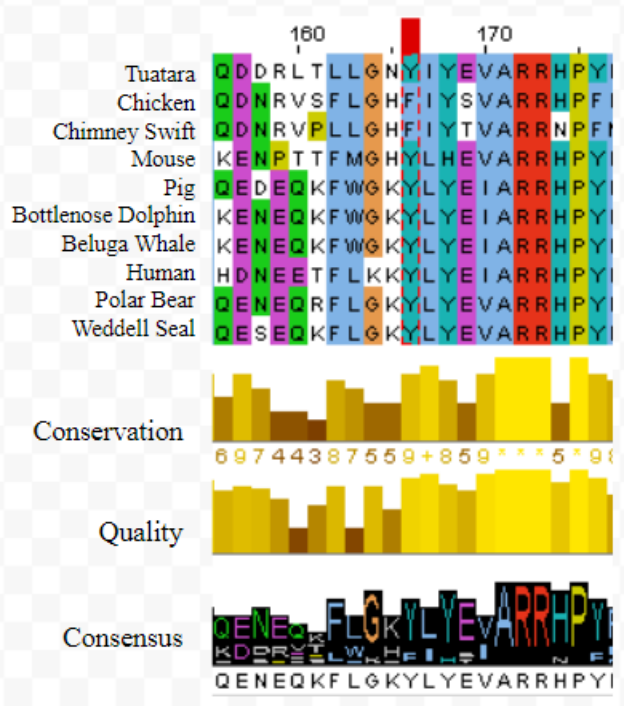


Figure 5 Clustal alignment of 10 species at position 138 with measures of conservation, quality, and consensus.

The next relative position examined was isoleucine 142 (Ile) (**Figure 6**). Once again, this region seems to show a degree of conservation. The majority of mammals possess the Ile residue with the exception of the mouse, polar bear, and Weddell Seal. Coincidentally, each of

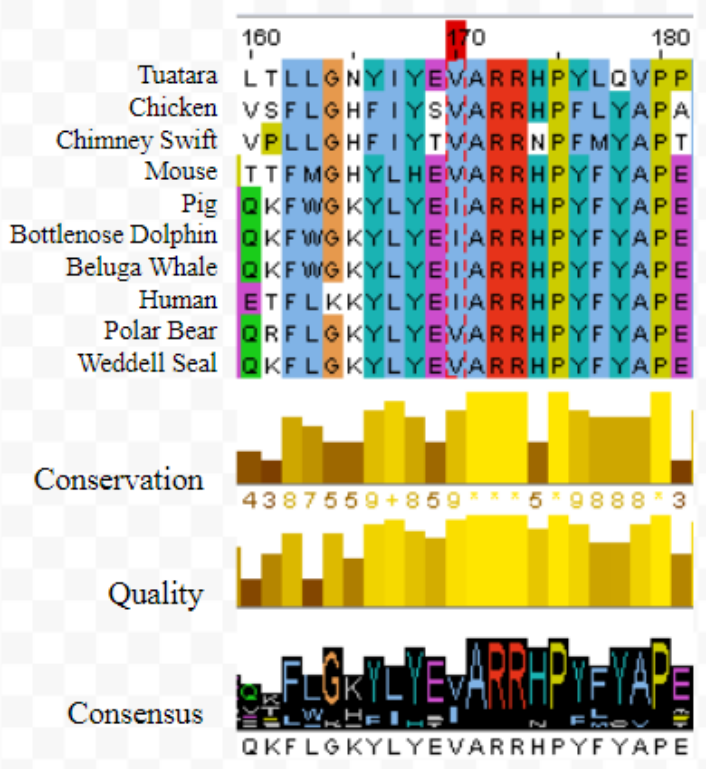


Figure 6 Clustal alignment of 10 species at position 142 with measures of conservation, quality, and consensus.

these species has instead the same amino acid as both bird species and the Tuatara; valine. This residue is very similar to Ile, consistent in every property save size (as it is slightly smaller). Regions before and after this residue are also relatively conserved, as all species possess alanine following valine and all non-bird species possess a large, negatively charged glutamate residue. Birds do show a slight variation in the preceding position, however, with a very small serine residue in chickens and a slightly hydrophobic threonine residue in the chimney swift. Both bird species do not have negative residues in this preceding position which may be indicative of binding variance.

The following position, histidine 146 (His) is localized in the most conserved region yet (**Figure 7**). This area is hydrophilic and all species with the exception of the Chimney Swift contain His at this region. The Chimney Swift, however, does contain a small, polar asparagine residue that is much different from the large, aromatic, and positively charged histidine residue in other species. Despite the species variance one might expect, the positions before and after the His residue are identical in every species, consisting of arginine and proline respectively.

Evidently the need for a positively charged region prior to, and a small neutral residue after this location is common throughout these species.

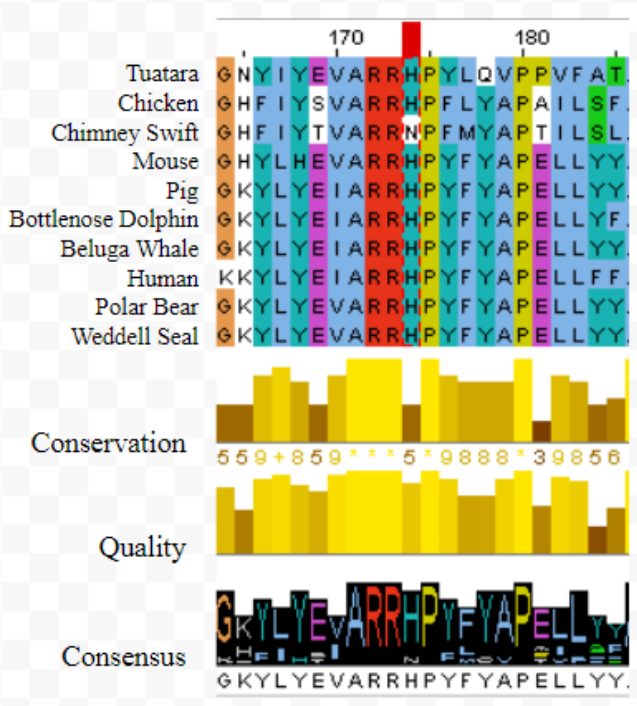


Figure 7 Clustal alignment of 10 species at position 146 with measures of conservation, quality, and consensus.

