An overview of sickle cell disease: analysis of the sickling process and current treatments

Biol126b - Protein Structure and Disease

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Who would have thought that one point mutation could give rise to such a crippling molecular disease\(^1\) as sickle cell anemia? Nearly anyone\(^2\) studying the sciences is familiar with this disease and will know that it is caused by the substitution of a valine for a glutamic acid residue at the sixth position of hemoglobin resulting in the characteristic sickling of a red blood cell. In this paper I will attempt to provide a brief history of the disease with a comprehensive discussion of its pathology and its associated hemoglobin structure as well as reporting on current and future therapies. As this marked the first instance of a genetic disease being linked to a specific protein and given the nature of this course, a thorough analysis of sickle cell anemia’s molecular underpinnings as opposed to its genetic basis will provide a better understanding of the sickling process and the suggested methods for treatment.

In order to understand how sickling occurs it is essential to consider the normal structure (HbA) and function of the hemoglobin molecule. As suggested above, this protein makes up a majority of the content within mammalian red blood cells and is responsible for transporting oxygen throughout the body. It is comprised of four subunits bound together each consisting of a heme group containing one iron (Fe\(^{2+}\)) atom that provides a site for free oxygen in the blood to bind to. Attached to each heme group is another polypeptide; all four polypeptides are collectively referred to as globin (two pairs of \(\alpha\)-globin and \(\beta\)-globin) and it has been shown that the resulting molecule exhibits pseudo 222 symmetry after the first low-resolution x-ray crystallography tests were conducted. While I will address oxygen dissociation with regards to sickle cell disease later in the paper, it is evident that the molecule will always be found in either of two states: deoxygenated or oxygenated hemoglobin. Additionally, the binding of a single oxygen atom and ultimately four in total, gives rise to a conformational

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\(^1\)Disregarding the genetics of sickle cell anemia, Linus Pauling refers to this disease as such in his seminal paper from 1949. I was going to begin my introduction with a discussion of Pauling’s reasoning behind this remark but I realized that Bunn [1] stole me idea.

\(^2\)Even physicists such as myself.
change in the protein’s overall structure as illustrated in figure 1(b). This observed change, more so than the drastically altered structure of hemoglobin S (HbS) under conditions of low oxygen concentration, will play a pivotal role in the rate limiting steps of the sickling process.

![Crystal structure of deoxyhemoglobin](PDB Id: 2HHB [2])  
![Overlay of oxyhemoglobin and deoxyhemoglobin chains](PDB Id: 1HHO [3])

Figure 1: Crystal structure differences between oxy- and deoxy-hemoglobin

As I previously stated, the sole cause of sickle cell anemia is due to a point mutation at the sixth position of the β-globin chain, a mutation that can be traced back in time and which displays certain geographic roots marked by extreme cases of malarial infection. This gene defect is more accurately shown to be the product of a single nucleotide polymorphism (SNP), denoted by the base change of a Tyrosine (T) for Adenine (A) base (GAG to GUG codon mutation), which results in the aforementioned substitution of the amino acid, valine, at this position. Initially, scientists believed this mutation originated in the Arabian peninsula during the Neolithic era and that the ensuing climate changes led to the migration of people carrying the affected gene. However, based on the analysis of chromosomal structures it has
been demonstrated that four independent mutational events, represented by four distinct haplotypes of the HbS gene, separately giving rise to the disease over approximately 70000-150000 years ago, have occurred. Of these four occasions, three haplotypes have been found to be indigenous to the African continent while a fourth appears to have developed in parts of the Middle East and India, and is appropriately named the Asian haplotype. “This can explain the observation made by many investigators that there is wide spread chromosomal heterogeneity of $\beta^S$ gene cluster haplotypes in United States as compared to the homozygous condition in Africa [4].” Furthermore, it has long since been held that the persistence of the mutation among black individuals, particularly within areas of Africa characterized by endemic malaria, is due to the fact that the malaria parasite is unable to consume polymerized hemoglobin in the sickled state while causing the red blood cell to rupture, and therefore incapable of reproducing, thus improving survival among carriers of the sickle cell trait [5].

