Kinetics of ATP and Hexokinase PII Binding Using TNP-ATP as a Probe in the Presence and Absence of Glucose



Molly Brinser

Allegheny College Department of Chemistry
Meadville, Pennsylvania
Class of 2016

Senior Comprehensive Project
Submitted to the Allegheny College Department of Chemistry in Partial Fulfillment of the Requirements for the Degree of Bachelor of Science

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Committee Members:

Alice Deckert, Ph.D.
Ryan M. Van Horn, Ph.D.
Kellie Brosius, Ph.D.

I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor Code and to maintain the integrity of both myself and the College community as a whole.

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Abstract

The glucose binding site in hexokinase is well known, but the ATP binding site is not as well understood. There is controversy over whether ATP prefers to have glucose present in order for binding to occur. Hexokinase and ATP binding was then studied in the presence and absence of glucose using TNP-ATP as an ATP analog. The kinetics of the association and dissociation reactions between hexokinase and TNP-ATP were monitored using absorbance stopped-flow. The association reaction in the absence of glucose ($k_{on,n}$) between hexokinase and TNP-ATP was second order and the rate constant was approximately 4000 $^{1}/_{sec}$. The dissociation reaction in the absence of glucose ($k_{off,n}$) between hexokinase and TNP-ATP was first order and the rate constant was 9.87 $^{1}/_{\mu Msec}$. The association reaction in the presence of glucose ($k_{on,g}$) between hexokinase and TNP-ATP was presumed to be second order with the rate constant > 9.87 $^{1}/_{\mu Msec}$. The dissociation reaction in the presence of glucose ($k_{off,g}$) between hexokinase and TNP-ATP was presumed to be first order with the rate constant > 4000/s. The fact that the rate constants for the glucose reactions were too large to measure, suggests that ATP binding prefers for glucose to be present.

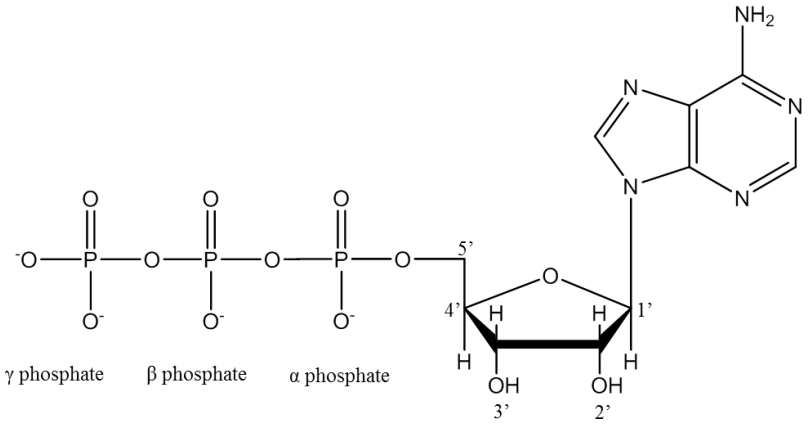
Introduction

An essential role proteins perform in all living organisms is catalyzing reactions that are required to sustain life.[1] Hexokinase PII, a necessary protein in glycolysis in *Saccharomyces cerevisiae*, catalyzes a reaction between glucose and adenosine triphosphate (ATP).[2-5] There is much controversy on whether glucose and ATP bind to the hexokinase randomly or if glucose is required to bind before ATP.[3] This project will focus on ATP binding to Hexokinase PII by studying the kinetics of the association and dissociation reaction between 2',3'-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate (TNP-ATP), an ATP analog which acts as a probe, and Hexokinase PII in the presence and absence of glucose using the stopped flow technique. Determining the reaction rate for both the association and dissociation reactions will enhance the current knowledge of ATP binding and help to answer the question about the order of binding. This knowledge will be able to serve as a basis for studying ATP binding in more complex proteins.

Proteins are critical biomolecules that are needed for many functions in living organisms. Various functionalities that proteins can have include binding to foreign particles as antibodies, acting as messengers to transmit chemical signals to stimulate specific biological processes, being major structure and support components for the cells, and transporting biologically necessary molecules throughout the organism.[1] One major protein function that is of interest in this paper is its ability to catalyze essential chemical reactions needed to support life. These proteins are called enzymes.[1] Without proteins that function as enzymes, the majority of chemical reactions within an organism would not occur fast enough under the temperature and pressure conditions found within the organism.[6] A vital chemical reaction that is catalyzed in many organisms is phosphorylation, which involves the transfer of a phosphate group from an

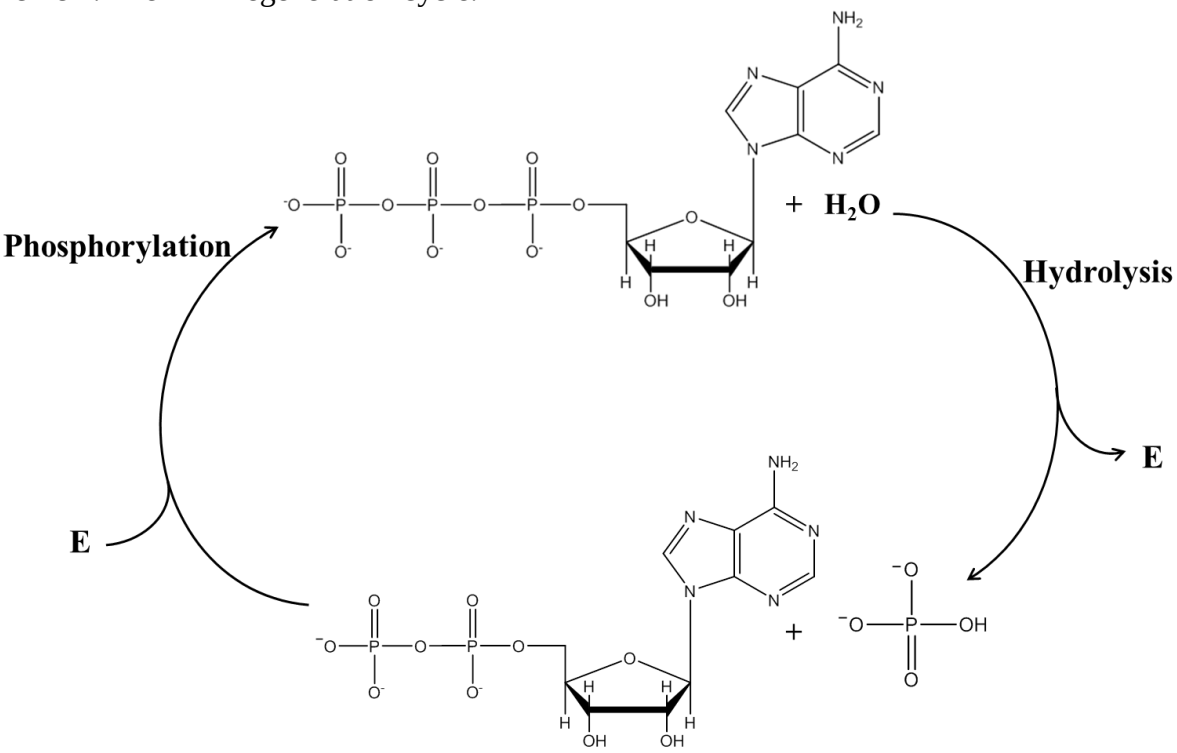
ATP molecule to a specific substrate. ATP is present in all living organisms and is often considered the energy currency in a cell since it has the ability to transport and store energy needed for many biologically necessary reactions. Chart 1 displays the structure of ATP, which contains an adenine nucleotide with three phosphate groups attached to the sugar component.[7]

Chart 1. Structure of ATP.



Dephosphorylating and reforming the ATP molecule are part of the ATP regeneration cycle as seen in Scheme 1. Dephosphorylation of ATP occurs via hydrolysis where the bond between the γ-phosphate and the β-phosphate groups is broken to form adenosine diphosphate (ADP), which is similar to ATP except it only retains the α and β phosphoryl groups while releasing an inorganic phosphate as shown in Scheme 1. Dephosphorylating the ATP molecule releases energy that the cell harvests for cellular work, which involves driving life required endergonic reactions.[8-10] ATP hydrolysis is an exergonic reaction, where the overall ΔH and ΔG values are negative and vary based on Mg^{2+} concentration, ionic strength, and physiological conditions.[9,10] Though the hydrolysis of ATP is known to provide the cell with energy, there is much controversy over the stage at which the energy is released and if it is released all at once.[10] ADP is recycled back to ATP through an endogenic phosphorylation reaction. The cell uses energy produced from metabolic processes to drive the reaction as shown in Scheme 1.