Another Tyr residue at position 161 in HSA is related to heme binding in subdomain IB, and also seems to have relative conservation (**Figure 8**). All species have the Tyr residue excluding the Chicken, which contains the hydrophobic and aromatic residue phenylalanine. Despite the discrepancy, this residue is similar in function to Tyr save its lack of polar character. Adjacent regions, however, do show a fairly low similarity. HSA position 160 contains an arginine residue and the comparable region in other species includes: glutamine in the Polar Bear, Weddell Seal, Bottlenose Dolphin, Mouse and Beluga whale, aspartate in the Chicken and Chimney Swift, and leucine and isoleucine in the Tuatara and Pig respectively. Leucine and isoleucine have similar properties, glutamine is relatively inert asides from polar character, and aspartate is small, polar, and negative. Although the amino acid itself varies, a retention in properties is again evident (aside from the hydrophobicity of leucine and

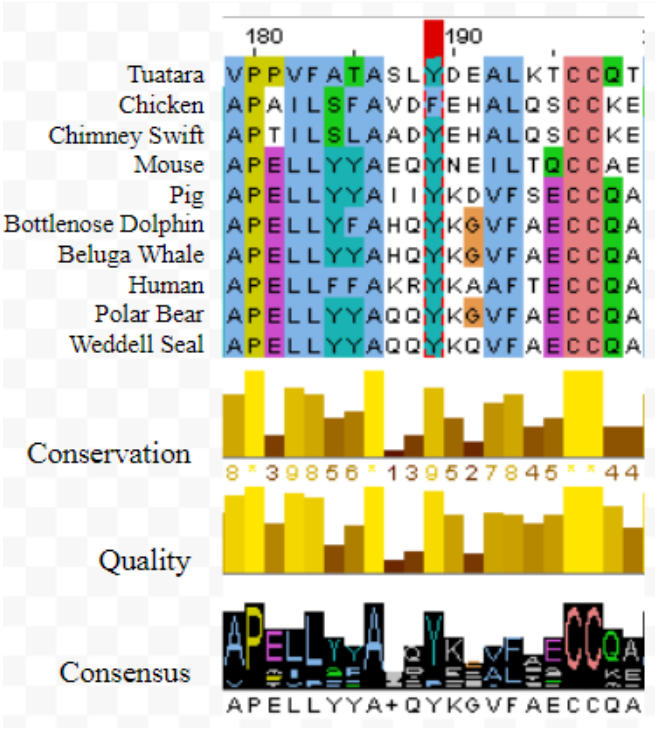


Figure 8 Clustal alignment of 10 species at position 161 with measures of conservation, quality, and consensus.

isoleucine). The position following Tyr161 also shows similarities, predominantly consisting of lysine save glutamate residues in bird species, asparagine in the Mouse, and aspartate in the Tuatara (which are also similar aside from the neutrality of asparagine).

Though deemed a relevant site for heme binding in HSA, Lys190 is minimally conserved in other species (**Figure 9**).[2] The most common amino acid in this region is actually leucine, which varies from lysine in charge and aliphatic character. Once again, the Chimney Swift is unique, possessing a small and polar alanine residue in this position. Despite this variation which is consistent before and after position 190, this region is a largely hydrophobic area, indicating the propensity for the formation of a hydrophobic binding pocket.

Although the amino acid sequence for each species is not in exact alignment, the clustal omega alignment by function shows a clear similarity at key heme interaction sites in HSA. If

these regions do not share the exact amino acid, they often possess an amino acid with a very similar size, charge, structure, or hydrophobicity. Each of the five binding regions showed high similarity across each species, with conservation values consistently around 90 percent. Admittedly, conservation throughout the protein is fairly prominent, often reaching values of 80 percent or higher. The first 50 amino acids, which is often a site of species specific protein modification, does show low conservation as one might expect. The Tuatara is represented as a blank in this region, starting alignment much farther down the protein sequences of other species (amino acid 161 in HSA for example is only 82 in the Tuatara). Since the Tuatara was chosen as an outgroup due to its genetic difference from mammals and unique variation from birds, it is not surprising that this area is different to the point of nonexistence. Based on this protein similarity, or lack thereof, Jalview is able to generate phylogenetic trees with or without relative branch distances. These trees are outlined in **Figure 10**. Although function of SA regions may be conserved between species, each tree is generated solely based on amino acid similarity. As expected, the Tuatara was an outgroup with less relative similarity to the rest of the mammals. In addition to this, the unique clade of bird species was most similar to the Tuatara. Aside from this, each mammalian species showed short branch lengths and decent similarity. A particular closeness was observed

