Analyzing the allosteric properties of *Cryptosporidium parvum* pyruvate kinase  
 under reducing conditions

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**Summary.** *Cryptosporidium parvum* is a parasite responsible for the waterborne disease cryptosporidiosis in humans.1 There is no cure, and the disease can cause death in immunocompromised individuals.2 *C.* *parvum* relies almost exclusively on glycolysis for energy because of its primitive mitochondria;3 therefore, its glycolytic enzymes are a potential treatment point. Moreover, its pyruvate kinase, which is a major control point in glycolysis, has several disulfide bridges that are not found anywhere else in nature.3 Two of these bridges connect monomers in the enzyme, and previous research in the Hayden group has found that they have little to no effect on the enzyme’s activity. But there is another set of disulfide bridges in the enzyme’s effector loops – a small string of amino acid residues near the allosteric binding pocket –that have been less thoroughly investigated. Consequently, my group will study the impact of removing those disulfide bridges in the effector loop. We will investigate how the enzyme’s activity changes in the presence of different allosteric modulators without the effector loop’s disulfide bonds. Our findings will help elucidate the role of the effector loop in the enzyme’s mode of action and may lead further research to focus on attacking the effector loop if its role is vital.

**Previous Research.** One difficulty in treating C*ryptosporidium parvum’*s pyruvate kinase (CpPyK) is that pyruvate kinases are highly conserved evolutionarily, so any treatment plan must target CpPyK without affecting any of the human pyruvate kinase isozymes.2 Pyruvate kinases are tetramers and consist of four identical subunits. Each subunit has three domains: the A-domain contains the active site and is a (β/α)8 barrel, the B-domain is near the active site and closes upon substrate binding, and the C-domain houses the allosteric site with a mix of α-helices and β-pleated sheets.4 Some mammalian pyruvate kinases also contain a short α-helix at the N-terminal called the N-domain whose function is mostly unknown. Between the monomers, however, CpPyK has two disulfide bridges that make the enzyme a dimer of dimers. No other pyruvate kinase has these bonds. These bridges form between Cys26 on the A-domain of one monomer and Cys312 on the A-domain of the other.1 Additionally, CpPyK has a unique disulfide bond in the effector loop of each monomer that forms when one of the substrates – phosphoenolpyruvate or ADP – binds. The effector loop is a small string of amino acids (#508-517) in the C-domain that occupies the allosteric site and swings out when a substrate binds.3 Given its location, the effector loop likely plays a significant role in the binding of various allosteric modulators; however, its exact importance is unknown.

The allosteric modulators of a pyruvate kinase are one area of differentiation that may create treatment opportunities. While all pyruvate kinases require a monovalent cation such as K+ and a divalent cation such as Mg2+, other modulators are less widely shared. For example, although most mammalian and bacterial pyruvate kinases are activated by fructose 1,6-bisphosphate (FBP), the Hayden group has found CpPyK to be unaffected by FBP and activated by the similar compound fructose 2,6-disphosphate (FDP).3 Moreover, CpPyK is inhibited by ATP like most pyruvate kinases, but AMP, which commonly activates pyruvate kinases, has a contested effect in CpPyK. Whether these rare allosteric behaviors of CpPyK are related to its disulfide bridges is unknown, but further study of these modulators may uncover important differences in CpPyK structure that could be used to treat cryptosporidiosis without harming human pyruvate kinases. Therefore, my group will investigate the effect fully reducing the enzyme has on its activity with various modulators present. Full reduction will remove disulfide bonds both between the monomers and in the effector loops and will enhance our understanding CpPyK’s effector loop and the allosteric site. Previous members of the Hayden group have tested the activity of the enzyme with the disulfide bonds between the monomers removed by point mutation and saw no change in activity. However, those bonds are relatively far from the active and allosteric sites,4 so their effect on substrate and allosteric binding may be diminished. The effector loop, on the other hand, is in the allosteric site, and breaking the disulfide bonds ought to profoundly change how allosteric activators and inhibitors impact the enzyme. One result may be that the enzyme’s activity will resemble that of a pyruvate kinase that lacks the disulfide bonds.

**My research.** Initially, I will determine the concentration of dithiothreitol (DTT) needed to fully reduce CpPyK. I will make up several solutions of 1 mg/mL CpPyK and various concentrations of DTT and run an SDS-PAGE analysis on each solution. According to protocols from AG Scientific and Interchim, 1-10 mM DTT is used to prevent new disulfide bonds and 50-100 mM DTT is used to fully reduce a protein for electrophoresis.5-6 Consequently, I will use concentrations of DTT ranging from 50-100 mM. Because of DTT’s structure (Figure 1), basic conditions must be used to deprotonate DTT’s thiols,7 so I will prepare and use Tris-HCl buffers at pH 7.5. A Tris-HCl buffer is chosen because previous research in the Hayden group has used it, and it is known not to impact the enzyme. After running the SDS-PAGE according to standard procedure, I will look for the band in each sample. A fully reduced sample will have broken the disulfide bonds between the monomers, so its band will be the monomer’s weight, 37 kDa. Conversely, unreduced protein will have bands at the tetramer’s and/or dimer’s weight, about 150 and 75 kDa respectively. While it is not certain that reducing the disulfide between the monomers will have reduced the effector loop disulfide, it is highly probable because both sets of disulfides are on the exterior of the protein and are easily accessed by the reducing agent. Nonetheless, to ensure that I study the fully reduced enzyme, I will use a concentration of DTT slightly higher than the minimum required to get the monomer’s molecular weight. The SDS-PAGE itself takes an hour to run, plus time preparing the buffers and solutions, so this step will take most of a single three-hour block.

