MOLECULAR ANALYSIS OF SICKLE CELL DISEASE

Introduction

Red blood cells contain large amounts of the protein called hemoglobin. This protein serves to transport oxygen from the lungs to tissues and organs of the body. Hemoglobin A (found in adult red blood cells) is a globular protein consisting of four polypeptide subunits (two $\alpha$ and two $\beta$ polypeptides, often presented as $\alpha_2\beta_2$). To each subunit is attached an iron-containing component called heme, and each heme group can bind a single molecule of $O_2$. Many changes in the structure of hemoglobin have arisen by point mutations occurring in the genes coding for the polypeptide subunits. These mutations frequently result in the substitution of one amino acid for another. Some of these mutations are harmless, while others can cause serious disease.

BEFORE COMING TO LAB: Use the NCBI web site (http://www.ncbi.nlm.nih.gov/guide) to determine the chromosome locus for the genes coding for the $\alpha$ and $\beta$ subunits of hemoglobin. Under Popular Resources (on the right side of the homepage), select <Genome>. Under the section titled Custom Resources select <Human Genome>. In the box titled 'Find A Gene', type in hemoglobin alpha 1 subunit in the <search for> field and select <Homo sapiens> from the drop down box. You will repeat this for the hemoglobin beta subunit.

Answer the Following Questions:

What is the official allele symbol for the hemoglobin alpha 1 subunit? __________

What is the location of the hemoglobin alpha 1 locus in the human genome (note chromosome number arm and location on that arm)? __________

What is the allele symbol for the hemoglobin beta subunit? __________

What is the location of the hemoglobin beta locus in the human genome (note chromosome number arm and location on that arm)? __________

A common hemoglobin variant is that known as sickle cell hemoglobin (hemoglobin S), which is present in individuals suffering from sickle cell disease (also known as sickle cell anemia). In hemoglobin S, a single glutamic acid (negatively charged at physiological pH) in the two $\beta$ chains is replaced by valine (uncharged at
physiological pH). This single amino acid substitution causes a change in the three dimensional shape of the hemoglobin molecule and consequently reduces its binding affinity for oxygen. In addition, red blood cells lose their characteristic flexible biconcave disc shape and assume an irregular crescent (sickle) shape. Sickled red blood cells can clump together and block blood flow through small capillaries. Blocked blood vessels, in turn, can cause pain, serious infections, and organ damage. The distorted cells are rapidly destroyed in the body contributing to anemia. Thus, changes in hemoglobin and red blood cell structure result in decreased oxygen levels within the body.

Sickle cell anemia is inherited as an autosomal recessive disorder. Heterozygous individuals produce both types of hemoglobin, with approximately 60% being hemoglobin A. This level of normal hemoglobin is generally enough to avoid serious disease, and individuals are said to have sickle cell trait (not to be confused with sickle cell disease). Individuals homozygous with the sickle cell allele produce only hemoglobin S, which leads to sickle cell disease. The responsible mutation is believed to have originated in a West African population. Heterozygous individuals appear to have a selective advantage, in that they appear to be resistant to one form of malaria. As a result, the mutant allele has reached a relatively stable equilibrium in the African population.

Several normal forms of human hemoglobin also exist. Each is produced (and functions) at a particular stage in human development. For example, hemoglobin type Gower 1 is produced by RBCs during embryonic and fetal development. It consists of two zeta chains and two epsilon chains (ζζεε). Hemoglobin F is also produced during fetal development and replaces Gower 1 by 8 weeks of gestation. After birth, RBCs produce hemoglobin A (ααββ) and hemoglobin A2. Hemoglobin A2 differs from hemoglobin A in that it is composed of two alpha chains and two delta chains (ααδδ). The delta chain differs from the beta chain by several amino acid substitutions, but it is equivalent in length to the beta chain (146 amino acids). While these substitutions affect overall surface charge of the hemoglobin protein, the ability to carry O2 in the bloodstream is not affected. Hemoglobin A2 is a minor hemoglobin component, accounting for less than 5% of total hemoglobin.

When doctors suspect sickle cell disease, they perform a number of diagnostic blood tests. Sickle-shaped red blood cells and fragments of destroyed red blood cells can be seen in a blood sample examined under a microscope. Another blood test called hemoglobin electrophoresis is also done. The hemoglobin electrophoresis test identifies the hemoglobins in a blood sample.