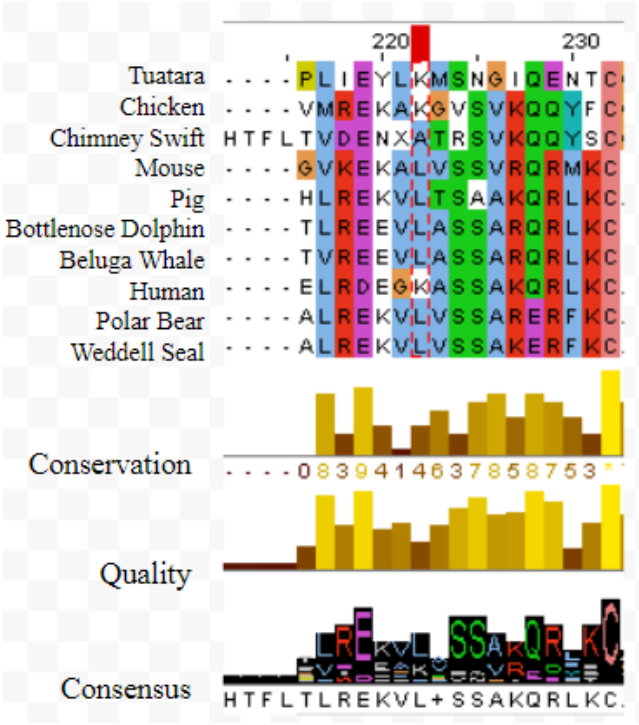


Figure 9 Clustal alignment of 10 species at position 190 with measures of conservation, quality, and consensus.

between the Bottlenose Dolphin and Beluga Whale, and the Polar Bear and Weddell Seal as these pairs are both clades based on protein similarity.

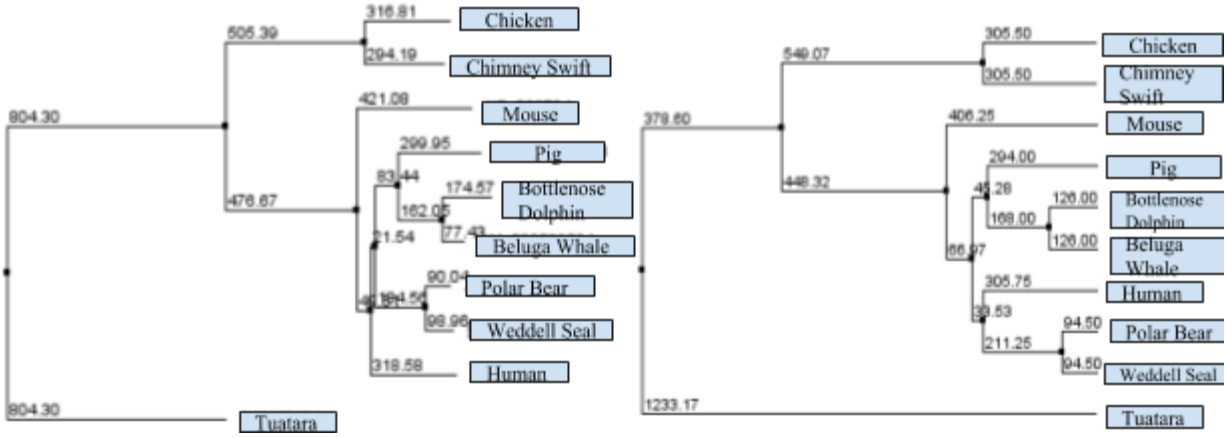


Figure 10 Phylogenetic tree of each analyzed species with and without relative branch lengths.

Ultimately the relevant regions outlined by Komatsu et al, 2004 and Carter et al., 1993 do seem to be relatively conserved throughout species at least in the properties of the amino acids.[2,8] This retention of function may indeed suggest that conservation of these regions is evolutionarily beneficial, but the propensity for SA to bind heme may have less of an impact on oxidative stress correction than other mechanisms. To examine this possibility, the next section will focus on blood chemistry of organisms with the oxidative stress mentioned previously. These include the stresses of hibernation, migration, and sea diving.

Blood Chemistry

To support the notion of SA's function in handling oxidative stress, one must consider the blood chemistry of species which accommodate such stress and possible mechanisms of doing so. Though most of these mechanisms relate to modulation of metabolic rate and careful regulation of various enzymes and antioxidants, the function of such mechanisms could shed light on the potential functioning of SA as an accommodating factor. Though each species in the

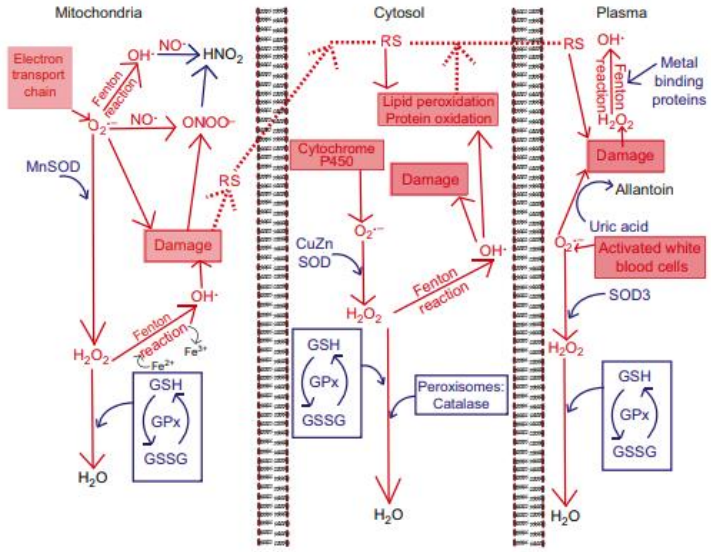
alignment analysis will not be examined for stress accommodation, species with similar physiology which have the same behavioral tendencies (diving, migration, hibernation) will be used as models.

When analyzing the possible routes of handling oxidative stress, it is important to recognize that oxidative stress may exist for a variety of different reasons, and many corrective pathways exist besides increasing oxygen carrying ability. Storey, 1996 outlines a few methods of oxidative stress accommodation across species.[9] In most species, it is not only a lack of oxygen (anoxia) which is associated with oxidative stress, but the influx of reactive oxygen species once an individual re-enters an oxygen rich environment.[9] Storey places focus on 3 predominant ways to deal with oxidative stress: preventing ROS formation, using free radical scavengers or antioxidant enzymes, and repairing damaged cellular components. Theoretically, SA could be affiliated with two of these options, preventing ROS formation by aiding in oxygen transport or using its inherent scavenging ability to neutralize ROSs.

