**Biochemistry Learning “Problem” – Aggregate versus case-oriented thinking (maybe…)**

I teach Introduction to Biochemistry (BCMB / BIOL / CHEM 3100), an upper level course for life science majors, most of whom will pursue careers in healthcare, nutrition, and exercise science. In the process of teaching about how protein structure and function are influenced by pH, I noticed that students appeared to be conceptualizing pH as a “switch,” such that changes in pH (availability of hydrogen ions) lead to protonation (binding of hydrogen ions) or deprotonation (unbinding of hydrogen ions) of all available protein molecules, rather than as a probabilistic and dynamic phenomenon.

On the following pages, I have included example problems from assignments and exams where I have seen students struggling. I have also included the keys / grading rubrics for some questions in blue font. To answer these questions, my sense is that students have to (generally in order):

1. recall or recognize key amino acid residues in proteins (hemoglobin was used for most of the examples since it is well characterized and many mutations have been identified and analyzed)
2. interpret the table of amino acid pKas on page 6
3. apply pKa information to identify the ratio of protonated to deprotonated amino acids at particular pHs
4. apply knowledge of protonation state to predict behavior of a protein in physiological or experimental scenarios (e.g., how the protein would move during gel electrophoresis, whether a protein would be likely to bind its substrate or assume a particular conformation)

I have not collected or analyzed student work systematically, but students’ responses to the questions on the following pages suggested that they thought that:

* there was some pH at which all protein molecules would be protonated or deprotonated, and
* once a protein was protonated or deprotonated, it would stay that way.

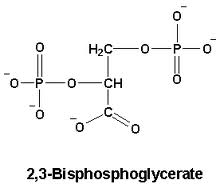
These conceptions limited students’ abilities to:

* describe the structure and function of a protein based on its amino acid composition and environmental surroundings (i.e., local pH),
* predict how structure and function would change based on environmental changes, and
* generalize structural and functional changes to predict outcomes at a physiological level.

For example, students would have difficulty explaining the relationship between the micro-level structures of a large number of hemoglobin molecules likely to be observed at a particular pH (i.e., of the millions of hemoglobin molecules in a single red blood cell, how many are likely to adopt a relaxed versus tense conformation at a given pH?) and the macro-level physiological outcomes (e.g., enough oxygen being delivered to and carbon dioxide being removed from tissues in the body). In trying to help students understand this phenomenon, a graduate TA offered this “trick:” if pH < pKa, the amino acid will be protonated; if pH > pKa, the amino acid will be deprotonated. To me, this trick seems like a symptom of the problem – that the system is not probabilistic or dynamic.

Although understanding this particular concept / set of concepts may not be critical, probability and dynamicity are foundational to many biological processes, including evolution, cell signaling, and disease. I would like to understand how undergraduates’ recognition and reasoning about the probabilistic and dynamic nature of biological phenomena influences their ability to understand and solve biochemical problems.

**Exam Question 1.** The affinity of purified hemoglobin for oxygen is much greater than its affinity in red blood cells. This is because red blood cells carry an allosteric regulator molecule, 2,3-bisphosphoglycerate (2,3-BPG). 2,3-BPG binds to hemoglobin in a pocket created in the middle of the His2, Lys82, and His143 residues on the two beta chains (i.e., His2, Lys82, and His143 on the beta 1 chain form one side of the pocket, and His2, Lys82, and His143 on the beta 2 chain form the other side of the pocket). The structure of 2,3-BPG is shown on the right.



**Part A (1 point):** What ratio of His side chains would you expect to be protonated versus deprotonated at physiological pH (i.e., pH 7.4)?

So-so answer: 1 protonated to 10 deprotonated (0.5 pts)

Ideal answer: 1 protonated to 50 deprotonated (1 pt)

**Part B (1 point):** What ratio of Lys side chains would you expect to be protonated versus deprotonated at physiological pH (i.e., pH 7.4)?

1000 protonated to 1 deprotonated (1,100 protonated to 1 deprotonated is also acceptable, and even a better answer but 1000 to 1 is close enough)

**Part C (2 points):** Explain why His and Lys would form a good binding site for 2,3-BPG. Be sure to address the protonation state of each amino acid’s side chain in your response.

Most Lys would be positively charged at physiological pH, and a good portion of His would be positively charged at physiological pH (hemoglobin structure shifts pKa toward physiological pH so even more likely to be protonated). Thus, the positive charges on their side groups would enable charge-charge interactions with 2,3-BPG. 1 pt for explaining that both Lys and His would carry positive charge, and 1 pt for noting that this would allow charge-charge interactions with 2,3-BPG (which carries a lot of negative charge).

