The Effect of Subunit Mutation on Cooperativity in Tetrameric Scapharca Hemoglobin

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5/28/2010

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Introduction

Hemoglobins are proteins that bind and transport oxygen in red blood cells. Specifically, oxygen molecules bind to the heme group, an iron complex present in all hemoglobin. Many varieties of hemoglobin exist, with many variations between species. Many of the variants are comprised of multiple subunits, leading them to have many useful and interesting properties. Among these properties is cooperative binding: when the chemical affinity increases with the amount of bound substrate. Understanding the mechanisms behind cooperativity has been a goal of researchers for some time.

The hypothesis of this project is that structural changes to one subunit of a cooperative complex affect the structural changes of other subunits and impact the cooperativity of the entire protein. This hypothesis will be explored through oxygen-binding experiments on mutants of *Scapharca inaequivalvis* tetrameric hemoglobin. Changes to the structure of the protein implemented by site-directed mutagenesis will be analyzed for their effect on the oxygen affinity and cooperativity of the protein.

Background

DNA is made up of four basic components, called bases: adenosine, tyrosine, cytosine, and guanine. The ordered combination of these four molecules forms the genetic blueprint for an organism. DNA’s physical structure is a double-stranded helix. Its main function is to hold the genetic information used to create proteins. The process of turning this sequence of bases into a large biomolecule has two main stages: transcription, which is the copying of genetic information, and translation, which is turning this information into functioning proteins.
Proteins are large biomolecules comprised of many smaller molecules. These smaller molecules are called \textit{amino acids}. There are 20 such amino acids occurring in nature. When these amino acids are connected in sequence according to the genetic code established in DNA, the molecules interact, which causes them to fold into complex 3D shapes. Proteins serve a variety of functions in organisms. Some proteins catalyze, or accelerate, the speed of chemical reactions. These are called enzymes. Other proteins perform transport functions, like hemoglobin, or serve in the structure of cells.

Proteins have three, potentially four, levels of structure. The first level is its \textit{primary structure}: the sequence of amino acids that comprise the protein. From the primary structure arises the \textit{secondary structure}. This is formed by interactions between the side chains of amino acids and their backbones, and typically results in either an alpha helix, or beta pleated sheet. Next comes the \textit{tertiary structure}: the physical 3D arrangement of the protein. The tertiary structure of a protein is primarily determined by interactions between the amino acids that comprise it, and specifically interactions between the hydrophobic elements. A protein’s \textit{quaternary structure} is the arrangement of multiple subunits of a protein, if they exist. Only proteins that are comprised of multiple smaller proteins have quaternary structures.

Hemoglobins are proteins with a specific function: to bind and transport oxygen to where it is needed, and then release it. Many hemoglobins are proteins with multiple parts, called subunits. Proteins with two subunits are called dimers; those with four are called...
tetramers, etc. Human hemoglobin, HbA, is an example of this. The key component of hemoglobin is the heme group. The heme group is an iron ion held in place by other molecules, and is what actually binds to oxygen. Hemoglobin is found in red blood cells in humans.

A commonly occurring statistic when discussing hemoglobin is its oxygen affinity. This is the tendency of oxygen to bind to the protein. A hemoglobin protein with high oxygen affinity is one that binds oxygen very easily and quickly; low affinity indicates the opposite. Oxygen affinity is primarily based on the structure of the protein, as most chemical and biological reactions are. The accessibility of the heme group, the shape of the interface where the bonding occurs, and many other factors influence how easily oxygen can bind.

Often, a protein such as hemoglobin can have multiple conformations that have differing attributes. For example, hemoglobin has an R conformation and a T conformation. The R, or relaxed, conformation is the one with significantly higher oxygen affinity, but is energetically unfavorable. Therefore, the protein commonly exists in the T, or tensed, conformation, which while having less affinity to bind its ligand, is a much more energetically favorable state.

When hemoglobin proteins are comprised of multiple subunits, oftentimes their function is altered by cooperative binding. When a hemoglobin protein is bonded to oxygen, it undergoes structural changes that increase its oxygen affinity. In cooperative binding, when one binding site increases its oxygen affinity, other binding sites also increase their affinity, causing them to bind oxygen more readily. Plainly stated, it is the connection between increase in bound ligand and increase in affinity. The first binding in a cooperative hemoglobin sets of a chain reaction that allows the rest of the oxygen to bind more quickly. This benefits the hemoglobin by making it more efficient, as a hemoglobin molecule not carrying its full capacity of oxygen is wasting space.
In experimenting with protein and other biological molecules, various elements of biotechnology are employed. One of the most important involves modification of a naturally occurring structure. **Plasmids** are circular rings of DNA, single or double-stranded, that serve as part of the genetic code of bacteria. They serve the same purpose as DNA in any other cell, but are mobile. They can replicate, produce mRNA, and serve as important parts of bacterial cells’ functionality. Also, they are capable of infecting other bacterial cells, a process called **transformation**.