A large group of mammals analyzed (such as the Bottlenose Dolphin, Beluga Whale, Weddell Seal, and Polar Bear) face a sizeable amount of oxidative stress when deep sea diving. Vazquez et al., 2011 examined this stress in deep-sea diving hooded seals.[10] It seems as though seals have a physiological reaction which is effectively the mammalian diving reflex in humans. This is characterized by vasoconstriction in the periphery and slowed heart rate (bradycardia) to minimize oxygen consumption.[10] However, it seems as though the biggest challenge of anoxia is presence of ROSs and overall oxidative damage. In most diving mammals, it seems as though an increase in antioxidant production is the most prevalent protective factor. Antioxidants consist of enzymatic and non-enzymatic species such as enzymatic Superoxide dismutases, catalase, glutathione peroxidases, and peroxiredoxins, and

non-enzymatic Glutathione (GSH).[10] Vazquez et al. also discovered that there is a transcription factor, Nrf2, which up-regulates the production of these enzymatic antioxidants in diving mammals.[10] Although these protective mechanisms are not associated with oxygen carrying ability, the need for oxygen transport and ROS binding would still likely be necessary in these species. For this reason, it is not unrealistic to think that a retention of this functionality in SA would not be evolutionarily selected for in diving mammals.

Other sources of oxidative stress are associated with the variations in blood flow associated with hibernation (which is seen in pregnant female Polar Bears) and the high activity and low oxygen concentrations seen in migration. Carey et al., 2000 examined a model of hibernation in Ground Squirrels.[11] Once again, a focus seems to be placed on the activation of oxidative stress pathways rather than oxygen carrying efficiency. Another transcription factor, NfkB, is redox sensitive and alters regulation of antioxidant enzymes in concert with the stress prevalent protein GRP75.[11] This method has also been mimicked in migratory birds as described by Cooper et al., 2016. The predominant source of dealing with oxidative stress is accommodating reactive species, (oxygen or nitrogen) and this is done by endogenous antioxidant increase or a dietary increase in antioxidants.[12] **Figure 11** outlines ways in which reactive species are generated and neutralized within the cell and blood plasma. Within the blood, there is a clear antioxidant effect of GSH similar to what is seen in diving mammals.[12] Uric acid and SOD3 also function as antioxidants in the blood plasma of this organism and are unique to birds and other uricotelic organisms.[12] Based on this, these species may use this unique



physiological component to adjust for oxidative stress rather than a unique form of SA. Despite this, birds (both migratory and non-migratory) and reptiles included in the Clustal Omega alignment had areas of conservation similar to HSA and other mammals.

Figure 11 Generation and neutralization of reactive species.[12]

Ultimately, methods of coping with oxidative stress seem to focus less on oxygen carrying manipulation, and more on the accommodation of reactive species which are generated in hypoxic conditions or a return from these conditions. Though regions that allow for heme binding in HSA seem to be retained with an exact amino acid or similar function across species, this does not appear to be chief factor in oxidative stress accommodation. Perhaps further analysis of SA as a free radical scavenger may be more relevant to dealing with oxidative stress across species than oxygen binding ability. Based on this, it is evident that structural variation or conservation in SA across each species does not appear to be directly linked with the physiological traits of that species.

Chapter 3 Mutations On Human Serum Albumin: An Experimental Approach

Overview and Goal

The purpose of this project, based of the work of Komatsu et al., is to generate the mutations outlined in Chapter 1. These mutations (which consist of introducing a stabilizing histidine residue in position 142 and a smaller leucine residue in position 161) will serve to facilitate heme binding in HSA. A variation in procedure will attempt to overcome limitations with *P.pastoris* expression, and generated mutant protein may be tested for heme binding capabilities.

The procedure to generate these mutants will use a site-directed mutagenesis kit in which the mutant plasmid will be introduced into *E. coli*. Despite Komatsu et al. using linearization and electroporation into yeast (*P. pastoris*) to produce the variant for isolation, I have proposed the use of Glutathione-S transferase (GST) which will allow the isolation and purification of this protein (tagged with GST) directly from *E. coli*. Although this eukaryotic protein will be produced in prokaryotic yeast, I am confident that the stability associated with HSA's tertiary globular structure will prevent any unwanted post-translational modification in *E.coli*.[1] Since the use of HSA as a heme binding oxygen carrying protein may have some future medical application, I am most interested in the expansion of this process to improve its efficacy. I hope that these alterations may allow expansion of this project, hypothetically even in association with future gene therapy to counteract the oxygen carrying limitations seen in diseases associated with myoglobin and hemoglobin deficiencies.

Materials and Methods

The focus of this project is to develop a series of mutant HSA genes that will be utilized in later experiments that investigate the nitrite reductase activity in HSA variants that are reconstituted with heme.. Based on the proposed functionality of Komatsu et al., 2004 and my co-advisor Dr. Galinato, two mutants, I42H and Y161L, were designed using the QuikChange II Site-directed Mutagenesis Kit. Primers for both mutants were synthesized using the web-based QuikChange Primer Design Program. Using the genetic code of HSA, the 142nd base pair was determined and mutated from isoleucine to histidine in mutant I142H, and the 161st base pair tyrosine mutated to leucine in mutant Y161L. The QuickChange program designed both a forward and a reverse primer sequence for each mutant, which was annealed with the thermally cycled HSA template (to denature) and extended using *PfuUltra* DNA polymerase. DNA without the desired mutation was degraded using Dpn I Digestion, leaving only the desired HSA mutant vector. Additionally, the two site QuikChange II Mutagenesis kit may also be used to create the double mutation, Y161L/I142H by the same process.