The hemoglobin electrophoresis test identifies hemoglobins in a sample based on overall molecular charge, which is determined by the amino acid sequences of the component polypeptides. In electrophoresis, molecules are distinguished from one another by their differing mobility in a porous gel when an electrical current is applied. All forms of hemoglobin have a net negative charge at pH 9.2, thus all forms will migrate toward the positive pole in an electric field. Even one amino acid difference
can potentially affect overall charge and, as a result, affect the rate of movement through a gel. The hemoglobin form with the greatest overall negative charge will migrate the fastest. Note that in this exercise agarose gel electrophoresis will be used to separate proteins of the same size that have differing overall (or net) electrical charge. Given the information provided thus far concerning the type of electrophoresis used and amino acid differences between hemoglobin A and S, make predictions about the relative migration rates for these two forms of hemoglobin. Which will have the fastest mobility?

In this exercise you will use the hemoglobin electrophoresis test to determine the hemoglobin composition of a blood sample obtained from a young boy (Dan) suspected of having sickle cell disease, his parents (Ben and Jennifer) and two of his three siblings (his sister Gail and his brother Paul). His brother Matt was overseas at the time of the testing. Blood tests are often performed on relatives of individuals with or suspected to have the disorder, because they may have the sickle cell trait. Discovering the trait in people can be important for family planning to determine their risk of having a child with sickle cell disease.

In an effort to save time, the agarose gel that you will use has already been prepared for you, and thus you will begin with Sample Analysis. You will, however, prepare a gel for the next lab section to use.

**Procedure**

**A. Sample Analysis**

1. You and your lab partner will 'share' a gel set-up with the lab group in your bench area. Place a gel tray (containing an agarose gel) into the gel tank so that the wells in the gel are oriented at the cathode end of the gel tank (the end with the black electrical connection). Note that the gel has two rows of wells. You and your lab partner will use one row of wells, and the other lab group will use the other row.

2. Fill the buffer reservoir with TG buffer (Tris-glycine buffer, pH 9.2), covering the gel to a depth of about 2 mm (i.e. the gel is just covered with buffer). If the combs are still in the gel, remove them carefully by grasping at both sides and pulling straight up.

3. You instructor will give your bench area the following samples (in microtubes): two standards - hemoglobin A (labeled Hb A) and hemoglobin S (labeled Hb S); and five individual samples - Ben (labeled B), Jennifer (labeled J), Dan (labeled D), Gail (labeled G), and Paul (labeled P). Note that there is enough sample in each tube for both lab groups (i.e. you and your lab partner will share these
tubes with the other lab group also using your gel; both lab groups will load these seven samples).

4. Load 25 μl of each sample into a separate well on one gel (i.e. load one sample per well). NOTES: Use a new pipet tip to load each sample; dispose of used tips in a biohazard bag or waste collection beaker at your bench; make sure you have a record of well location for each sample. Each hemoglobin sample has been diluted with glycerol to increase its density and cause it to fall to the bottom of a well during gel loading.

5. Place the tank top on the gel tank and press it down in order to connect electrode wires to electrodes. NOTE: Wells of the gel should be oriented towards the black (negative) electrode; Match the black electrode wire with the black electrode).

6. Plug the gel rig electrode wires into the power unit (match electrode wire colors with receptacle color – red with red and black with black). Turn the power unit on. Select constant voltage and set display to 100 volts. Press the button with the runner symbol. If the rig was set up properly, as soon as you push the “runner” button you should begin to see small bubbles arise from the thin silver wire attached to the electrodes (the wire is covered with electrode buffer and runs across the width of the gel rig at each end).

7. Perform the electrophoresis at 100 volts until the hemoglobin samples have migrated approximately 1/2 of the length of the gel (approximately 1 1/2 hours).

8. During the electrophoresis, prepare an agarose gel by following the procedure in Part B below. This gel will be used by the next lab section. If lab time had permitted, you would have prepared this gel first to use in the electrophoresis of your samples.

9. When the hemoglobin samples have migrated approximately ½ the gel length, press the runner to stop the electrical current, turn the power unit off, and (wearing gloves) remove the gel from the electrophoresis unit. Visualize the hemoglobin forms present in each sample; it may help to place the gel on a white light transilluminator.