**Part D (2 points):** In the Providence variant of hemoglobin, Lys82 is substituted by asparagine. Explain how this substitution would affect binding of 2,3-BPG by hemoglobin.

Side group of Asn cannot be protonated, so binding would be less stable. 1 pt for noting that side group of Asn is neutral. 1 pt for noting that this would decrease affinity for 2,3-BPG / make 2,3-BPG binding less stable.

**Part E (3 points):** In the Providence variant of hemoglobin, the Asn82 substitution can spontaneously undergo deamination to form an aspartate. Would you expect hemoglobin with a Lys82 to Asp substitution to differ from Lys82 to Asn in its binding of 2,3-BPG? Why or why not?

Side group of Asp can be ionized so that it is negatively charged, making an even poorer binding site for 2,3-BPG because of the charge-charge repulsion. 1 pt for noting that side group of Asp would be negative at physiological pH. 1 pt for noting that this would be worse for 2,3-BPG binding than Asn. 1 pt for explaining that this is because of charge-charge repulsion (or some language that indicates that they understand that two negative charges will repel).

**Exam Question 2.** A new mutant, hemoglobin Cowtown, has been found in a man and his father, both of whom have erythrocytosis (i.e., a larger proportion of blood volume occupied by red blood cells). The abnormal hemoglobin is not detectable via electrophoresis in alkaline buffers, but it resolves distinctly during electrophoresis at pH 6.0. Amino acid analysis of the beta chain reveals that the C-terminal histidine residue (beta 146) has been replaced by leucine.

**Part A (3 points):** Explain why this substitution would cause the abnormal hemoglobin to be detectable during electrophoresis at pH 6.0, but not in alkaline buffers. Include a drawing / drawings of gel(s) to help illustrate your points.

At pH 6, approximately 50% of the side groups of His146 in the wild-type hemoglobin will be protonated – these will be in equilibrium, with any given His146 being protonated / deprotonated 50% of the time. This will generate a single band that moves more quickly toward the (-) electrode than a hemoglobin variant with a neutral substitution, such as Leu146 (Cowtown variant), which will not be protonated at any pH. Thus, an unaffected individual will have one band representing both His146 alleles, and an affected individual will have two bands (one that represents the His146 allele and the other than represents the Leu146 allele), with the Leu146 band more distant from the (-) electrode. At alkaline pH, His146 will be more likely to be deprotonated, and thus will overlap with the Leu146 band in the gel (alkaline pH = one band each for unaffected and affected individual, that are comprised of His146 for affected and a combination of His146 and Leu146 variants for affected individual).

1 pt for noting that WT (His variant) will migrate more quickly than Cowtown (Leu) toward negative (-) electrode at pH 6 (with associated drawing). 1 pt for determining that His is more likely to be deprotonated at alkaline pH (with Leu / Cowtown variant being unaffected by pH changes). 1 pt for noting that WT and Cowtown will migrate at the same rate at alkaline pH (with associated drawing).

**Part B (2 points):** Would this substitution cause hemoglobin to favor the R or T state? Be sure to address which interactions would be disrupted.

This substitution will prevent salt bridge between His146 and Asp94 from forming in deoxyhemoglobin, and stabilizing T state. Thus, Cowtown variant more likely to be in the R state. 2 pts for mentioning salt bridge and that R state would be favored. 1 pt for either. 0 pts for neither.

**Part C (3 points).** Explain how the oxygen affinity of hemoglobin Cowtown would compare to wild-type hemoglobin. Draw oxygen binding curves for Cowtown and wild-type hemoglobins to help illustrate your points.

1 pt for showing both curves as sigmoidal (generally sigmoidal is fine, just shouldn’t be hyperbolic or straight line). 1 pt for both curves ultimately reaching the same asymptote. 1 pt for Cowtown curve being shifted to the left of the WT curve, showing greater affinity for oxygen.

**Practice Exam Question 1.** Family members from four generations were found to have erythrocytosis (i.e., a larger proportion of blood volume occupied by red blood cells) resulting from the Syracuse variant of hemoglobin. In this variant, histidine at residue 143 in both the α2 and β2 chains is replaced by a proline. When deoxygenated hemolysate of affected individuals was analyzed by gel electrophoresis in oxygen-free conditions, two bands were visible.

**Part A:** Explain why this substitution would cause the abnormal hemoglobin to migrate differently during electrophoresis. Include a drawing of a gel to help illustrate your explanation.