While I would prefer to concentrate on the molecular basis of sickle cell anemia’s pathology, I would be remiss to neglect the means by which the disorder is inherited.\footnote{This will prove useful for commenting later on how the disease is expressed under various conditions and genotypes.} Due to the prevalence of sickle cell anemia within the black community (∼8% or approximately one in every 600 people within the United States), Taliaferro and Huck, not long after the disease was originally documented and subsequently studied clinically, suggested that the sickling phenomenon was the result of a single autosomal recessive gene. Although there was no clear distinction at the time between individuals displaying no apparent physical signs of anemia, referred to as having sicklemia or the sickle cell trait, and those whose erythrocytes frequently sickle, this difference was simply revealed to be the classification of the heterozygous (HbA HbS) and the homozygous (HbS HbS) states respectively [6]. This theory was vastly supported by the work of Pauling, who showed in the same year of Neel’s publica-
tion on the occurrences of the sickle cell trait within several populations, that the “existence of normal hemoglobin and sickle cell anemia hemoglobin in roughly equal proportions in sicklema hemoglobin preparations is obviously in complete accord with this hypothesis [7].” In concurrence, Neel demonstrated in a study of 42 parents of 29 patients with the disease, that all parental red cells within sealed blood samples collected, deprived of an oxygen-rich environment, ultimately sickled and therefore in agreement with the dominant gene hypothesis. A calculation of the gene frequency for sickling in African Americans \( (p) \) is found by applying the following formula: \( 2p(1 - p) = 0.8 \); the resulting \( p \) value is 0.042 corresponding to a ratio among individuals with sicklema to those developing sickle cell anemia of approximately 44:1 and within acceptable limits for actual population data considering the increased mortality rates for those with SCD [6]. There are a host of influences that contribute to the variability of the disease, several of which I will discuss shortly, but two important genetic factors include thalassemia, a particular form, \( \beta \)-thalassemia which reduces the production of normal hemoglobin such that an individual carrying one gene of both sickle cell disease and \( \beta \)-thalassemia (sickle \( \beta \)-thalassemia) can potentially develop symptoms associated with the homozygous condition for the disease itself as well as hemoglobin C which when combined with HbS (hemoglobin SC disease) has similar degrees of severity.

The substitution of a valine amino acid results in a series of biochemical changes leading to structural deformations of one’s red blood cells. These asymmetric differences between the red cells of normal and diseased individuals were first reported in 1910 by James Herrick whose intern observed ‘peculiar elongated and sickle shaped’ cells in the blood of an anemic black medical student admitted to the hospital [5]. Since Pauling’s work in 1949, there were numerous experiments during the decades to follow that attempted to understand the

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4Pauling’s experiment actually determined by means of electrophoretic analysis, that a composition of 40 percent sickle cell anemia and 60 percent normal carbonmonoxyhemoglobin had similar mobilities to the material extracted from individuals with sicklema. It was discovered, on the basis of electrophoresis, that the difference between sickle cell anemia and normal hemoglobin is 2–4 more net positive charges.

5Based on my research, it appears that the most important breakthroughs and discoveries occured not
molecular pathogenesis of sickle cell disease. Following the steps outlined in figure 2, the process of HbS polymerization is initiated upon the generation of deoxyhemoglobin due to the unbinding of all four oxygen atoms from oxyhemoglobin; the globular $\alpha_2\beta_2^S$ hemoglobin tetramer is depicted as a flat circle while the corresponding red blood cell is shown on the right side of the diagram. When HbS is subject to deoxygenation, the incorporated valine residues at the sixth position of the $\beta$-globin chains induce hydrophobic interactions with neighboring hemoglobin molecules as well as a separation of the $\beta$ subunits as shown in figure 3(b).\(^6\)