10. Make a rough sketch of your gel. In your sketch note 1) the orientation of the + and – electrodes (the anode and cathode, respectively), 2) the standard or sample that was loaded into each well, and 3) the relative location of each band visible in each lane (the area beneath a given well). Note that each band detected for a sample will co-migrate with that of a given standard.

11. Clean your bench area as follows: discard your gel (NOT the gel tray or comb), gloves, pipet tips, and any unused sample in the biohazard bag provided. Carefully pour the electrophoresis buffer into a collection container and rinse the
gel tank and tray with tap water. Leave these at your lab bench to dry. Wipe up any buffer spills with a wet sponge.

12. Interpret your results for the five blood samples (Ben, Jennifer, Dan, Gail, Paul), giving 1) hemoglobin composition, 2) phenotype, and 3) genotype.

13. Create a pedigree that outlines sickle cell disease for the individuals examined. If necessary, consult your textbook for the standard symbols used in pedigrees.

14. Questions: What is Dan's diagnosis based on this test? What was the probability that the parents would have had a child with sickle cell disease? The Smith's have an older son (Matt) who, by all appearances, is phenotypically normal and has not yet been tested for sickle cell trait. What is the probability that he is not a carrier?

15. Question: Below is a karyotype for a woman who has sickle cell trait. Using the following allele symbols, note the allele locations for both the alpha and beta subunit genes. Allele symbols: HBA1 – normal alpha subunit allele; HBB - normal beta subunit allele; HBS – sickle cell allele. Note that you will need the information requested (for chromosome locus; page 57) from the NCBI website.
B. Preparation of Gels

The gel you used was prepared for you in advance due to the time constraint imposed by a two hour lab period. You and your lab partner must now prepare a gel for use by the next lab section.

1. Place your gel tray into a casting tray with a gel tray from the neighboring lab group (i.e. two lab groups must share a casting tray). Orient the gel trays such that the open ends of the tray are facing the rubber gaskets that line the two sides of the casting tray. Turn the knob at the top of the casting tray to seal the ends of each gel tray with the rubber gaskets.

2. Place two combs in your gel tray – one at an end of the gel tray and the other in the middle of the tray. There will be groves on the gel tray for the combs to fit into.

3. Prepare 40 ml of a 0.8% agarose solution as follows: Weigh 0.32 g of agarose into a weigh boat and transfer this to a 125 ml Erlenmeyer flask. If the available balance has a maximum weighing capacity that will allow you to do so, you can weigh the agarose directly into the flask. Using a 50 ml graduated cylinder, measure 40 ml of electrophoresis buffer (Tris-glycine buffer, pH 9.2) and add this to the flask containing the agarose. Swirl to mix. NOTE: The agarose will not dissolve.

4. Gently stuff a clean KimiWipe into the mouth of the flask. Dissolve the agarose by heating the solution in a microwave. Begin by heating for 30 seconds. Remove and swirl flask contents – note that the agarose has not completely dissolved. Heat the solution again for 15 seconds. Watch the flask carefully and stop the microwave when solution begins to boil violently and upward out of the flask. Remove the flask from microwave and carefully observe solution - no solid particles should be visible (check carefully, for the agarose will become transparent as it melts). It will probably be necessary to heat the flask one or two more times for 15 seconds. NOTE: IF two flasks are heated at once, a minimum of three 30 second heating periods will be needed to completely melt the agarose.

5. Allow gel solutions to cool for 5 minutes. (NOTE: To determine if the melted solution is cool enough to pour, touch the bottom of the flask to inside of your wrist – when it is hot (just uncomfortable, but not yet not “warm”) to touch the solution is ready to pour; Do not pour an agarose solution that has begun to solidify into a gel mold).

6. Pour the liquid agarose solution into the gel tray/mold. Be sure the combs are in place!
7. Allow the solution to cool and solidify until it is firm (about 15 - 30 minutes). The agarose will turn a semi-translucent white when it is solidified.

8. Remove mold with gel from the casting tray. Remove combs, rinse and leave them at your bench. DO NOT THROW COMBS AWAY. Place the mold with gel into a gel storage box at your bench. You will share this box with the other lab group at your bench. Add approximately 50 ml of TG buffer (to prevent the gel from drying out) and cover the box with its lid. Your gel will be stored in the refrigerator and used by the next lab section.