Scheme 1. The ATP regeneration cycle.

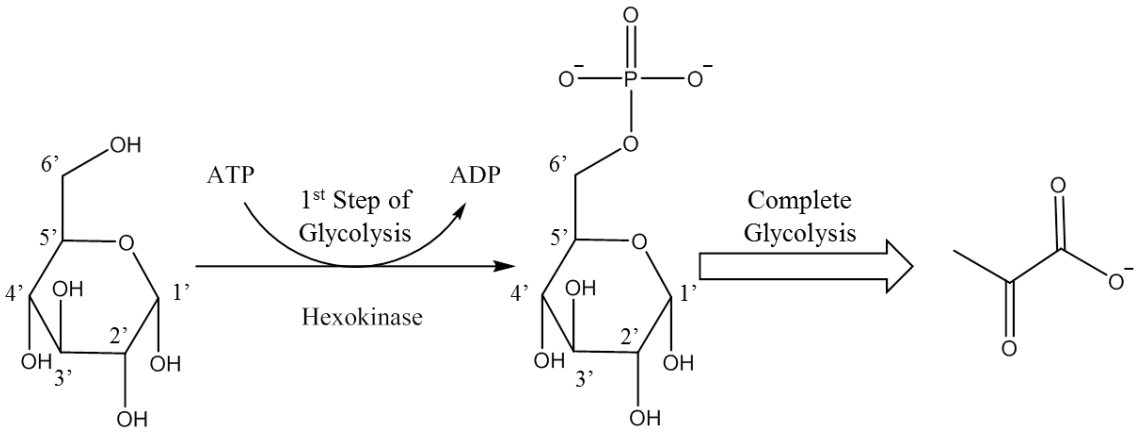


Phosphorylation reactions control a wide range of processes within the cell including metabolism regulation. Enzymes that catalyze phosphorylation reactions are called protein kinases.[8] Protein kinase is a large family of proteins that includes sugar kinases, which catalyze the transfer of a phosphate group onto a specific sugar molecule. This phosphate transfer aids in the metabolism of the specific sugar molecule. Metabolism of the sugar molecule releases energy that the organism can harness for other cellular processes. Specific sugar kinases include galactokinase, ribokinase, and, of importance in this paper, hexokinase.[11]

The need to break down glucose into a useable energy source is required in all living organisms because glucose is a readily available food source. Scheme 2 shows glycolysis, where glucose is converted into pyruvate, another biologically necessary compound required in other metabolic pathways that include the synthesis of ethanol, lactic acid, and carbon dioxide.[12] Hexokinase plays a valuable role in glycolysis because it catalyzes the first step, where the γ-

phosphate from ATP is transferred to the 6’ hydroxyl position on the glucose ring to produce glucose-6-phosphate and adenosine diphosphate.[2-5] Therefore, hexokinase has a direct, essential relationship with glycolysis and an indirect, essential relationship with other biological processes involving pyruvate.

Scheme 2. The glycolysis pathway.



The yeast species *Saccharomyces cerevisiae* has two types of hexokinase, of which PII is the focus protein of this paper.[3] Figure 1 shows the structure of Hexokinase PII, which is divided into two general regions: the small and large lobe. These regions play critical roles in glucose and ATP binding. The dividing point between the small and large lobes is called the cleft, which contains the glucose binding site. Despite knowing the glucose binding site, the ATP binding site location is not as well understood. There is no crystallized structure of hexokinase with ATP and structural studies on model systems suggest two possible binding sites for ATP in hexokinase.

Stoham and Steiz[13] successfully crystallized hexokinase with 8-bromoadenosine, an adenine analog[5]. The adenine molecule from 8-bromoadenosine was situated in a hydrophobic area in the large lobe of the hexokinase. Additionally, the ATP binding site in heat shock protein Hsc70 is found in a hydrophobic region that is structurally similar to those in hexokinase. Both of these observations suggest that ATP binds in the hydrophobic region of hexokinase’s large

lobe. Kabsch et. al.[5] believed that the adenine from ATP binds to the hydrophobic region in α-helices present in the large lobe of hexokinase based on its similarities to the ATP binding site in actin protein.

Other evidence suggests that ATP can bind to a β-sheet within the small lobe. Arora *et. al*.[14] developed a peptide chain containing the amino acids present in the small lobe β-sheet and found that this specific region bound to an adenine analog, suggesting the ATP binds to the small lobe. Because of all the evidence present on ATP binding to hexokinase, it is possible that ATP can bind to the hydrophobic region within the large lobe or the β-sheet within the small lobe or both regions.

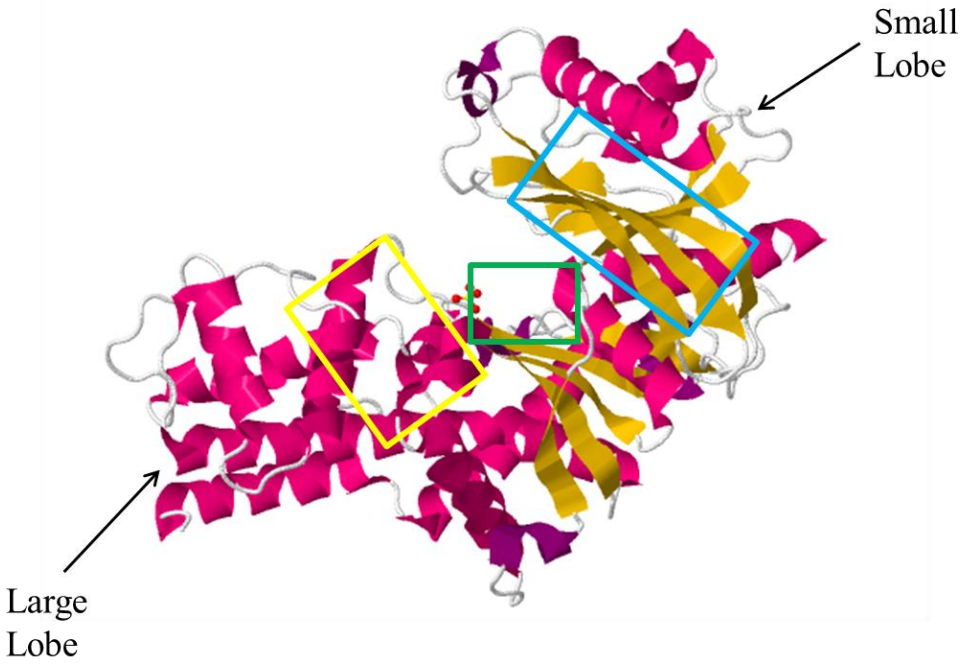


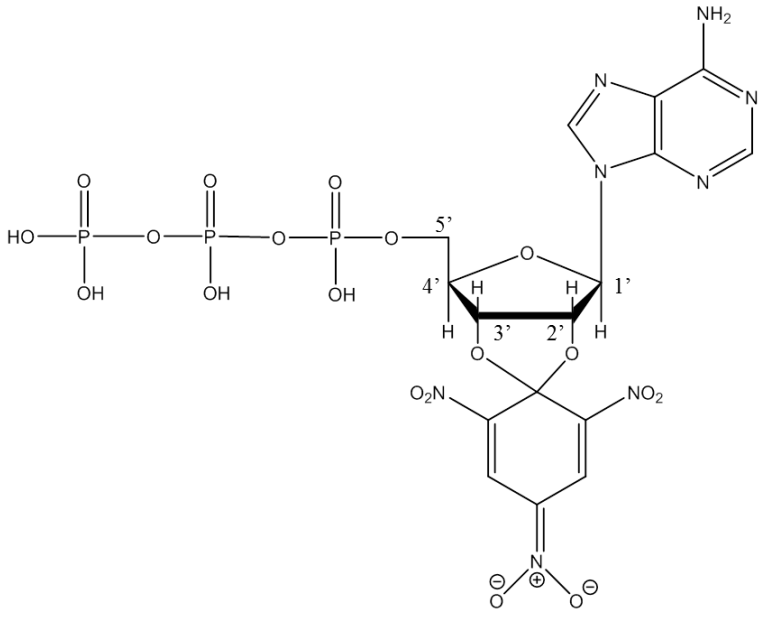
Figure 1. The crystallized structure of Hexokinase PII, from *Saccharomyces cerevisiae*, (PDB # 1IG8) indicating the α-helices (pink) and the β-pleated sheets (orange).[3] The cleft region, where glucose binds, is represented by the green box. The large lobe is located in the bottom left hand corner and the hydrophobic region within the α-helices responsible for ATP binding is indicated by the yellow box. The small lobe is located in the top right hand corner and the β-pleated sheet responsible for ATP binding is indicated by the blue box.