These hydrophobic patches, depicted as a projection out from one of the $\beta$-globin subunits will “bind to a complementary hydrophobic site” (shown as an indentation) from another hemoglobin tetramer [1]. As more tetramers associate themselves with neighboring hemoglobins, a long polymer is assembled along the cell’s long axis, and inevitably a 14-strand helical fiber with a double strand composed of staggered hemoglobins being the essential building block. The time scales governing this process, given as a delay time ($t_d$), between HbS deoxygenation and the formation of this ‘twisted rope-like structure,’ is “inversely proportional to the intracellular hemoglobin concentration (C) raised to about the 15th power” with $k$ being an experimental constant and which can range from milliseconds to seconds before these “polymers can be detected, after which they accumulate rapidly [1] [9].” As the fiber continues to grow and alignment of the individual polymers occur, the red blood cell assumes the form of the elongated, ‘sickle’ shape. In actuality, this ‘banana’ or ‘sickle’ motif is not the only configuration scientists have found among affected individuals; electron microscopy has identified bundles of HbS fibers oriented with each projection in the long after Pauling’s and that very little worthwhile analysis of the disease (treatments notwithstanding) took place afterwards; you may feel otherwise, but as more information was made available, scientists studying the disease found themselves standing on the shoulders of giants.

\(^6\)I attempted to overlay the x-ray crystallographic structures of both deoxy and deoxy-S hemoglobin forms but they appeared nearly identical, which as I later determined was more or less true when minimal levels of oxygen being present.
Figure 2: Polymerization of deoxygenated HbS. Image taken from [1]
Figure 3: Important features to note are the gray clusters which represent noncovalently bonded heme groups as well as the red box containing the region where the sixth residue, glutamic acid, is located. Images taken from [http://www.ornl.gov/sci/techresources/Human_Genome/posters/chromosome/hbb.shtml](http://www.ornl.gov/sci/techresources/Human_Genome/posters/chromosome/hbb.shtml)

Case of 'holly-leaf' shaped cells.

The structure and behavior of sickled cells is controlled by a variety of factors including oxygen saturation, intracellular hemoglobin concentration and hemoglobin F. Hemoglobin F, or fetal hemoglobin, is known to inhibit polymerization because of a specific glutamine residue which hinders an important lateral contact from being made with the sickle cell fiber. Additionally, the sickle cell trait has been proven asymptomatic since the concentration of HbS is too low in heterozygous individuals for polymerization to occur and polymers almost never form in the cell during flow through smaller portals such as capillaries and arterioles as there is insufficient time compared to $t_d$. It has also been observed that the rate of deoxygenation plays an important role in the development of sickled cells: quick deoxygenation has no physical effects on a red blood cell’s appearance presumably since multiple polymerization events act independently of one another and cannot coalesce with any degree.

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80 percent of the time.
of success whereas acclimation to gradually decreasing $O_2$ levels will result in an aggregation of HbS molecules entering the sickling process. Consequently, I find that the property most influencing the kinetics of sickling is oxygen dissociation, the widely drawn sigmoidal curve recognizeable to any student studying biology and/or physics as hemoglobin has been the stand-alone model for demonstrating cooperative or allosteric binding. In an effort to further our understanding of sickle cell anemia, I plan to elucidate\(^8\) the cell’s responses to deoxygenation as a function of $O_2$ partial pressures as well as comment on why the ‘sickle’ shape is energetically preferred.\(^9\)

Addressing the reason why the valine amino acid substitution ultimately forces the hemoglobin tetramers to polymerize and ergo adopt the ‘sickle’ shape, I looked at the results of molecular dynamics simulations on a lateral contact involving HbA and deoxygenated HbS (from their crystal structures) performed by Kuczera \textit{et al.} \cite{10}. Regretfully, my own attempts to calculate the free energy difference between normal and sickle cell hemoglobin (and the developing fiber) proved a more difficult task than I initially expected due to a variety of interactions, some of which are not known. For the purposes of discussion, and the cited paper, we consider isolated hemoglobin tetramers as the ‘monomer’ while interacting pairs shall be ‘dimers;’ there is understandably a free energy difference relating to the association of these two components. As HbS undergoes polymerization, each tetramer particpates in two asymmetric lateral contacts (as an acceptor and as a donor for the valine side chain).

Some of the remarkable conclusions, in my opinion, drawn from these simulations indicate that “it is not the stabilizing hydrophobic interaction of valine in HbS that is the dominant factor in the [free energy] difference,\(^{10}\) but the loss of the destabilizing [electrostatic]

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\(^8\)Yes. I used the dreaded word but this will likely be the only instance and I believe it to be impossible to write a scientific paper without incorporating such technical (and verbose) terms as deoxygenation, polymerization, dissociation etc.