Following generation of the mutant HSA vector, the process of electroporation may be used to introduce the vector to *E.coli*. Electrocompetent *E.coli* cells were cultured, placed in cuvettes with plasmid, and electroporated at 250 mVs. After a period of incubation, cells were plated on nutrient broth Ampicillin plates (as ampicillin resistance was used as a selection mechanism) and left to grow overnight. Colonies which had taken up the plasmid were cultured and used in a plasmid prep procedure to attain working stocks of mutant plasmid. Once isolated, quantified, and purified, the plasmid was sequenced to confirm the desired mutation.

In order to amplify the mutant HSA region within the plasmid, the plasmid was linearized and the HSA region was amplified using PCR. If this region can be amplified, the GST system

may be utilized to induce protein expression. By including the GST moiety at the N-terminus of the mutant protein, protein folding and subsequent elution may be followed by non degradative affinity purification. This could be done with the GST vector pGEX 4T-1, which includes a specific orientation of restriction sites within its multiple cloning site. In the aforementioned PCR amplification, primers for HSA amplification were designed with hanging ends that incorporate restriction sites. Each of these sites match those found in the multiple cloning site of the pGEX 4T-1 vector, and cutting this vector and the amplified HSA segment allows for incorporation of the mutant HSA into the pGEX vector. Once generated, this fusion vector may be introduced into *E.coli* using electroporation. Colonies with the plasmid may be selected for using Ampicillin resistance, cultured, and processed using the GST processing kit procedure. Mutant protein may be isolated as described above using an affinity column, and this protein may be tested for oxygen binding ability.

Discussion

Unfortunately, this project reached a point of stagnancy once mutant HSA plasmid was generated. Although primers were checked for equivalent annealing temperatures, amplification in the proper reading frame, and a lack of incorrect landing sites, the PCR reaction did not amplify the HSA region. Despite numerous revisions to the PCR cycle times and temperatures using wild type control HSA plasmid, no amplification occurred. A tentative PCR program which was the last variation to be tested has been outlined in **Figure 7**. If mutant HSA could have been amplified, ligation and incorporation of this material into pGEX and column purification should be relatively straightforward to yield mutant protein. It seems as though this

PCR amplification is the predominant limiting factor in this experiment. Possible corrections for this limitation would likely be associated with continued variation of the PCR program. Using PCR mix specifications, annealing or extension temperatures and cycle lengths must be optimized through trial and error.

94°C	94°C	50°C	68°C	68°C	4°C
5mins	3 seconds	3 seconds	5 seconds	5 minutes	∞
	35 cycle				

Figure 12 PCR cycle times and temperatures for HSA amplification.

Comparison between Nature and Research

Positions 161 and 142 have been outlined by Komatsu et al. and Carter et al. as relevant locations for heme binding, and the adjustment of these amino acids to better facilitators of heme binding is the focus of this project.[2,8] Though these sites have been examined for conservation throughout various species in Chapter 2, it is possible that amino acids which vary may serve to have the facilitating binding effect of leucine and histidine in Y161L and I142H respectively. Leucine in position 161 is advantageous because of its smaller size. Interestingly enough, the Chicken, Chimney Swift, Tuatara, and pig each have a smaller amino acid residue in this relative position (aspartate in bird species and leucine and isoleucine in the Tuatara and pig respectively). Although these species (excluding the migratory Chimney Swift) do not experience higher than average rates of oxidative stress, the possession of this smaller amino acid may indeed be beneficial in heme binding to some degree and retained for the benefit of increased oxygen carrying ability. Position 142 relies on histidine's unique residue structure to stabilize the heme group. Though the species analyzed may not possess an isoleucine in this position, the amino acid which is substituted is valine, which is small and similar in function to

isoleucine. The residue of this amino acid will not have the stabilizing effect of histidine, being no different functionally than isoleucine.

Although SA in each species analyzed does not line up identically with HSA, it shows regions of functionality which are very similar to HSA. This relative conservation seems to indicate some form of evolutionary advantage in SA which may indeed be associated with heme binding and oxygen carrying capabilities. Position 161 does show and amino acid variance which is comparable to the heme binding facilitating mutations attempted in this project. Further analysis in other species which experience oxidative stress may be necessary to show a statistically significant correlation between evolutionary SA modification and functional oxygen carrying ability.