There is much controversy on whether glucose is necessary to facilitate ATP binding to hexokinase. There are three possible mechanisms that dictate the order in which the ATP and glucose substrates bind to the hexokinase: random addition, ordered addition with glucose binding before ATP, or a pathway that is random but has a strong tendency for the glucose to bind first. If glucose binds before ATP, then the cleft closes and moves the β-sheet present in the small lobe. This movement is believed to bring the small lobe β-sheet in a position so that the ATP can interact with both the small lobe β-sheet and the large lobe hydrophobic pocket. If this is the primary mechanism, then it would corroborate that both binding sites for ATP are important. If ATP binds before glucose, it can bind to either the β-sheet in small lobe or hydrophobic pocket in the large lobe. It is likely that the ATP bound to the small lobe will eventually interact with the large lobe and the ATP bound to the large lobe will eventually interact with the small lobe. This would cause the cleft, which is where the glucose binds, to partially close resulting in glucose addition to be sterically unfavorable. This theoretical argument suggests that glucose has to bind before ATP.[5] Despite the argument stated above, there is not much research on ATP binding to hexokinase and there is only a little bit of evidence that favors glucose being present for binding to occur.

Because of the necessary functions of ATP that include making unfavorable reactions thermodynamically favorable, regulation of biological processes through phosphorylation, and providing an energy source for cells, it is crucial that ATP binding to various proteins is well understood. Two methods of detecting kinetic and equilibrium constants of proteins include fluorescence and absorbance. Fluorescence methods can be favorable when studying proteins because of their high sensitivity. In order for those methods to work, compounds with spectroscopic properties that can imitate ATP binding have to be utilized.[16] In 1973, Hiratsuka

and Uchida[17,18] synthesized a colored ATP analog called 2',3'-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate (TNP-ATP) and used this analog as a probe to study changes in the conformation of the protein heavy meromyosin ATPase after binding of ATP. As seen in Chart 2, TNP-ATP is an adenosine molecule with three phosphate groups attached to the 5' position on the sugar and a 2,4,6-trinitrophenyl group attached to the 2' and 3' positions on the sugar. TNP-ATP is a valuable ATP analog because it is easy to produce and absorbs light in the visible region.[19] Generally, TNP-ATP absorbs at wavelengths between 400 and 550 nanometers and fluoresces between 530 and 560 nanometers.[16] TNP-ATP is weakly fluorescent when in aqueous environments, such as being unbound in a buffer solution, but its fluorescence is heightened when in nonpolar environments, such as being bound to a protein.[16,20] This means that the fluorescence intensity increases when the analog is bound to a protein active site, allowing for it to be a fluorescence probe.[16,20,21] Because TNP-ATP absorbs in the visible region, it can also act as an absorbance probe.[19] For most proteins and enzymes, TNP-ATP binds tighter than its ATP counterpart, which allows for it to be a competitive inhibitor.[16,20]

Chart 2. The structure of TNP-ATP.



The most common application of TNP-ATP has been to study the kinetic and equilibrium constants of ATP bound to various proteins. These constants are usually measured by the fluorescence or absorbance change of TNP-ATP, which is substituting as ATP, as it associates and dissociates with the protein.[16] The association rate constant is called k_{on} and the dissociation rate constant is called $k_{off.}$ The association and dissociation reaction between TNP-ATP and a protein are shown in Scheme 3. Equation 1 shows how the association and dissociation constants can be used to determine the equilibrium constant for TNP-ATP and protein binding.

Scheme 3.

$$\text{TNP-ATP + Protein} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \text{TNP-ATP*Protein}$$

$$\frac{k_{on}}{k_{off}} = K_{eq} \qquad (1)$$

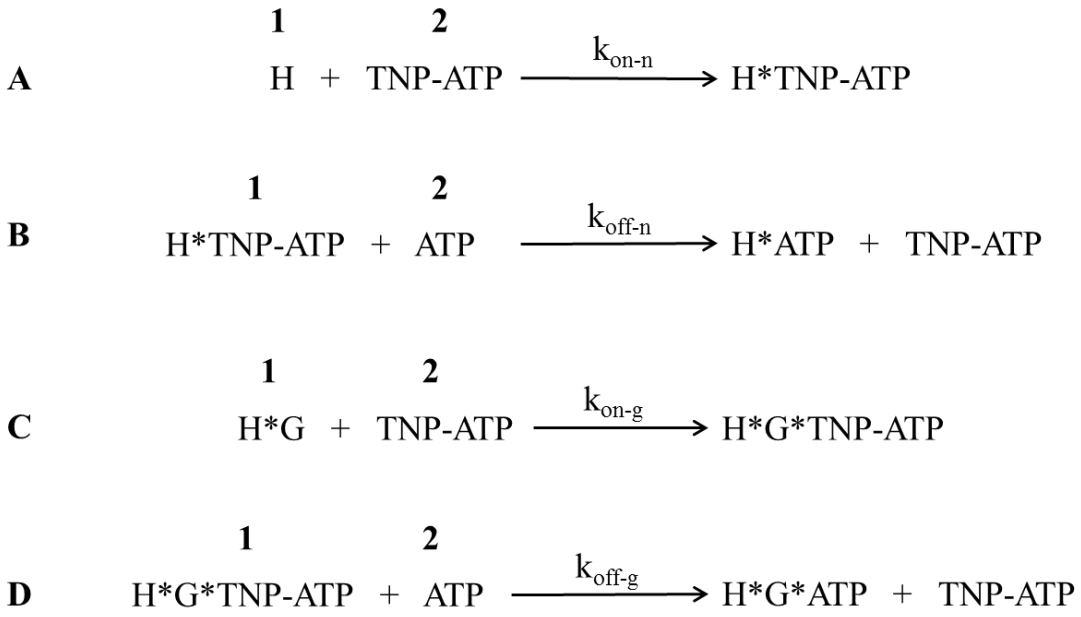
Because TNP-ATP is a useful ATP analog, it can be utilized to study binding between TNP-ATP and hexokinase and provide a basis on how ATP would bind. Both k_{on} and k_{off} influence binding affinity between hexokinase and TNP-ATP. The forward reaction is favored due to the vast intermolecular attractions formed between the substrate and the protein when tight binding (large K_{eq}) is present. In the opposite case, proteins that loosely bind to the substrate ($K_{eq} \sim 1$) will have more similar association and dissociation rate constants, when compared to tight binding, since there are fewer intermolecular interactions between the two.[20] The dissociation rate is usually easier to measure than the association rate because it tends to be slower. However, both should be studied in order to fully understand the binding affinity. In

cases where the association rate constant cannot be determined, the equilibrium and dissociation constants can be measured. Using these two values in Equation 1, the association rate constant can be calculated.

Because glycolysis is a necessary metabolic reaction found across various living organisms, we would expect the binding of ATP to hexokinase to occur relatively fast. Thus, an instrument that can mix and take readings rapidly would be required to study a fast rate constant. An instrument that fulfills both of these requirements is stopped flow.