\(^9\)This will be my only foray into the quantitative biology aspect of the disease. I would once again be remiss if I didn’t include at least some equations or if I denied myself the opportunity to perform some mathematical analysis which I believe I have kept to a minimum with respect to the overall content of the paper.

\(^{10}\)\(\Delta \Delta G = \Delta G^{{HbS}}_{assoc.} - \Delta G^{{HbA}}_{assoc.} = \Delta G^{{HbS}}_D - \Delta G^{{HbA}}_D\) where “\(\Delta G_D\) is the free energy of transfer of the $\beta 6$
interactions of glutamic acid in HbA [10].” These electrostatic energy terms dominate in the calculation of $\Delta \Delta G$ found to be -15kcal/mol; specifically they represent a gain in entropic energy, greater in the dimer, to account for the large loss in enthalpy-driven stability whose energies are shown to be stronger between the monomer and its water environment than they are for glutamate’s interactions with neighboring water molecules and proteins from the dimer. Van der Waals energies can be neglected as they are an order of magnitude smaller. Without going into unnecessary details regarding the protein interactions, the inter-chain energies arising from the association of two tetramers yields a difference of -3.6kcal/mol favoring the valine residue over glutamic acid thus stabilizing the lateral contact or dimer, promoting HbS polymerization. Since the electrostatic energies play a critical role in the formation of the dimer states, it has been suggested that the addition of cations, to dampen the electrostatic repulsions from Glu-$\beta$6, may encourage the production of HbA aggregates and possibly HbS-like fibers [10]. While there has been minimal work to date in determining these values experimentally, the calculations are consistent with reported energies pertaining to charged-neutral biochemical/protein structures.

To better evaluate the biochemical differences between sickle cell and normal hemoglobin, I considered the widely applicable Adair model for ligand binding processes which reveals a noticeable difference with respect to the binding affinities of HbS at varying levels of oxygen concentration. I would like to point out that I chose to analyze oxygen saturation’s affects on hemoglobin using this particular model as opposed to the standard MWC model of today or, specifically, Pauling’s since 1) the wide availability of published data including known adair constants and 2) this model accounts for multiple body interactions with energies that may substantially differ from the typical pairwise interactions associated with Pauling’s tetrahedral hemoglobin molecule. While many can recall Adair’s equation (4) from memory, I feel compelled to derive it from first principles of elementary thermodynamics.\(^{11}\)

\(^{11}\)I will be leaving out several steps but everything follows systematically. I have observed that biochemists
Applying the Adair model, the output for normal hemoglobin is consistent with recent experiments (P(HbA)\textsubscript{50%}) under native conditions while the adair constants used for generating the HbS sigmoid, via a computer fitting algorithm, were based on oxygen binding curves made by stepwise deoxygenation of HbA and HbS at various solute concentrations in buffer (apparently both forms of hemoglobin behave similarly in solution) [11]. According to figure 4, as the concentration of \(O_2\) (directly proportional to its partial pressure) decreases to extremely low levels (and vice versa), HbA and HbS overlap; this clearly supports earlier findings that for prolonged periods of deoxygenation or at very low levels of oxygenation, sicklemia hemoglobin can polymerize as HbS fibers and subsequently 'sickle.'
Figure 4: Scaled oxygen dissociation curve for HbA and HbS using their adair constants ($k_1 = 0.0517$, $k_2 = 0.0398$, $k_3 = 0.453$, $k_4 = 6.9$) and ($k_1 = 0.0743$, $k_2 = 0.0271$, $k_3 = 0.0966$, $k_4 = 0.489$) listed in [12] and [11] respectively. $P(HbA)_{50\%} = 3.85\text{mmHg}$ and $P(HbS)_{50\%} = 12.2\text{mmHg}$ from graph.

Furthermore, and arguably the most significant aspect of this analytical treatment of sickle cell anemia, the graph, above, displays an obvious shift to the right for HbS (commonly observed in patient crisis events when their $O_2$ levels are analyzed, usually by pulse oximetry [13]) indicating that the hemoglobin of an individual with SCD, when compared to the red cells of an unaffected person, will have drastically different binding affinities over a wide range of pressures,$^{12}$ marked by lower percentage of HbS oxygen saturation, and resulting in an increased risk for an anemic episode.