Appendix A

Supplementary Data

CLUSTAL O (1.2.4) multiple sequence alignment

```
AAM46104          ------------------------------------------------------------      0
NP_990592         MKWVTLISFIFLFSSATSRNLQRFARDAEHKSEIAHRYNDLKEETFKAVAMITFAQYLQR      60
XP_010001931      MKWVTLISFIFLFSSATSRNLQRVARGAEHKSEIAHRYNDLKEETFKAVTMITFAQYLQR      60
NP_033784         MKWVTFLLLLFVSGSAFSRGVF---RREAHKSEIAHRYNDLGEQHFKGLVLIAFSQYLQK      57
NP_001005208      MKWVTFISLLFLFSSAYSRGVF---RRDTYKSEIAHRFKDLGEQYFKGLVLIAFSQHLQQ      57
XP_019788269      -----------MTCSAYSRGVF---RRDTHKSEIAHRFNDLGEENFKGLVLIAFSQYLQQ      46
XM_022599584      MKWVTFISLIFLFSSAYSRGVF---RRDTHKSEIAHRFNDLGEENFKGLVLIAFSQYLQQ      57
CAA23754          MKWVTFISLLFLFSSAYSRGVF---RRDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQ      57
XP_008691428      MKWVTFISLLFLFSSAYSRGVT---RREAQQSEIAHRYNDLGEEHFRGLVLVAFSQYLQQ      57
XP_006729869      MKWVTFISLLFLFSSAYSRGVM---RREAQQSEVAHRYNDLGEEHFRGLVLVAFSQYLQQ      57


AAM46104          ------------------------EDPTCLKSLDTIFLDEICHEEGFAAKYD-LAACCAK      35
NP_990592         CSYEGLSKLVKDVVDLAQKCVANEDAPECSKPLPSIILDEICQVEKLRDSYGAMADCCSK      120
XP_010001931      CSYEGLSKLVKDVVDLAQKCVADEDAAGCSKPLPSIFLDEICQVEKLRDSYGAMADCCSK      120
NP_033784         CSYDEHAKLVQEVTDFAKTCVADESAANCDKSLHTLFGDKLCAIPNLRENYGELADCCTK      117
NP_001005208      CPYEEHVKLVREVTEFAKTCVADESAENCDKSIHTLFGDKLCAIPSLREHYGDLADCCEK      117
XP_019788269      SPFDEHVKLVNEITDFAKTCVADESAANCDKSLHTLFGDKLCAVASLRETYGEMADCCGK      106
XM_022599584      SPFDEHVKLVNEITEFAKTCVADESATNCDKSLHTLFGDKLCAVASLRETYGEMADCCVK      117
CAA23754          CPFEDHVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAK      117
XP_008691428      CPFEDHVKLAKEVTEFAKGCAADQSGADCDKSLHTLFGDKLCTVASLREKYGELADCCEK      117
XP_006729869      CPFEDHVKLAKEVTEFAKGCAADQSGADCGKSLHTLFGDKLCTVASLREKYGELADCCEK      117
                                         .   * * : ::: *::*    :   *. :* ** *


AAM46104          AEVERKECLLAHKNATPGFIPAFQRPGIEVSCKLYQDDRLTLLGNYIYEVARRHPYLQVP      95
NP_990592         ADPERNECFLSFKVSQPDFVQPYQRPASDVICQEYQDNRVSFLGHFIYSVARRHPFLYAP      180
XP_010001931      ADPERNDCFLSFKIPQPDFVPPYERPSSDVICKEYQDNRVPLLGHFIYTVARRNPFMYAP      180
NP_033784         QEPERNECFLQHKDDNPSL-PPFERPEAEAMCTSFKENPTTFMGHYLHEVARRHPYFYAP      176
NP_001005208      EEPERNECFLQHKNDNPDI-PKL-KPDPVALCADFQEDEQKFWGKYLYEIARRHPYFYAP      175
XP_019788269      QDPERNECLLKHKDDNPDL-PKL-KPDPETFCTEFKENEQKFWGKYLYEIARRHPYFYAP      164
XM_022599584      QEPERNECLLKHKDDNPDL-PKL-KPDPDTLCAEFKENEQKFWGKYLYEIARRHPYFYAP      175
CAA23754          QEPERNECFLQHKDDNPNL-PRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAP      176
XP_008691428      QEPERNECFLKHKDDNPGF-PPLVTPEPDALCAAFQENEQRFLGKYLYEVARRHPYFYAP      176
XP_006729869      QDPERNECFLTHKDDNPGF-PPLVTPEPDAMCAAFQESEQKFLGKYLYEVARRHPYFYAP      176
                   : **::*:* .*   *.:      *   . *  :::.   :  :::: :***:*:: .*


AAM46104          PVFATASLYDEALKTCCQTADKATCFHPRIP----PLIEYLKMSNGIQENTCGILKKFGE      151
NP_990592         AILSFAVDFEHALQSCCKESDVGACLDTKEI----VMREKAKGVSVKQQYFCGILKQFGD      236
XP_010001931      TILSLAADYEHALQSCCKEADVNACLDEKVHHTFLTVDENXATRSVKQQYSCGILQKFGE      240
NP_033784         ELLYYAEQYNEILTQCCAEADKESCLTPKLD----GVKEKALVSSVRQRMKCSSMQKFGE      232
NP_001005208      ELLYYAIIYKDVFSECCQAADKAACLLPKIE----HLREKVLTSAAKQRLKCASIQKFGE      231
XP_019788269      ELLYFAHQYKGVFAECCQAADKGACLIPKIE----TLREEVLASSARQRLKCTSIQKFGE      220
XM_022599584      ELLYYAHQYKGVFAECCQAADKGACLIPKIE----TVREEVLASSARQRLKCTSIQKFGE      231
CAA23754          ELLFFAKRYKAAFTECCQAADKAACLLPKLD----ELRDEGKASSAKQRLKCASLQKFGE      232
XP_008691428      ELLYYAQQYKGVFAECCQAADKAACLTPKID----ALREKVLVSSARERFKCASLQKFGD      232
XP_006729869      ELLYYAQQYKQVFAECCQAADKAACLTPKID----ALREKVLVSSAKERFKCASLQKFGD      232
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AAM46104        RTLKATKLVQMSQKFPKADFATINKLVEDITHMHTECCRGDTLECLRDREALTEYTCSHK      211
NP_990592       RVFQARQLIYLSQKYPKAPFSEVSKFVHDSIGVHKECCEGDMVECMDDMARMMSNLCSQQ      296
XP_010001931    RIFKAQKLAIMSQKYPKAPLTELVKVVNDIADIHKECCSGDMVECMDDRAELVTYICSKQ      300