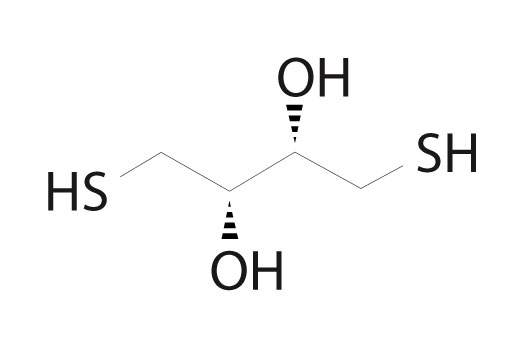
The second step of my research will be a series of kinetics assays to study the activity of fully reduced CpPyK. For controls and to compare the activity, I will also run kinetics on wild type CpPyK, and double mutant CpPyK (disulfide bonds between the monomers removed). The double mutant CpPyK is the primary control, as differences between it and the fully reduced enzyme can only be attributed to the disulfides broken in the effector loop; meanwhile, testing the kinetics of wild type CpPyK will connect our data to previous trials that studied wild-type and double-mutant CpPyK and found them to have the same kinetics. My results should match this and will confirm that the data I am collecting is sound. Finally, data from the literature on rabbit pyruvate kinase will be studied because it has no disulfide bonds between the monomers or in the effector loop, and removing all of those disulfide bonds in CpPyK may result in comparable activity.

In carrying out these assays, I will use several molecules known to allosterically affect pyruvate kinases. Pending time constraints, I hope to test each enzyme with no modulator and with fructose 1,6-bisphosphate (FBP), ATP, and AMP. No modulator will be the control for the activity of each enzyme. FBP will be the main focus because it is known to activate rabbit pyruvate kinase8 but does not affect the activity of wild type CpPyK.1,3,9 Consequently, my hypothesis predicts the activity of fully reduced CpPyK to be much greater than wild-type or double-mutant CpPyK. ATP and AMP are other modulators that may affect CpPyK. ATP is known to inhibit it by feedback inhibition, and studies of AMP’s effect have been ambiguous. If I end up having a bit of extra time, I may also test fructose-6-phosphate (F6P) or glucose-6-phosphate (G6P), two compounds that do not affect CpPyK but are known to effect other pyruvate kinases.9 All of these tests will enhance our understanding of CpPyK by delving into the role of the effector loop in CpPyK’s mode of action.

For all of these trials, I plan to use one of the HP 8452A spectrophotometers connected to Olis Globalworks software available at the institution. I will prepare a cuvette containing Tris-HCl buffer, pH 7.5 as the blank. Then I will add PEP, ADP, NADH, and LDH according to the concentrations in Table 1. PEP and ADP are the substrates for pyruvate kinase, and the presence of NADH and LDH couples the production of pyruvate with the reduction of NADH by LDH. NADH has a significant absorbance at 340 nm, and measuring the rate at which its absorbance drops *reveals* the activity of the pyruvate kinase. Lastly, the CpPyK enzyme – wild-type, double-mutant, or fully reduced – will be added, and the assay will be run for 1.3 minutes (Table 1). For assays of the fully reduced enzyme, wild-type CpPyK incubated at the necessary concentration of DTT will be used, and the same concentration of DTT will be added to the buffer to prevent any dilutions. Factoring in the preparation of the cuvette, each trial should take 4-5 minutes. For confidence, each assay will be run in triplicate, and a water bath will be used to keep the temperature at 25 OC for all trials. Thus, the total number of expected trials is 36 (three enzymes and four allosteric conditions, all in triplicate). Even under conservative estimates of one trial per five minutes, the two weeks (six hours) allocated to these assays will be sufficient.

Finally, I will allocate a few hours to analyze the data collected by the kinetics assays. Some analysis will be done earlier, but this will be a time to compile all of the data to look for trends and draw conclusions. My hypothesis is that fully reducing CpPyK will drastically alter its activity, likely to resemble a pyruvate kinase that does not have those disulfide bridges to begin with. To evaluate this, I will work up the Olis data into plots of absorbance over time using Microsoft Excel. The slopes of these lines are directly proportional to the rate of NADH reduction, which is directly proportional to the activity of the CpPyK enzyme. Hence, comparing the slopes is a comparison of the enzyme’s activity with a higher slope indicating increased activity. If my hypothesis is accurate, the fully reduced enzyme will have a much higher activity with FBP present than with no allosteric modulator while wild-type and double-mutant CpPyK activity will be unchanged. This is because FBP strongly activates rabbit pyruvate kinase, which lacks any disulfide bonds in the effector loop, but does not activate wild-type CpPyK. Similarly, the presence of AMP or ATP should cause the fully reduced enzyme’s activity to vary significantly from wild-type and double-mutant CpPyK. Such results would indicate that the effector loop plays a major role in the binding of various effectors. The AMP assay may be especially useful, for previous studies in the Hayden group have been ambiguous – one study found that AMP activates the wild-type enzyme while another saw no effect. Our data may shed some light on the interaction while also determining if the effector loop plays a role. Of course, the data may also find that fully reduced CpPyK has the same activity as wild-type and double-mutant CpPyK, in which case we would conclude that the disulfide bridges in the effector loop do not play a major role.