$^{12}$Approximately between the two extremes: $1\text{mmHg}$ and $148\text{mmHg}$, according to figure 4.
In this last section, I plan to discuss the nature of these crises I have just alluded to and the methods of treatment being suggested as well as those that are currently in use. Since SCD is a chronic illness, the acute medical episodes typically characterized by severe pain to the chest and extremities, will tend to occur throughout a patient’s life and may last up to several days and even weeks at a time. The cause is explicity rooted with the structure of the disease; the distorted shape of affected red blood cells due to the polymerization of HbS fibers makes the individual prone to maladies involving the obstruction of blood flow. These crises are usually triggered by the accumulation of sickled red cells, provided $t_d$ is less than the time for transit within blood vessels, which adhere more readily than normal cells to endothelial surfaces leading to vaso-occlusion, see figure 5. In extreme cases, patients, commonly children, may receive blood transfusions or bone marrow transplants in the hopes of rapidly reducing the ratio of HbS and total hemoglobin concentration to less than 30% [9]. Less invasive procedures intended to ameliorate the disease’s effects include the “chemical inhibition of hemoglobin S polymerization, reduction of the intracellular hemoglobin concentration, and pharmacological induction of hemoglobin F [1].” Currently there are no reliable anti-sickling drugs while there has been renewed interest in the research of antifugal drugs, such as Clotrimazole which is engineered to block (Gardos) cation-transport channels, particularly $K^+$ and loss of water in order to limit intracellular hemoglobin concentration and control cellular dehydration.

Arguably the most effective treatment being employed today has been the administration of hydroxyurea, designed to promote hemoglobin F production which has been extensively shown to decrease the severity and rates of annual crisis events as reported in 1995 following a national clinical trial conducted with 299 adults [14]. The mechanism by which hydroxyurea
and other antitumor drugs influence hemoglobin F stimulation is not known; however, it is believed that “by selectively killing cells in the bone marrow, hydroxyurea increases the number of erythrocytes that produce hemoglobin F [9].” As stated earlier, hemoglobin F, lacking β-globin chains, inhibits HbS polymerization at each lateral contact due to the interference by a key glutamine amino acid located at γ87, thus the likelihood for vaso-occlusion events and related episodes diminish as there are fewer and less deformed red blood cells capable of attaching to capillary walls. Moreover, while certain studies found evidence for reduced adhesion, they also reported an increase in the time required for polymerization. Regarding the clinical trial I referred to, Charache et al. made use of a ‘randomized, double-blind, placebo-controlled’ study that was concluded before the planned 24 months were completed since nearly all patients showed significant improvement and reduced frequency of painful crises, see figure 6(a), with no apparent short-term side effects [14].

![Graphs showing data](image)

(a) Median times from the start of treatment to the first reported painful crisis  
(b) Measurements of F Cells

Figure 6: Data according to the Treatment Group from [14]

Although intervention with hydroxyurea resulted in a 44% reduction in the median annual painful crisis rate suggesting hemoglobin F as the principle mediator, this was only chiefly demonstrated for the first 3 months from the onset of therapy, whereas a more convincing theory, supported by the documented decline of neutrophil populations (neutropenia) as well
as the number of reticulocytes following treatment with the drug throughout the duration of the trial, purports these changes as the more likely explanation for hydroxyurea’s ‘efficacy’ [1].

In conclusion, there is no known cure for sickle cell anemia and the average lifetime expectancy for a patient harboring the disease is between 50 to 60 years in industrialized countries. One of the most promising techniques developed in recent decades is gene therapy, but despite its potential to inactivate the HbS gene or promote the expression of hemoglobin F and other supporting regulator molecules, scientists believe this route to be extraordinarily difficult. Hydroxyurea is currently the leading candidate for universal SCD treatment and while it is approved by the FDA for certain types of leukemia and other cancers, research is still being conducted to determine the magnitude of both its beneficial and unwarranted effects of which the latter appear to be minimal. In this paper I have attempted to understand sickle cell anemia by analyzing the structure of the hemoglobin S tetramer and fiber as well as investigating the sickling process which all together reveals an active science community engaged in determining the cause of this disease and hopefully rationally developing better treatments and/or possible cures.
Bibliography