NP_033784       RAFKAWAVARLSQTFPNADFAEITKLATDLTKVNKECCHGDLLECADDRAELAKYMCENQ      292
NP_001005208    RAFKAWSLARLSQRFPKADFTEISKIVTDLAKVHKECCHGDLLECADDRADLAKYICENQ      291
XP_019788269    RALKAWSVARLSQKFPKADFAEVSKIVTDLTKVHKECCHGDLLECADDRADLAKYICENQ      280
XM_022599584    RALKAWSVARLSQKFPKADFAEVSKIVTDLTKVHKECCHGDLLECADDRADLAKYICENQ      291
CAA23754        RAFKAWAVARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQ      292
XP_008691428    RAFKAWSVARLSQRFPKADFAEVSKVVTDLTKVHKECCHGDLLECADDRADLAKYMCENQ      292
XP_006729869    RAFKAWSIALMSQKFPKADFAEVSKLVTDLTKIHKECCHGDLLECADDRADLAKYMCENQ      292
                * ::*  :  :** :*:* :: : *.. *   ::.*** ** :**  *   :    *.::

AAM46104        DAISSKLPTCCEKSVLERGECIVRLENDDKPADLSERIAEYIEDPHVCDHLAKEQDAFLA      271
NP_990592       DVFSGKIKDCCEKPIVERSQCIMEAEFDEKPADLPSLVEKYIEDKEVCKSFEAGHDAFMA      356
XP_010001931    DVFSSKIKECCEKPVVERSQCIIESEYDDKPEDLPSLVEKYVQDKEVCNGFQKDHDGFMS      360
NP_033784       ATISSKLQTCCDKPLLKKAHCLSEVEHDTMPADLPAIAADFVEDQEVCKNYAEAKDVFLG      352
NP_001005208    DTISTKLKECCDKPLLEKSHCIAEAKRDELPADLNPLEHDFVEDKEVCKNYKEAKHVFLG      351
XP_019788269    ATISSKLQKCCHKPLLEKSHCISEVEKDELPENLSLLAADFAEDKEVCKNYNEAKDVFLG      340
XM_022599584    ATISTKLKKCCDKPLLEKSHCIAEVEKDELPENLSPLAADFAEDKEVCKNYNEAKDVFLG      351
CAA23754        DSISSKLKECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLG      352
XP_008691428    DSISSKLKECCDKPVLEKSQCLSEVERDELPGDLPPLAADFVEDKEVCKNYQEAKDVFLG      352
XP_006729869    DSISSKMKECCDKPLLEKSHCLTEVERDELPGDLSPIAADFVEDKEVCKNYQEAKDVFLG      352
                  :* *:  **.* ::::..*: . : *  * :*     .: :. .**.     :. *:.

AAM46104        KFLYEYSRRHPELSTQILLGVGKGYQELLERCCKTDNPPECYGQAEADLKKHIAQFQELV      331
NP_990592       EFVYEYSRRHPEFSIQLIMRIAKGYESLLEKCCKTDNPAECYANAQEQLNQHIKETQDVV      416
XP_010001931    EFLYEYSRRHPEFSTQLILRIAKGYEALLEKCCKTDNPAECYGNAQEELNKHVQETQEVV      420
NP_033784       TFLYEYSRRHPDYSVSLLLRLAKKYEATLEKCCAEANPPACYGTVLAEFQPLVEEPKNLV      412
NP_001005208    TFLYEYSRRHPDYSVSLLLRIAKIYEATLEDCCAKEDPPACYATVFDKFQPLVDEPKNLI      411
XP_019788269    TFLYDYARRHPEYSVSLLLRIAKGYEATLEDCCAKDDPPACYATVFEKLRPLVEEPKNLI      400
XM_022599584    TFLYEYARRHPEYAVSLLLRIAKGYEATLEDCCAKDDPPACYATVFEKLQPLVEEPKNLI      411
CAA23754        MFLYEYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLI      412
XP_008691428    TFLYEYSRRHPEYSVSLLLRLAKEYEATLEKCCATDDPPTCYSKVLDEFKPLVEEPQNLV      412
XP_006729869    TFLYEYSRRHPEYAISLLLRLAKEYEATLEKCCATDDPPTCYGKVLDEFKPLVEEPQNLV      412
                 *:*:*:****: :  ::: :.* *:  ** **   :*  **. .  .:.  : : ::::

AAM46104        QQNCDLYNTLGGYLFHNALLIRYTKRMPQLTSEELIFYT-RITKAASRCCEVSVDKKLPC      390
NP_990592       KTNCDLLHDHGEADFLKSILIRYTKKMPQVPTDLLLETGKKMTTIGTKCCQLPEDRRMAC      476
XP_010001931    KTNCELLNTHGEADFLKSLLIRYTKKMPQVSTETLLEIGKKMSTVGTKCCQLPEDRRLPC      480
NP_033784       KTNCDLYEKLGEYGFQNAILVRYTQKAPQVSTPTLVEAARNLGRVGTKCCTLPEDQRLPC      472
NP_001005208    KQNCELFEKLGEYGFQNALIVRYTKKVPQVSTPTLVEVARKLGLVGSRCCKRPEEERLSC      471
XP_019788269    KQNCELFEKLGEYQFQNALIVRYTKKVPQVSTPTLVEVSRNLGRVGSKCCKNPESERMSC      460
XM_022599584    KQNCELFEKLGEYLFQNALIIRYTKKVPQVSTPTLVEVSRNLGRVGSKCCKNPESERMSC      471
CAA23754        KQNCELFKQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPC      472
XP_008691428    KSNCELFEKLGEYAFQNALLVRYTKKVPQVSTPTLVEVSRKLGKVGTKCCKKPESERMSC      472
XP_006729869    KTNCELFEKLGEYGFQNALLVRYTKKVPQVSTPTLVEVSRKLGKVGTRCCKKPDSERMPC      472
                : **:* .  *   * :::::***:: **: :  *:    .:   .::**     .:: *

AAM46104        TEGYVDFVLGQICQRHQRSSINVNVCQCCSNSYALRSLCITSLGGDEKFVPIEFSADLFT      450
NP_990592       SEGYLSIVIHDTCRKQETTPINDNVSQCCSSSYANRRPCFTAMGVDTKYVPPPFNPDMFS      536
XP_010001931    SEGYLSIVIQDMCRRQETTPINDNVSQCCSNSYADRRPCFTKMGVDTKYVPPAFDPNMFN      540
NP_033784       VEDYLSAILNRVCLLHEKTPVSEHVTKCCSGSLVERRPCFSALTVDETYVPKEFKAETFT      532
NP_001005208    AEDYLSLVLNRLCVLHEKTPVSEKVTKCCTESLVNRRPCFSALTPDETYKPKEFVEGTFT      531
XP_019788269    AEDYLSLVLNRLCVLHEKTPVSERVTKCCTESLVNRRPCFSALTVDETYEPKAFDEKTFT      520
XM_022599584    AEDYLSLVLNRLCVLHEKTPVSERVTKCCTESLVNRRPCFSALTVDETYEPKAFDEKTFT      531
CAA23754        AEDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFT      532