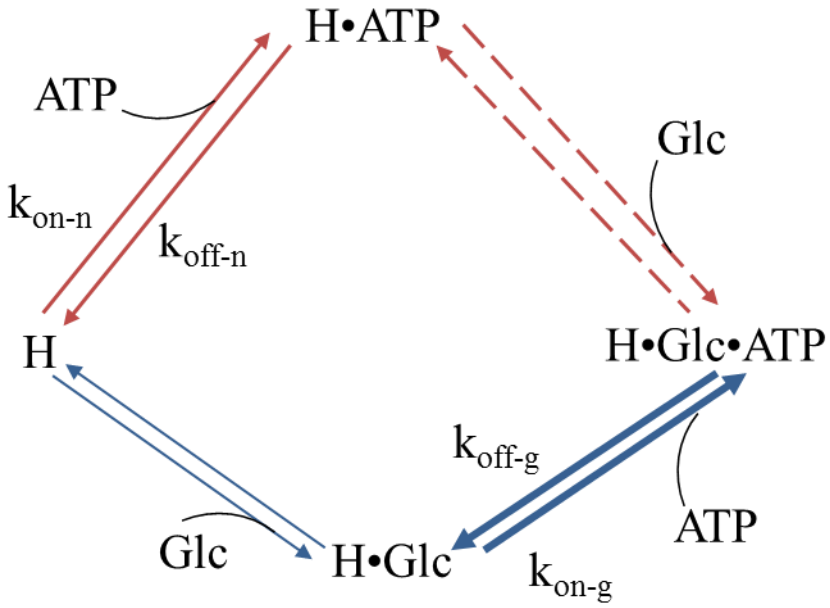
The stopped flow instrument is widely used as a rapid chemical kinetics detector. As a result of rapidly mixing two solutions (1 and 2 in Scheme 4) and observing fluorescence or absorbance intensity changes very quickly, an accurate reading for a rate constant can be determined. In this study, the k_{on} and k_{off} values will be determined for TNP-ATP and Hexokinase PII binding in the presence ($k_{on\text{-}g}$ and $k_{off\text{-}g}$ in Scheme 4) and absence ($k_{on\text{-}n}$ and $k_{off\text{-}n}$ in Scheme 4) of glucose. Scheme 4 shows the reactions that will be performed in this study, and their corresponding rate constants. For the association reaction, the TNP-ATP solution is added to the hexokinase solution. For the dissociation reaction, the TNP-ATP will be complexed to the hexokinase and chased with ATP. Because TNP-ATP can act as a competitive inhibitor, ATP is added in excess with the expectation that it will shift the equilibrium to favor removal of TNP-ATP and binding of ATP to the hexokinase.

Scheme 4.



The kinetic data given from stopped flow can be further analyzed to determine what reaction order the association or dissociation reaction has. Scheme 5 displays how the information given by k_{on} and k_{off} for both the no glucose and glucose reactions can help infer whether glucose is required for ATP binding and which mechanism is preferred. It would be ideal to study the association and dissociation reactions between hexokinase and glucose and between hexokinase-ATP and glucose but there is no feasible method for doing so. Thus, only the association and dissociation reactions between hexokinase-glucose and TNP-ATP and between hexokinase and ATP can be studied currently. That does not prevent inferences from being made about what mechanism ATP binding prefers. If $k_{on-g} > k_{on-n}$, then, it can be inferred that the glucose binding first, or least sterically hindered, mechanism is favored. If $k_{on-n} > k_{on-g}$, then the ATP binding before glucose mechanism would likely be favored. Other factors that could influence the reaction kinetics of ATP binding in the presence and absence of glucose, such as temperature and pH, can also be explored using stopped flow.

Scheme 5. The overall mechanism of ATP and glucose binding to hexokinase



Hexokinase is found in many different species of living organisms including humans, mice, rats, yeast, cows, and bacteria.[2,13,14,22] Hexokinases constitute a set of isoenzymes, which are enzymes that differ in amino acid sequence but catalyze the same reaction that is tissue specific.[2,3] Since hexokinases are isoenzymes, gaining information on how ATP binds to a species or tissue specific hexokinase in the presence and absence of glucose will provide useful knowledge for the binding kinetics in other types of species or tissue specific hexokinases.

Experimental

Absorbance and Fluorescence

As mentioned before, TNP-ATP can both absorb and fluoresce in the visible light region. Because of this property, both absorbance and fluorescence can be utilized to determine the rate constant for each of the four proposed reactions in Scheme 4. After determining that measuring the absorbance change would be most suitable, the stopped-flow technique can measure the change in absorbance for any of the four reactions. The collected absorbance data is graphed and the curve is fitted with the appropriate equation. For both $k_{off\text{-}n}$ and $k_{off\text{-}g}$, the data was fitted with a 1st order integrated rate law, given by Equation 2:

$$A=\Delta A e^{-k_{obs}t}+A_{\infty} \quad (2)$$

where A is the absorbance at any time, A_{∞} is the absorbance at long time, ΔA is the change in absorbance from t=0 to t= ∞, t is time, and k_{obs} is the observed rate constant. For both $k_{on\text{-}n}$ and $k_{on\text{-}g}$, because equal concentrations of H or H*G and ATP were used in the reaction, the data was fitted with a second order integrated equation given by Equation 3:

$$A= \frac{\Delta A}{1+k_{obs}A_o t}+A_{\infty} \quad (3)$$

where A is the absorbance at any time, A_o is the initial absorbance, $A_{\infty}$is the absorbance at long time, ΔA is the change from t=0 to t=∞, t is the time, and k_{obs} is the observed rate constant.

Preparing a Tris-KCl Buffer Solution

A Tris-KCl buffer was prepared using Epure water (17.5 MΩ), 53 mM Tris, 26 mM KCl and adjusted with 0.1 M aqueous hydrochloric acid to pH 7.5. The buffer was stored at room temperature.

Sample Preparation

The ATP, TNP-ATP, and hexokinase stock supplies were stored in a minus 18°C freezer without automatic defrosting. Hexokinase solutions were stored in a 4°C refrigerator for up to a week. ATP and TNP-ATP solutions were made fresh daily by allowing the stock supply to defrost at room temperature for five minutes. After defrosting, the ATP stock supply was vortexed before being diluted. Any solution containing TNP-ATP was covered in foil because the compound is photosensitive. All solutions were kept on ice when not in the refrigerator.

Determination of Stopped Flow Wavelength

In order to use either absorbance or fluorescence stopped flow, the wavelength with the greatest absorbance or fluorescence change was first determined.

Using an OceanOptics USB200 UV-Vis spectrometer, the absorbance reading of 0.500 mL of 18 μM TNP-ATP was collected in a glass quartz cuvette. Then an additional absorbance reading was collected after each of eight 0.100 mL additions of 14.7 μM hexokinase. The absorbance readings were exported into Microsoft Excel and each spectra was multiplied by the dilution factor. The hexokinase-TNP-ATP spectra were compared to the TNP-ATP spectra to determine the wavelength with the greatest absorbance change.

The Stopped-Flow Method

Shown in Figure 2, the stopped-flow instrument has the ability to rapidly mix equal volumes of two samples while taking precise measurements over short time scales. The progress of the reaction is measured by taking absorbance or fluorescence readings at a selected wavelength as a function of time. For this experiment, absorbance on the KinTek SF300X

Stopped-Flow was measured and the selected wavelength was 407 nm. Before taking absorbance readings, the dark currents and the zero reference using Epure water were set.

To study $k_{on\text{-}n}$, two separate solutions were made, one of which contained hexokinase in buffer while the other contained TNP-ATP in buffer. To study $k_{on\text{-}g}$, two separate solutions were made, one of which contained hexokinase and glucose in buffer while the other contained TNP-ATP in buffer. The hexokinase or hexokinase-glucose solution was rapidly mixed with the TNP-ATP solution to induce TNP-ATP binding to hexokinase. To study $k_{off\text{-}n}$, two separate solutions were made, one of which contained hexokinase and TNP-ATP in buffer while the other contained ATP without TNP attached in buffer. To study $k_{off\text{-}g}$, two separate solutions were made, one of which contained glucose, hexokinase, and TNP-ATP in buffer while the other contained ATP without TNP attached in buffer. The hexokinase-TNP-ATP or glucose-hexokinase-TNP-ATP solution was chased with the ATP solution to induce replacement of the bound TNP-ATP with ATP binding to hexokinase. Stopped flow takes absorbance readings as a function of time.

Samples were carefully loaded into the instrument to ensure that no air bubbles were present. Each sample was housed in a temperature controlled environment before, during, and after an injection. The temperature was regulated by the circulation of water. Once the desired temperature was reached, the instrument was primed with 5 injections of both samples. To determine the appropriate temperature and time ranges, the k_{on} and k_{off} reactions were run at varying temperatures and time intervals while keeping the concentration constant.

All four reactions depicted in Scheme 4 were run on stopped-flow under the following parameters: flow rate = 8 mL/sec, total volume per shot = 40 μL, and λ = 407 nm. The optimal temperature range for all four reactions was 16°C. The optimal time intervals for $k_{on,n}$, $k_{on,g}$, and

$k_{off,n}$ was 0.05 seconds, 0.05 seconds, and 0.5 seconds, respectively. The samples were injected 10 times for each collection and the curves were averaged.