Because plasmids are small and mobile, they are easily altered and adapted by biologists. The alteration of genetic information is called **mutagenesis**. When this is directed at a specific section or sequence of genetic code, it is referred to as **site-directed mutagenesis**. A common technique among biologists and biochemists is to add genetic material to plasmids, usually for mass production of a protein. When plasmids are used in this manner, they are known as **vectors**.

When adding genetic information to a plasmid, **restriction enzymes**, also known as restriction endonucleases, are invariably used. Restriction enzymes are types of proteins that recognize specific sequences of DNA. When it comes across that sequence of DNA, it breaks the DNA chain at that point. Some restriction enzymes leave **sticky ends** when they cut DNA. This is when the enzyme leaves one strand of the DNA longer than the other. The significance of a sticky end is that it allows the DNA to reconnect with another end that was cut by the same restriction enzyme. Since the
same enzyme cut both ends, it left the same offset in the DNA, allowing the two sides to fit together and reconnect.

**Structure and Function of HbII**

This project uses homodimeric and heterotetrameric hemoglobins from the blood clam *Scapharca inaequivalvis*. These two proteins (ScHbI and ScHbII) are well-documented examples of cooperative hemoglobin function, and therefore are excellent systems in which to study the mechanisms and functionality of oxygen affinity and cooperativity in hemoglobin.

*Scapharca* hemoglobin is similar in many ways to human hemoglobin (HbA). Like HbA, it exists as a heterotetramer: a tetramer with two variants of subunits. In HbA, these are known as α and β. The tetramer consists of two of each. The tetramer produced by these acts cooperatively, for maximum efficiency. The most significant differentiating factor between human hemoglobin and *Scapharca* hemoglobin lies in the interface in which it binds oxygen. Human hemoglobin relies on interaction between α and β subunits. In ScHbI and ScHbII, however, the binding interface is created by interactions between the E and F helices of each subunit. In both, ligand-binding involves significant structural transformation, making *Scapharca* an excellent resource for exploring a wide range of globins. Where hemoglobin differs from many other oxygen-binding proteins is in its cooperativity. Many of these other proteins either have non-cooperative quaternary structure, or are monomers.

*Scapharca inaequivalvis* hemoglobin exists in both dimer and tetramer forms, as previously mentioned. The tetramer form of the protein has two homodimers, commonly labeled as subunits A and B. While
the dimeric form of this hemoglobin is often the target of experimentation, here the focus is on the
tetrameric form. This is because subunits of a homodimer cannot be modified independently of each
other. For experimentation to be done concerning differing subunits, each must be able to be altered
individually; hence the usage of a dual-homodimeric tetramer.

Before the specific mutations can be addressed, it is important to understand the structural
conformations that occur during oxygen binding in hemoglobin. When liganded, the protein’s heme
group moves, Phe 97 undergoes conformational changes, and there is change in the arrangement of
water in the interface. These structural changes are integral to the function and cooperativity of HbI and
HbII. The most prominent of these is the change to Phe 97. In unliganded HbI, Phe 97 is tucked into the
heme pocket, and restricts the heme iron from being accessible. In this T (tensed) conformation, the
oxygen affinity of the hemoglobin is lowered. When liganded, however, this Phe swings outward,
allowing the heme iron to descend. This shifts the complex to its R (relaxed) conformation, increasing its
oxygen affinity. When Phe 97 is replaced by a tyrosine, however, the protein is locked in its high-affinity R
state. The hydroxyl group of Tyr creates a larger side chain, preventing it from tucking into the histidine pocket. This keeps the heme iron accessible, greatly increasing its affinity. As seen in Figure 5, crystallography shows that this change does not have extreme effect on the shape of the hemoglobin; the change in affinity comes from locking it into a high-affinity state. Tetrameric HbII is tested with a single subunit locked in a high-affinity state, and the results are compared to the same protein with both subunits mutated, and neither mutated.