**Qualifications**. I am one semester away from completing a B.S. in chemistry from Birmingham-Southern College and have plenty of laboratory experience and enzyme knowledge. I have worked in Dr. Stultz’s research lab both full-time over the summer and ten hours a week this fall. In this lab, I have extensively analyzed ruthenium reductions with UV/Vis spectroscopy and High Performance Liquid Chromatography; hence, I am comfortable with UV/Vis and reduction analysis. I have also run several SDS-PAGEs and am familiar with enzyme kinetics and inhibition. This project will broaden my research background by adding biochemical research to my inorganic experience and will further prepare me for the advanced research that I will carry out in graduate school and in my professional life. Additionally, I will present my findings to the rest of the Hayden group at the end of the study and may coauthor an official manuscript if the findings are valuable.



**Figure 1.** The molecular structure of dithiothreitol (DTT). Deprotonating the thiol groups allows it to act as a powerful reducing agent.

**Table 1.** Plan for preparation of cuvettes for kinetics assays.

|  |  |  |  |
| --- | --- | --- | --- |
| **Reagent** | **[Sample]** | **[Stock]** | **Microliters of Stock Solution** |
| Tris-HCl buffer, pH 7.5 | 50 mM | 50 mM | 1 mL |
| ADP | 5 mM | 600 mM | 8.33 μL |
| PEP | 1 mM | 100 mM | 10 μL |
| NADH | 0.1 mM | 100 mM | 5 μL |
| LDH | 4 μg/mL | 1 mg/mL | 4 μL |
| Enzyme | 1 μg/mL | 1 mg/mL | 1 μL |

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**Budget:**

|  |  |  |  |
| --- | --- | --- | --- |
| Reagent | Source | Amount | Cost |
| Salary | N/A | $25/hr; 27 hrs | $675 |
| UV/Vis Spectrophotometer | N/A | 6 hours | $300 |
| Dr. Hayden, consultant | N/A | 1 hr | $300 |
| Wild-Type CpPyK | Dr. Hayden | 100 μL | Gift ($0.00) |
| Double-Mutant CpPyK | Dr. Hayden | 100 μL | Gift ($0.00) |
| DTT | P212121 | 25 g | $75.00 |
| SDS-PAGE Gels | BIORAD | 10 gels | $116 |
| Tris-HCl | Gold-Bio | 100 g | $31.00 |
| KCl | Flinn Scientific | 100 g | $5.80 |
| MgCl2 | Carolina | 100 g | $12.50 |
| 6M HCl | Carolina | 1 L | $13.95 |
| NaOH | Carolina | 500 g | $8.95 |
| ADP | Sigma-Aldrich | 100 mg | $39.00 |
| PEP | Sigma-Aldrich | 1 g | $291.00 |
| NADH | Gold-Bio | 1 g | $43.00 |
| LDH | Sigma-Aldrich | 5000 units | $44.70 |
| Fructose-1,6-bisphosphate | Santa Cruz Biotechnology | 1 g | $50.00 |
| AMP | Sigma-Aldrich | 5 g | $46.30 |
| ATP | Sima-Aldrich | 1 g | $56.80 |
| **Total** |  |  | **$2,109.00** |

**Justification**. I and my two partners will work at least three hours a week for three weeks. A 25 dollars an hour salary is reasonable for laboratory research. A UV/Vis instrument will be needed for the two weeks of kinetics assays, and we may need to consult with Dr. Hayden during the project either to troubleshoot problems or to help us interpret our results. Wild-type and double-mutant CpPyK have been generously donated by Dr. Hayden and will not need to be purchased or synthesized. DTT was cheapest at P212121 and was cheaper than β-ME and other reducing agents. 25 g is more than enough for 100 mL of 1 M DTT, which will plenty for various dilutions and experiments. One precasted gel is needed for the SDS-PAGE analysis of DTT reduction concentration, and 10 gel cassettes was the lowest amount available. Tris-HCl, HCl, and NaOH are needed to prepare Tris-HCl buffers at pH 7.5, and KCl and MgCL2 will be added to the kinetics assays buffer to boost pyruvate kinase activity. ADP, PEP, NADH, and LDH are needed for the kinetics assays: ADP and PEP are substrates, and NADH and LDH allow for coupling with LDH and measurement of loss of NADH. While only a few microliters of these species will be needed per trial, the budgeted amounts are the lowest available. Lastly, FBP, AMP, and ATP are allosteric modulators that will be tested, and like the previous species only a few microliters are needed. The above amounts and costs were the lowest that could be found.