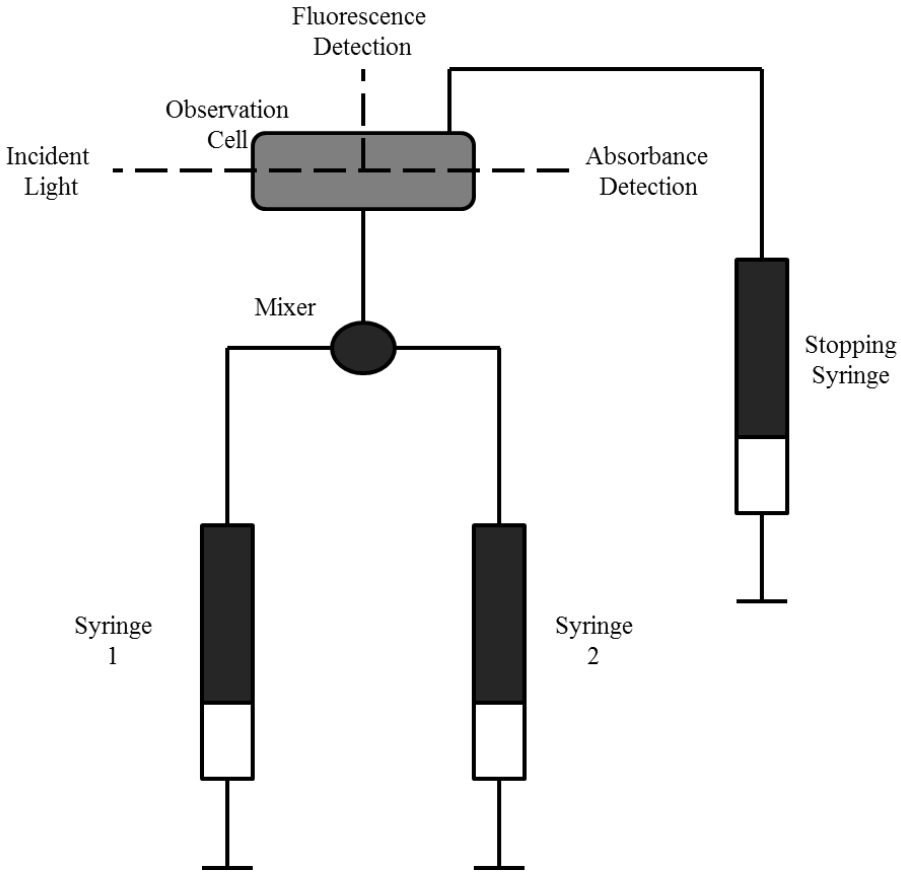


Figure 2. Schematic diagram of a stopped flow instrument. Small volumes of the solutions are injected into a mixer from syringes A and B. The resultant volume is rapidly mixed and displaced into an observation cell. Solution entering the observation cell is fresh, being just milliseconds old. Here, ultraviolet and visible light is shined on the solution and either absorbance or fluorescence spectroscopy can be used. The volume that is injected into the observation cell is controlled by the stop syringe, hence the name "stopped flow."

Results and Discussion

The main objective of this study was to determine the observed rate constant for each of the four reactions depicted in Scheme 4. Studying the k_{on} and k_{off} constants for when glucose is and is not present will allow inferences to be made about the order of ATP and glucose binding shown in Scheme 5. Before that was done, the wavelength with the greatest absorbance and fluorescence change was determined.

Figure 3 displays an experiment where 100 µl aliquots of 14µM hexokinase were added to a 18µM TNP-ATP solution. The absorbances before and after the eight hexokinase aliquot additions in the absence of glucose were found using ultraviolet-visible spectroscopy. There was a change in absorbance at 376nm, 407 nm and 472 nm. Interestingly, the absorbance at 376 nm increased as more protein was added. Because the absorbance change at 407 nm was the most prominent out of the three, 407 nm was chosen to be the detection wavelength for absorbance stopped-flow.

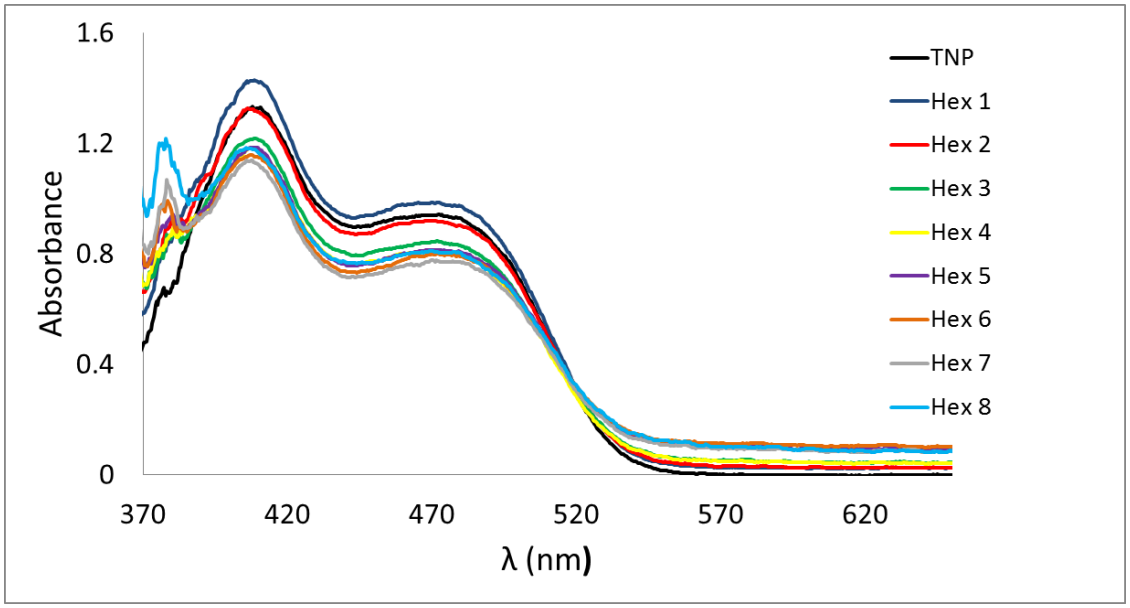


Figure 3. The absorbance spectra of an 18 µM TNP-ATP solution before and after eight 100 µL additions of 14 µM hexokinase. All solutions were made in aTris-KCl buffer. The peak absorbance intensities were located at 376 nm, 407 nm, and 472 nm.

The next step was to determine the appropriate temperature and time ranges under which to observe the k_{on} and k_{off} reaction. Three temperatures tested were 16°C, 25°C, and 35°C, with 16°C providing the only case where the reactions were observable. Thus, it was used throughout the study. Because k_{on} and k_{off} are two completely different reactions, they may have different time spans where the reaction can be seen. To determine the time span for $k_{on\text{-}n}$, a stopped-flow experiment facilitating this reaction was performed where the concentration of hexokinase and TNP-ATP were both 1.0 μM at a constant temperature value of 15.7°C over different time ranges. The determined optimal time range was 0.05 seconds. To see this graph, refer to Figure 4A. For other time intervals, see the Appendix. Looking at Figure 4A, a potential trend is evident at 0.05 seconds. The trend at 0.05 seconds makes sense based off the data presented in Figure 3. In Figure 3, as hexokinase binds to TNP-ATP, there is a decrease in absorbance. Thus, it is expected for free TNP-ATP to have a higher absorbance than bound TNP-ATP, which is evident in the data presented. This time interval was used throughout the rest of the study for both k_{on} reactions. To determine the time span for $k_{off\text{-}n}$, a stopped-flow experiment facilitating this reaction was performed where the hexokinase concentration was 4.0 μM, the TNP-ATP concentration was 3.8 μM, and the ATP concentration was 6 mM. The concentrations were held constant and the reaction was observed at a temperature of 16.2°C at different time spans. The determined optimal time range was 0.5 seconds. To see this graph, refer to Figure 6. For other time intervals, see the Appendix. In Figure 6, there is an upward trend that is expected because Figure 3 depicts free TNP-ATP as having a higher absorbance than bound TNP-ATP. Thus, a 0.5 second interval provides the best time range to observe $k_{off\text{-}n}$ reactions and was used throughout the rest of this study for both k_{off} reactions.

. After the optimal temperature range (16°C) and time range (~0.05 seconds) for the k_{on} reactions were determined, $k_{on\text{-}n}$ and $k_{on\text{-}g}$ reactions were run on stopped-flow. Figure 4 displays the $k_{on\text{-}n}$ experiment where the concentration of hexokinase and TNP-ATP were varied over 0.05 seconds at a constant temperature of 16.4°C. Hexokinase and TNP-ATP had the same concentration in each reaction and the observed k values determined when the concentrations were 1.0 μM, 0.5 μM, and 0.25 μM were 3097 $^{1}/_{sec}$, 3198 $^{1}/_{sec}$, and 1107 $^{1}/_{sec}$, respectively. The $k_{on\text{-}n}$ reaction was fitted with Equation 3 because it is expected that the reaction would depend on the concentration of hexokinase and TNP-ATP. The absorbance spectra for all three graphs made sense because free TNP-ATP had a higher absorbance the bound TNP-ATP.