Figure 5: Demonstrates the structural effect of the F97Y mutation
Mutagenesis

The desired mutations to the wild-type *Scapharca inaequivalvis* HbII hemoglobin require amino acid replacements. The structural change on the A subunit is achieved by replacing the phenylalanine at the 97th position with a tyrosine. On the B subunit, this same substitution occurs at the 99th position. These changes are abbreviated as F97Y and F99Y, respectively.

To produce this mutation, XL1-Blue E. Coli cells will be transformed with recombinant HbII genes. Two mutants are designed: a single mutant, with a wild-type A subunit and a mutant B subunit, and a double mutant, with both subunits mutated. The mutants are labeled A(F97Y)B(WT) and A(F97Y)B(F99Y). This is made possible by the heterotetrameric nature of HbII. Because there are differing subunits, coded for by different genes, mutations may be made to only some of the subunits, allowing observation of the effect on the final protein. Oligonucleotide primers, short DNA sequences containing the desired mutation, are designed from the *Scapharca* hemoglobin genes, according the specifications outlined in the *QuikChange* protocol (see Appendix). These short pieces of DNA serve as starting points for later replication.

The plasmids that will be used as vectors are double-stranded, so two oligonucleotides must be made for each mutation. These are sense and antisense, one for each direction of the double-stranded DNA plasmid. To switch the Tyr at the 97th position for a Phe, the codon for Tyr must be replaced with one for Phe. Multiple codons for Tyr exist, however, so one must be selected. This is done by obtaining data on which codons occur most often in wild-type E. coli. For phenylalanine, this is TAT. The switch from Tyr to Phe requires only a single point mutation, as only one base differs between the codons. The sequence for the oligonucleotide is selected for a specific melting point, and other conditions favorable to annealing and replication.
Once obtained, the oligonucleotide primers are then annealed to plasmids, and extended in a polymerase chain reaction as outlined in the QuikChange protocol (see Appendix). Once the plasmid containing the desired mutation is sufficiently replicated, it is then prudent to check the product for error using gel electrophoresis. Once the samples are checked, the paternal DNA is digested using DPN I endonuclease, leaving only the recombinant DNA. This is possible because the paternal DNA is methylated, while the newly replicated plasmids are not. Digesting the paternal material leaves only the replicated, recombinant genes in plasmid form. The remaining plasmids are then transformed into XL1-Blue supercompetent E. coli cells and grown on LB-ampicillin agar plates. Once colonies develop on the plates, the surviving mutated colonies are selected, and 6 liter quantities of the cells containing the mutated hemoglobin are grown.

**Purification**

Once quantities of transformed E. coli cells are grown, they are purified to obtain mutated *Scapharca* hemoglobin. The cells were broken open in a pressure cell, to release the intracellular proteins, along with the desired mutant hemoglobin. The mutants must be separated from the rest of the cell debris and protein. The first step of purification is to run the cells through a nickel column. This takes advantage of a “His tag”, or series of histidine residues, that was added to the vector beforehand. Histidine tags bond easily to nickel, so by running the contents of the broken cells, the mutated proteins will stick to the nickel column. Other materials, such as cell components or proteins, likely lack this bonding capacity and will run through. Such a technique would have been unavailable in the past, before mutagenesis became available, as scientists were forced to rely on a protein’s natural characteristics when purifying.

Once the mutated proteins are bonded to the nickel column, the column is washed with an excess of imidazole. Imidazole is a compound that bonds more readily to the His tags than the Ni of the column,
and therefore disrupts the bonds between the protein and the column. Released, anything that was bound to the column can run free.

This is not sufficient to purify the protein, however, because of all the other compounds and proteins that are likely present. Therefore, the His tags are cut off of the hemoglobin proteins using thrombin, a restriction enzyme. The added gene was designed with a site recognized by thrombin, so that it could be easily removed at this stage. This results in the mutant hemoglobins no longer readily binding to the nickel column, unlike the rest of the product collected from the column. The results of the previous purification step are then run again through a nickel column, with the result being purified HbII protein.