**Part B:** This substitution in the β chain prevents the formation of salt bridges that are important for stabilizing the T-state of hemoglobin. Explain why replacing a histidine with a proline would disrupt the formation of salt bridges.

**Part C:** Explain how this substitution would affect the affinity of Hemoglobin Syracuse for oxygen. Draw oxygen binding curves for the wild-type and Syracuse variants to help illustrate your points.

**Homework Problem Set:** In Carbonic Anhydrase Deficiency Case Part 1, we studied how this disease can affect CO2, pH, and bicarbonate levels in the body. In this final case of the Protein Structure and Function Unit, we will connect CO2 and pH levels to hemoglobin structure and function, including the impacts of the Hb Hiroshima variant and carbonic anhydrase deficiency. Earlier in the unit, we learned in the Fast-moving Hemoglobin cases that Hb Hiroshima has a His to Asp substitution at residue 146 of the β chain. Remind yourself:

1. What interaction does this substitution disrupt?

Residue 146 is at the C-terminus of the B chain. His pKa is 6. His146 forms salt bridges with Asp94 at lower pH (because His more likely to be protonated).

1. How does this substitution affect hemoglobin structure? Does this substitution favor the R or T state?

Salt bridge changes conformation of hemoglobin to T state. Mutation of His146 prevents this salt bridge from forming, so hemoglobin assumes R state.

1. How does this substitution affect oxygen affinity?

R state has higher affinity for O2, so HB Hiroshima mutation has increased O2 affinity.

The function of His146 is also affected by changes in pH. We know that the side chain of His can be protonated or deprotonated. Its pKa is 6.0.

1. What is the ratio of protonated to deprotonated His side chains at pH 6.0?

One to one.

1. What is the ratio of protonated to deprotonated His side chains at pH 7.0?

1 protonated to 10 deprotonated.

1. Consider how pH levels compare in lungs versus tissues. How would the ratio of protonated to deprotonated His side chains compare in the two locations?

pH higher in lungs than tissues. Hemoglobin more likely to be protonated in tissues than lungs.

1. How does the protonation state of His146 affect hemoglobin structure? Is His146 protonated or deprotonated in R state? In T state?

The more hemoglobin that is deprotonated, the fewer form salt bridges. Hemoglobin assumes R state, and has higher affinity for O2. The more hemoglobin that is protonated (like in tissues), the more that will assume the T state, lower affinity for O2.

1. How does the protonation state of His146 affect hemoglobin’s affinity for oxygen?

Protonated = lower affinity. Deprotonated = higher affinity.

Some patients with carbonic anhydrase deficiency experience metabolic acidosis, which is caused by the failure of carbonic anhydrase in the kidney to convert H2O + CO2 to H2CO3 to allow for secretion of H+ in the urine.

1. How would metabolic acidosis affect the structure and function of hemoglobin? Be sure to address the protonation state of His146 and affinity for oxygen in your response.

Blood pH would be lower in individuals with metabolic acidosis, thus hemoglobin would be more likely to be protonated, assume the T state, show lower affinity for O2.

Patients with carbonic anhydrase deficiency may also experience higher than normal CO2 levels in their blood.

1. To which hemoglobin residue does CO2 bind?

CO2 binds C-terminal residue (His146), forming carbamate adduct.

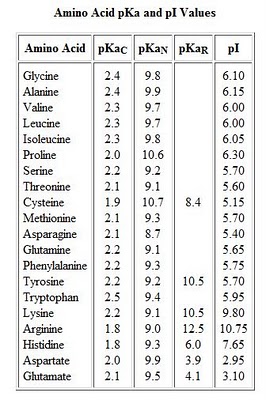
1. Does CO2 binding favor the R or T state of hemoglobin? What CO2 interactions favor this state?

Carmabates of oxyhemoglobin are more stable than carbamates of deoxyhemoglobin. Thus, when CO2-bound hemoglobin returns to lungs, carbamate is destabilized, CO2 is released and exhaled.

1. How would you expect the oxygen binding curve of hemoglobin from a patient with carbonic anhydrase deficiency to compare with an unaffected individual? Please explain the rationale for your response.

Oxygen binding curve of individuals with CA deficiency would be shifted to the right (lower affinity for O2). Lower pH means more likely to be protonated, more likely to be in T state and display lower O2 affinity.

Students are provided the following information on assignments and exams.



**Amino Acid Structures:** The following amino acid structures are listed in alphabetical order. Ionizable groups are shown in their neutral form - this implies absolutely nothing about the predominant form at any particular pH.