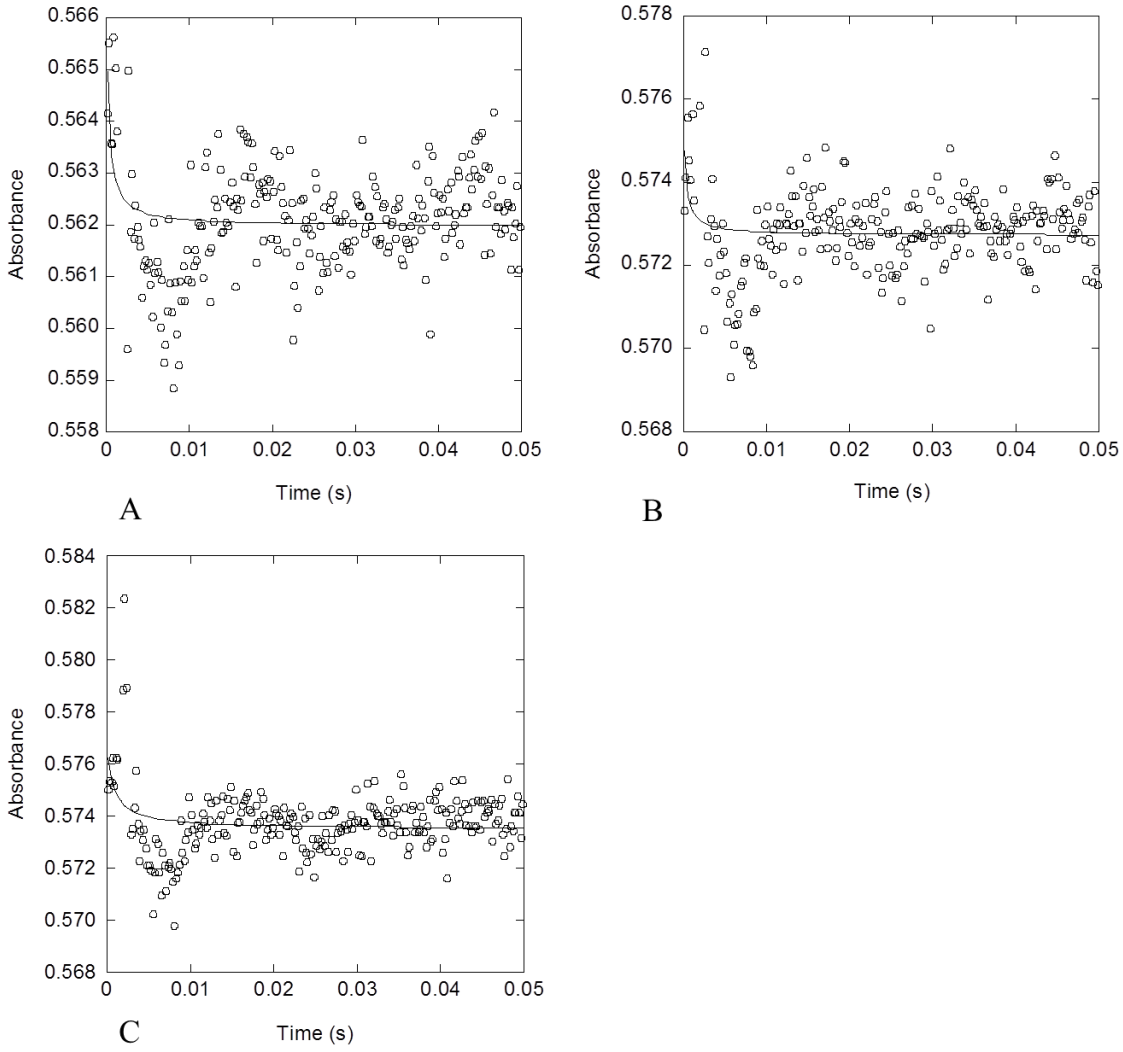


Figure 4. The stopped-flow absorbance spectra of three separate $k_{on\text{-}n}$ reactions with varying concentrations of hexokinase and TNP-ATP at 16.4°C over 0.05 seconds. Both hexokinase and TNP-ATP were either 1.0 μM (A), 0.5 μM (B), or 0.25 μM (C). The curves were fitted with a second order rate equation (Equation 3). The rate constants determined for 1.0 μM, 0.5 μM, and 0.25 μM were 3097.2 $^{1}/_{sec}$, 3198.9 $^{1}/_{sec}$, and 1107 $^{1}/_{sec}$, respectively.

Figure 5 shows the three k values being fitted with a linear trendline to find the overall observed rate constant for $k_{on,n.}$ This was found to be approximately 4000 $^{1}/_{\mu Msec}$. This is a limitation to the study because only three points were used to fit with a linear trendline. Also, the three points did not appear to follow a linear trend. Despite these limitations, fitting the points to a linear trendline can provide an approximate value for $k_{on,n}$ and give some insight on the reaction. The same experiment was performed again only this time the reaction was performed with 12.7 μM

glucose present and the hexokinase and TNP-ATP concentrations were either 1.0 μM, 0.5 μM, and 0.25 μM. Unlike with the no glucose treatment, the curves for the glucose treatment were unable to be fitted with any type of equation. To view reaction profiles, see the Appendix. All three graphs displayed the same type of uniformed sinusoidal scatter and no decrease in absorbance, which indicates that the reaction was not seen likely because it was too fast. Combining this with the fact that stopped-flow cannot take readings for time spans less than 0.05 seconds, the $k_{on\text{-}g}$ reaction is too fast to measure. Although an overall rate constant for $k_{on\text{-}g}$ was not found, the graphs in the no glucose and glucose treatments can be compared qualitatively. Not seeing the glucose treatment infers that it was too fast to observe. A faster reaction is proportional to a larger rate constant indicating that the glucose treatment likely had a larger rate constant than the no glucose treatment ($k_{on,g}>k_{on,n}$).

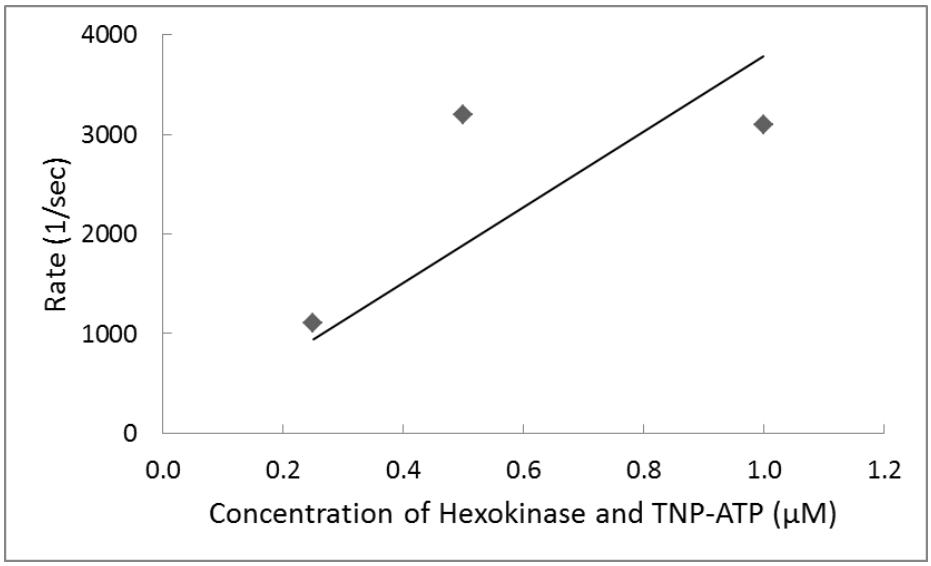


Figure 5. The three observed rate constants from Figure 7 were fitted with a linear equation $k_{obs}=3788[H*TNP\text{-}ATP]$ to determine the overall $k_{on,n}$ rate constant of approximately 4000 $^{1}/_{\mu Msec}$.