Further purification is required, however, because the result of the previous purification consists of both dimeric and tetrameric forms of the hemoglobin. Because the intended target of experimentation is the tetrameric form, any mutant hemoglobin that exists in dimer form must be removed. Both the A dimer, the B dimer, and the desired AB tetramer are present in the previous product. Also, any hemoglobin that may have oxidized in a previous step must be removed. For this, size-exclusion chromatography is used to extract the larger tetrameric form from oxidized or dimeric forms. Size-exclusion chromatography involves a column of a porous polymer. As a sample passes through the column, the smaller particles are able to enter more of the pores in the material, slowing their travel time. Inversely, larger particles travel a more direct route, and pass through the column quicker. This allows the separation of a solution based on molecular size. Used in conjunction with spectrographic techniques, this allows only the tetrameric form to be selected. By observing the absorption at a specific wavelength, which should differ between the tetramer, dimer, and any oxidized product, each can be identified. This size-exclusion chromatography is used to obtain pure tetrameric HbII hemoglobin for further use.
Experimentation

The purified hemoglobin is then analyzed in a series of oxygen binding experiments. These are done to assess the oxygen affinity of a particular mutation of hemoglobin. First, a sample of HbII is selected and fully deoxygenated. This is done by flushing the tenometer it is contained in with excess nitrogen, which releases any bound O$_2$ or CO. The tenometer is then sealed to prevent any contamination.

Using a spectrophotometer, absorption is measured at specific wavelengths between 500nm and 600nm. Oxygenated and deoxygenated hemoglobin have different absorption spectrums. Deoxygenated *Scapharca* hemoglobin has a single peak, while oxygenated has two (see Figure 6). The change in absorption values is predictable, based on the physical structure of the protein.

The selected wavelengths that are tested consist of 5 data points, and 4 wavelengths that are known not to vary as the hemoglobin shifts from oxygenated to deoxygenated, which are used to normalize the results. Specific amounts of air are then added to the tenometer. Smaller amounts are used in higher-affinity samples. After each addition, the sample is mixed for a period of ten minutes to allow the oxygen to bind. After each addition of air to the sample, absorption is measured at the same wavelengths. The rate of change in absorption values as the hemoglobin oxygenates and other factors allow for the affinity of the protein to be inferred. These experiments are conducted on the wild-type A(WT)B(WT), the single mutant A(F97Y)B(WT), and the double mutant A(F97Y)B(F99Y).

![Absorption Spectrums of wild-type HbII](image)

*Figure 6: Difference in absorption spectrum between deoxygenated and oxygenated wild-type HbII*
The data gathered from the absorption data is used to calculate various metrics of affinity and cooperativity. A p50 value, or amount of oxygen required for half-saturation, is generated to assess the hemoglobin’s oxygen affinity. A Hill coefficient is also generated. The Hill coefficient, first created to measure hemoglobin cooperativity, relates the concentration of ligand with the ratio of bound to unbound sites on the protein.

Results

Oxygen binding experiments on the HbII mutants demonstrate that the structural changes related to the T-to-R conformational change have significant impact on the cooperativity of the protein. Figure 7 demonstrates that while the oxygen affinity of the single mutant and double mutant are both higher than that of the wild-type, the cooperativity decreases dramatically. Also, the cooperativity of the double mutant is slightly higher than that of the single mutant. This points to two conclusions. First, that the structural mutation which prevents it from undergoing the full T to R transition decreases the cooperativity of the protein, indicating that the structural conformation is a key part of cooperative binding. Second, the single mutant having the lowest cooperativity shows that subunits which undergo similar structural modifications in the T to R transition are required to have a highly cooperative protein. The increase in cooperativity in the double mutant

![Figure 7: Hill plots, p50 values, and Hill coefficients for WT, single mutant, and double mutant HbII](image-url)
indicates that even with the highly diminished transition, having identical subunits results in more cooperativity than unmatched subunits.

The single mutant also demonstrates a strong susceptibility to oxidation. Oxygen affinity tests had to be repeated for the single mutant, due to rapid oxidation of test samples. The double mutant did not display the same instability, however. This suggests that mutation to a single subunit in the cooperative complex introduces instability into the entire model. This is likely caused by a mismatch between the subunits.

Discussion

Understanding and controlling the mechanisms of cooperativity and oxygen affinity in hemoglobin can lead to advances in many areas of science. One such area is the rapidly developing field of artificial oxygen carriers. With the demand for donating blood fast outpacing the supply in the United States, the need for a replacement is becoming increasingly apparent. It also addresses the issue of safety: while the United States blood supply is kept extremely safe, some diseases (prion-based ones, specifically) cannot be tested for. Many other countries’ blood supplies are not kept as safe as the United States’, especially in those areas ravaged by HIV. Developing an economical and practical artificial oxygen carrier would present a compelling emergency alternative to blood transfusion, without the need for type matching, maintaining a refrigerated supply, donations, and other problems.