XP_008691428    AEDYLSVVLNRLCVLHEKTPVSERVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFT      532
XP_006729869    AEDYLSVVLNRLCVLHEKTPVSERVTKCCTESLVNRRPCFSALEIDEAYVPKEFNAETFT      532
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AAM46104         FHEDLCHAAQDKLQERKQQMIVNLVKHKPNITKEQLQTVFGGFTKMTEKCCKAEDHEACF      510
NP_990592        FDEKLCSAPAEEREVGQMKLLINLIKRKPQMTEEQIKTIADGFTAMVDKCCKQSDINTCF      596
XP_010001931     FDEKLCSASPAEQELGQMKLLVNLIKRKPQMTEEQIKTIADGFTAMVDKCCKQADIETCL      600
NP_033784        FHSDICTLPEKEKQIKKQTALAELVKHKPKATAEQLKTVMDDFAQFLDTCCKAADKDTCF      592
NP_001005208     FHADLCTLPEDEKQIKKQTALVELLKHKPHATEEQLRTVLGNFAAFVQKCCAAPDHEACF      591
XP_019788269     FHADLCTLPENEKQIKKQIALVELVKHKPKVTEEQLKTVMGDFAAFVDKCCAADDKEACF      580
XM_022599584     FHADLCTLPENEKQIKKQIALVELVKHKPKVTEEQLKTVMGNFAAFVDKCCAADDKEACF      591
CAA23754         FHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCF      592
XP_008691428     FHADLCTLPEAEKQAKKQSALVELLKHKPKATEEQLKTVMGDFGAFVDKCCAAENKEGCF      592
XP_006729869     FHADLCTLPEAEKQVKKQSALAELVKHKPKATEEQLKTVMGDFGAFVEKCCAAENKEACF      592
                 *. .:*     : :  :   : :*:*:**: * **:::: ..*  : :.**   : : *:

AAM46104         GEEGPKLVAESQTALAA-- 527
NP_990592        GEEGANLIVQSRATLGIGA 615
XP_010001931     GEEGANLIVQGRAILGIGM 619
NP_033784        STEGPNLVTRCKDALA--- 608
NP_001005208     AVEGPKFVIEIRGILA--- 607
XP_019788269     ALEGPKLVVKTREAIA--- 596
XM_022599584     ALEGPKLVVSTREAIA--- 607
CAA23754         AEEGKKLVAASQAALGL-- 609
XP_008691428     AEEGPKLVATAQAALV--- 608
XP_006729869     AEEGPKLVAKAQAALA--- 608
                 . ** :::   :  :
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Note: “.” denotes large variance, “:” denotes small variance, and “” denotes high conservation.

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Academic Vita
Sean R. Artello

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EDUCATION

Penn State Erie, The Behrend College
Biology, Bachelor of Science
· Chemistry and Psychology minor
· Schreyer Honors Scholar

CLINICAL EXPERIENCE

North East PA Pet Emergency Center Assistant Technician December, 2016-Present
· Perform various technician duties such as blood work, surgery assistance, and patient restraint/care
· Cemented position as a paid team member as I effectively worked in sensitive emergency situations

AKC Dog Show Ambassador Erie, PA
· Foresaw and organized placement and set up of over 1000 show participants January, 2017
· Mediated owner and AKC conflict and diplomatically catered to participants needs and requests

RESEARCH EXPERIENCE

HSA Mutation Project Erie, PA
· Perform various point mutations in order to stabilize HSA Heme binding October, 2016-Present
· Record intricate notes, re-created failed procedures, and wrote grant proposals awarding $2500.

RELEVANT COURSEWORK

General Biology	Calculus I and II	General Chemistry I and II	Genetics
Cell Biology	Organic I and II	Abnormal Psychology	Biostatistics
Physics I and II	Microbiology	Personality Psychology	Biochemistry I and II

WORK EXPERIENCE

Honors Cell Lab Teacher's Assistant Erie, PA
· Aid with lab progress, answer student questions, grade student assessments August, 2016-Present
· Designed a lab to isolate and analyze the DNA of various dog breeds

Peek N' Peak Ski Instructor Erie, PA
· Taught beginners the basics, and returning customers more advanced skills December, 2009-Present
· Watched over and taught children age 4-12 in Peek N' Kids

VOLUNTEER EXPERIENCE

Assist ACS Sponsored events as Tri-beta member Erie, PA
· Aid in the organization and conduction of youth chemistry experiments October, 2016-Present
· Interact with and educate roughly 100 participants ages 5-15

Annual Roadside Cleanup as an Eagle Scout Erie, PA
· Organized a group of 16 Scouts to volunteer for the cleanup May, 2006-June, 2014
· Helped with Safety and picked up trash over the majority of Harborcreek

Activities

Sacristan at St. Mark's Church July, 2008-Present
Co-founed/VP of Campus Health and Fitness Club at Behrend November 2016-Present

VP of Disc Golf Club at Behrend August, 2016-Present