After the optimal temperature (16°C) and time range (0.5 seconds) for k_{off} reaction were determined, $k_{off\text{-}n}$ and $k_{off\text{-}g}$ reactions were run on stopped-flow. Figure 6 displays the $k_{off\text{-}n}$ trial at 16°C over 0.5seconds where the concentrations of hexokinase, TNP-ATP, and ATP were 4.0

μM, 3.8 μM, and 6 mM, respectively. The curve was fitted with a first order integrated rate law shown as Equation 2 because the only concentration that is affecting the reaction is that of TNP-ATP. The overall rate constant was found to be 9.87 $^{1}/_{sec}$. The $k_{off,n}$ value is significantly less than the approximated $k_{on,n}$ value.

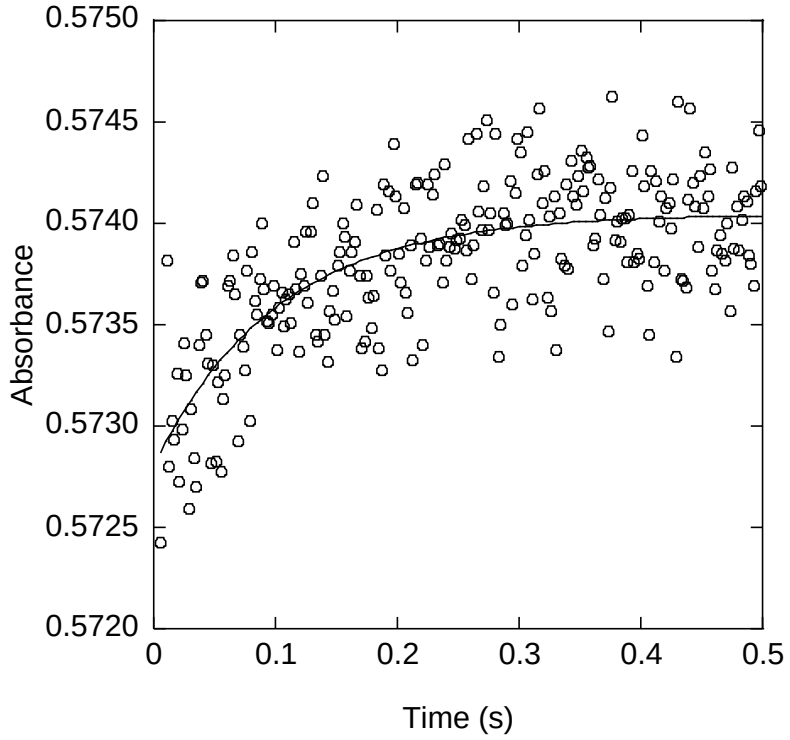


Figure 6. The stopped-flow absorbance spectra of the k_{off-n} reaction at 16°C over 0.5 seconds where the concentrations of hexokinase, TNP-ATP, and ATP were 4.0 μM, 3.8 μM, and 6 mM, respectively. The curve was fitted with an exponential decay equation (Equation 2).

The $k_{off,g}$ experiment was performed with 10 μM glucose present at 16°C over time intervals of 0.1, 0.5, 1.0, and 5.0 seconds where the hexokinase, TNP-ATP, and ATP concentrations were 1.0 μM, 0.9 μM, and 100 μM, respectively. Figure 7 displays the 0.5 second $k_{off,g}$ reaction. From observing the graph, no trend was present and the reaction was concluded to have already occurred. The same conclusion was made for the other three time intervals. This suggested that the koff,g reaction is too fast to measure. To view the reaction profiles of the other time intervals, see the Appendix. Similarly to what was done between the $k_{on,n}$ and $k_{on,g}$ treatments, the $k_{off,n}$ and

$k_{off,g}$ treatments can be compared qualitatively. It is important to note that the $k_{off,g}$ reactions were performed at smaller concentrations than the $k_{off,n}$ reaction. It is possible that the reaction can be observed at higher concentrations which would be something to do in the future. Despite the differences in concentrations, not seeing the $k_{off,g}$ reaction infers that it is too fast to measure. If it is too fast to measure, it is likely that the reaction has a large rate constant. Since a rate constant was observed for $k_{off,n}$ but not $k_{off,g}$, it can be tentatively concluded that the $k_{off,g}$ reaction has a larger rate constant than the $k_{off,n}$ reaction.

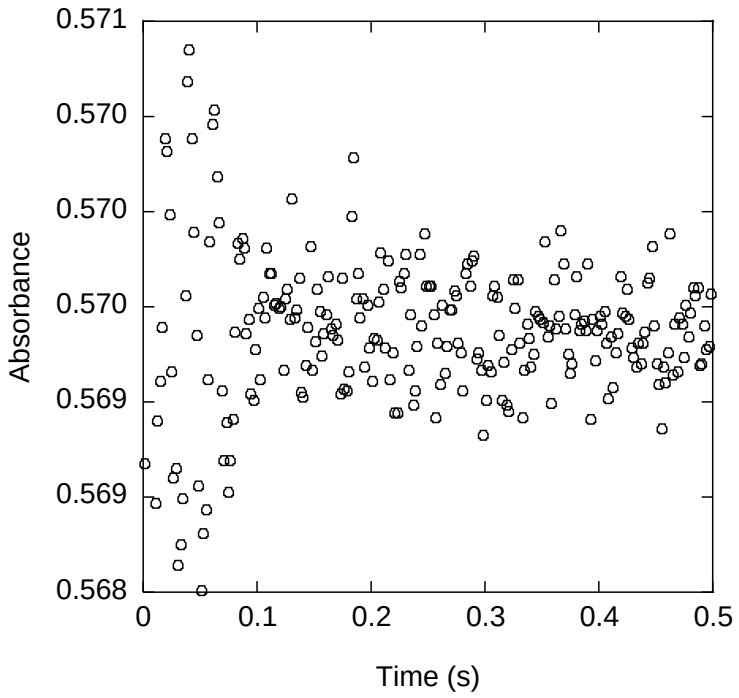


Figure 7. The stopped-flow absorbance spectra of the k_{off-g} reaction with 10 μM of glucose present at 16°C over 0.5 seconds where the concentrations of hexokinase, TNP-ATP, and ATP were 1.0 μM, 0.9 μM, and 100 μM, respectively.

Knowing that $k_{on,g}>k_{on,n}$, $k_{off,g}>k_{off,n}$, and $k_{off,n}<<k_{on,n}$ can help piece together a reaction profile for both the glucose and ATP first mechanisms. Figure 8 shows such a reaction profile. Here, the bolded lines represent what is being measured in this study while the dashed lines represent what is inferred but not measured in the present study. For both the glucose first and ATP first mechanism, the starting point is where there is free hexokinase, glucose, and ATP. The

reaction that appears to the left is where glucose binds to hexokinase first and the reaction that appears to the right is where ATP binds first.

Because glucose attached to hexokinase can be co-crystallized, this suggests that the glucose binds more tightly to hexokinase than does ATP. Because it can bind more tightly, this shifts the equilibrium between H+G and H*G to favor H*G. Thus, hexokinase with glucose attached is shown at lower relative energy than hexokinase with ATP. Previous studies have suggested that when ATP binds first, the small and large lobes interact which results in closing the glucose binding cleft. This suggests that binding glucose after ATP would sterically be unfavorable which would be expected to make the binding of glucose in the presence of bound ATP to be slower than without bound ATP. Thus, it is shown to have a higher barrier than the H+G→H*G reaction.