To address this need, two main possibilities are being explored. The first of these are hemoglobin-based replacements. These present problems of their own, however, as hemoglobin requires specific conditions in which to function effectively. Both human and other mammalian hemoglobin have been explored for this purpose. The second possibility is diverging beyond hemoglobin to other molecules. The leading contenders at the moment are perfluorocarbons. These are molecules composed of carbon
and fluorine that are able to bind oxygen. These also present difficulties, however, because they are not soluble in blood, and must be emulsified in water.

The primary barrier to effective hemoglobin-based artificial blood substitutes are the physiological effects of hemoglobin when outside of red blood cells. Free-circulating hemoglobin is known to cause renal failure, due to reactions between protein and a byproduct of urea. This prevents hemoglobin from being administered directly. Several alternatives have been devised. One product, Polyheme, is having success in clinical trials. It attempts to avoid the dangerous physiological consequences of pure hemoglobin by purifying and polymerizing it. A study found that while patients treated with Polyheme had slightly higher occurrence of myocardial infarction and other adverse events, “the benefit-to-risk ratio of PolyHeme is favorable when blood is needed but not available.”

Other hemoglobin-based alternatives are also being explored. HemoPure, which has been approved for use in South Africa, is developed from bovine hemoglobin. It employs two methods; first, polymerizing the protein; and second, cross-linking subunits of the hemoglobin to prevent the tetramer from dissociating. It boasts three-year shelf-life and universal compatibility, as well as increased affinity and efficiency.

In the realm of alternate molecules, perfluorocarbons are promoted for their oxygen capacity, lack of side effects, and wider availability compared to hemoglobin-based substitutes. The current leading PFC based oxygen carrier is Oxygent. It uses perfluorooctyl bromide (PFOB), which is a linear PFC. One characteristic of PFOB is that it is removed from circulation after time, stored, and exhaled by the body. This prevents any residual damage or negative effects.

A problem faced by all of these potential replacements is controlling their oxygen affinity. Several early versions of hemoglobin-based substitutes faced the issue of hemoglobin’s oxygen affinity being too high outside of the environment of the red blood cell. What some alternatives are trying to accomplish is to
genetically modify hemoglobin to better maintain its tetrameric structure, while simultaneously lowering its oxygen affinity and maintaining its cooperativity. This is where research such as that conducted here is potentially valuable. For a mutated variant of hemoglobin to be successful, the mechanisms behind cooperativity and oxygen affinity in hemoglobin must be fully understood.
Appendix

A(WT)B(WT)

Hill Coefficient: 1.8  p50: 10.0 mm Hg

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A(F97Y)B(WT)

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A(F97Y)B(F99Y)

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**Guidelines for Oligonucleotide Creation**

Primers should be between 25 and 45 bases in length, with a melting temperature \( T_m \) of $\geq 78^\circ C$. Primers longer than 45 bases may be used, but using longer primers increases the likelihood of secondary structure formation, which may affect the efficiency of the mutagenesis reaction. The following formula is commonly used for estimating the $T_m$ of primers:

$$ T_m = 81.5 + 0.41(\%GC) - 675/\%mismatch $$

For calculating $T_m$:
- $N$ is the primer length in bases
- Values for $\%$GC and $\%$ mismatch are whole numbers

The desired mutation should be in the middle of the primer with $\approx$10-15 bases of correct sequence on both sides.

The primers optimally should have a minimum GC content of 40% and should terminate in one or more C or G bases.

**Protocol for PCR Reaction**

1. Synthesize two complimentary oligonucleotides containing the desired mutation, flanked by unmodified nucleotide sequence. Purify these oligonucleotide “primers” prior to use in the following steps.
2. Prepare the sample reaction as indicated below:
   - 5 µl of 10x Pfu reaction buffer
   - 1.5 µl Sense oligonucleotide primer
   - 1.5 µl Antisense oligonucleotide primer
   - 1 µl dNTP mix
   - 3 µl DMSO
   - 1.2 µl DNA template
   - 3.6 µl H$_2$O
   - 1.2 µl PfuTurbo DNA polymerase (2.5 U/µl)

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3. Cycle each reaction using the cycling parameters outlined in the above table.
4. Following temperature cycling, place the reaction on ice for 2 minutes to cool the reaction to $\leq 37^\circ C$. 
References