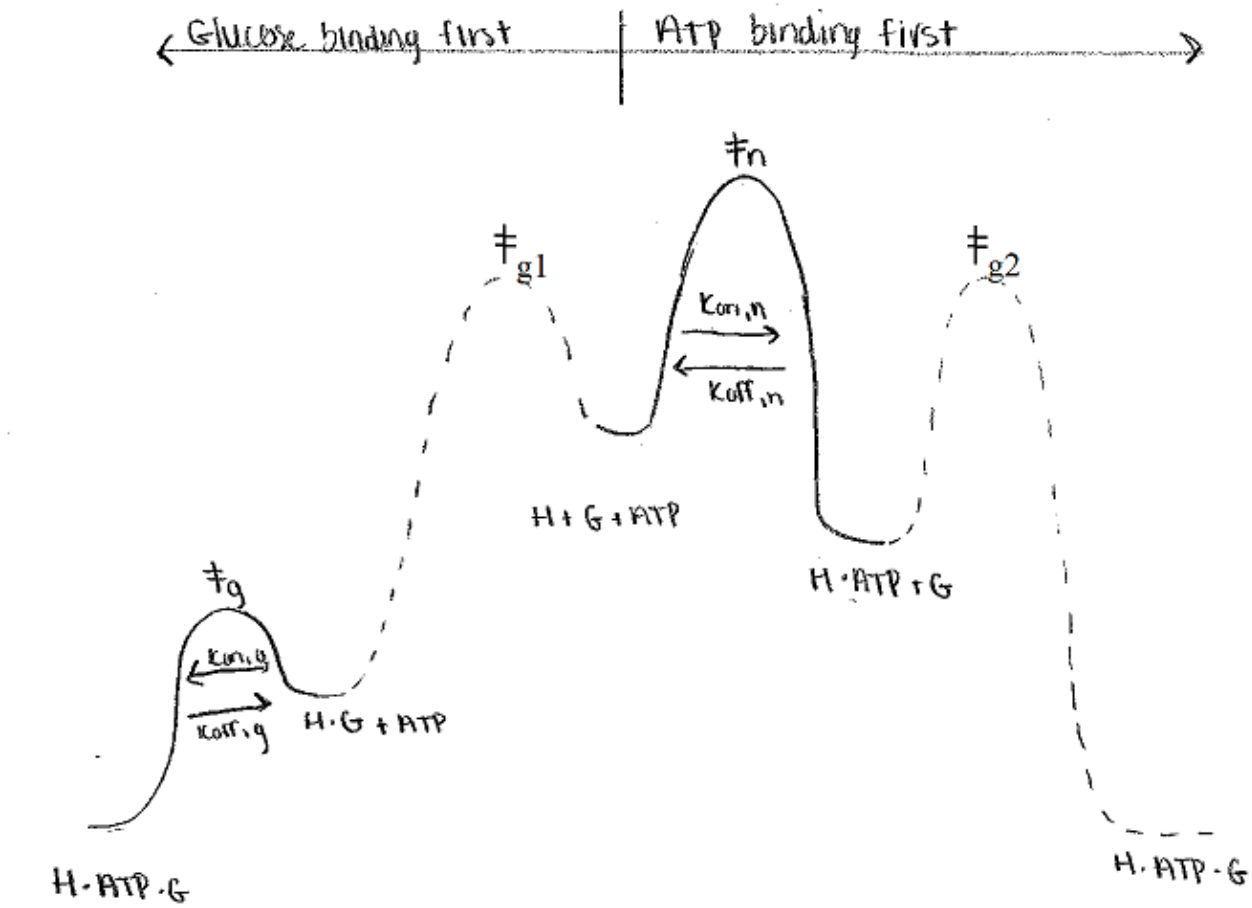


Figure 8. The reaction profile for ATP and glucose binding to hexokinase. The bolded lines display what can be measured while the dashed lines show what is inferred but not measured. The distance between H+G+ATP and H*ATP +G is directly proportional to $k_{on,n}/k_{off,n}$ and the distance between H*G +ATP and H*G*ATP is directly proportional to $k_{on,g}/k_{off,g}$. The process to left of center displays glucose binding first whereas the process to the right of center displays ATP binding first. $‡_g$ denotes the measurable glucose first pathway transition state, $‡_n$ denotes the measurable ATP first pathway transition state, $‡_{g1}$ denote the immeasurable glucose first pathway transition state, and $‡_{g2}$ denotes the immeasurable ATP first pathway transition state.

The main limitation to this study is that it is not possible to measure each transition state shown in Figure 8. Despite $‡_n$ and $‡_g$ being the only measurable transition states, inferences can be made to indicate what mechanism is most preferred. Though no numerical values were found for $k_{on,g}$ or $k_{off,g}$, the results suggest that $k_{on,g}>k_{on,n}$ and $k_{off,g}>k_{off,n}$. Because glucose can be co-crystallized with hexokinase, it is expected that the glucose first mechanism is probable. Furthermore, the co-crystallization argument also suggests that the $‡_{g1}$ barrier is not relatively

high enough to prevent glucose from associating with the hexokinase. It can be assumed that the glucose will then associate with the hexokinase. Although this study did not find numerical values for $k_{on,g}$ and $k_{off,g}$, it is inferred that the glucose reactions are faster than their respective no glucose reactions. Having a fast $k_{on,g}$ and $k_{off,g}$ reaction means that the ATP can readily associate and dissociate with hexokinase. Thus, ATP will likely bind to the hexokinase-glucose complex. This overall explanation suggests that the glucose first mechanism is a likely pathway for ATP binding.

It was found that $k_{on,n} >> k_{off,n}$ which suggests that hexokinase associated with ATP becomes stabilized. This alone suggests that ATP binding first is a probable mechanism. However, the barrier for $ǂ_{g2}$ is not known. This means that the energy barrier for $ǂ_{g2}$ could be relatively high. If the barrier is relatively high enough, then the process is essentially trapped as H*ATP+G. As stated above, the energy barrier for $ǂ_{g1}$ is not expected to trap H+G+ATP because co-crystallization occurs with hexokinase associating with glucose. Despite $k_{on,n}$ being greater than $k_{off,n}$, the potential barrier trap H*ATP+G could encounter at $ǂ_{g2}$ further suggests that the glucose first pathway is most likely.

Conclusion and Future Plans

Although this study was able to determine rate constants for $k_{off,n}$ (9.87 $^{1}/_{\mu Msec}$) and $k_{on,n}$ (~4000 $^{1}/_{sec}$) but unable to determine exact rate constants for $k_{off,g}$ and $k_{on,g}$, it was able to qualitatively compare the no glucose treatment to the glucose treatment to infer what mechanism ATP binding prefers. Because the glucose treatments had faster rate constants than the no glucose treatments and co-crystallization occurs with hexokinase and glucose but not ATP, it can be inferred that glucose binding to hexokinase first is preferred to occur before ATP binding first.

The kinetics of ATP binding to hexokinase in the presence and absence of glucose can also be determined using different parameters that can influence the k_{on} and k_{off} constants, such as varying the temperature and pH. Observing how ATP binds in the presence or absence of glucose at different pH and temperature values will provide a more complete understanding of ATP binding to hexokinase. Because hexokinase is an isoenzyme and is found in many species and tissue types, the knowledge of how ATP binds to one specific hexokinase can be applied to other hexokinases of different species or tissue origins.[2,3]

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Appendix

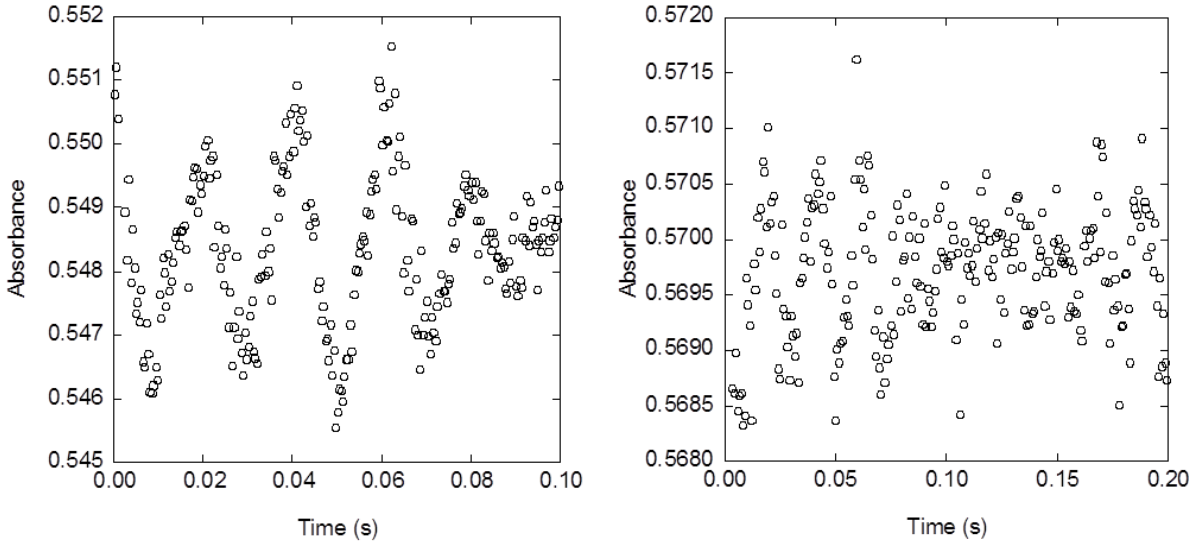


Figure A1. The stopped-flow absorbance spectra of two separate $k_{on\text{-}n}$ reactions at15.7^{0}C where the concentrations of hexokinase and TNP-ATP were both 1.0 μM. The left diagram displays the time interval of 0.1 seconds and the right diagram displays the time interval of 0.2 seconds.

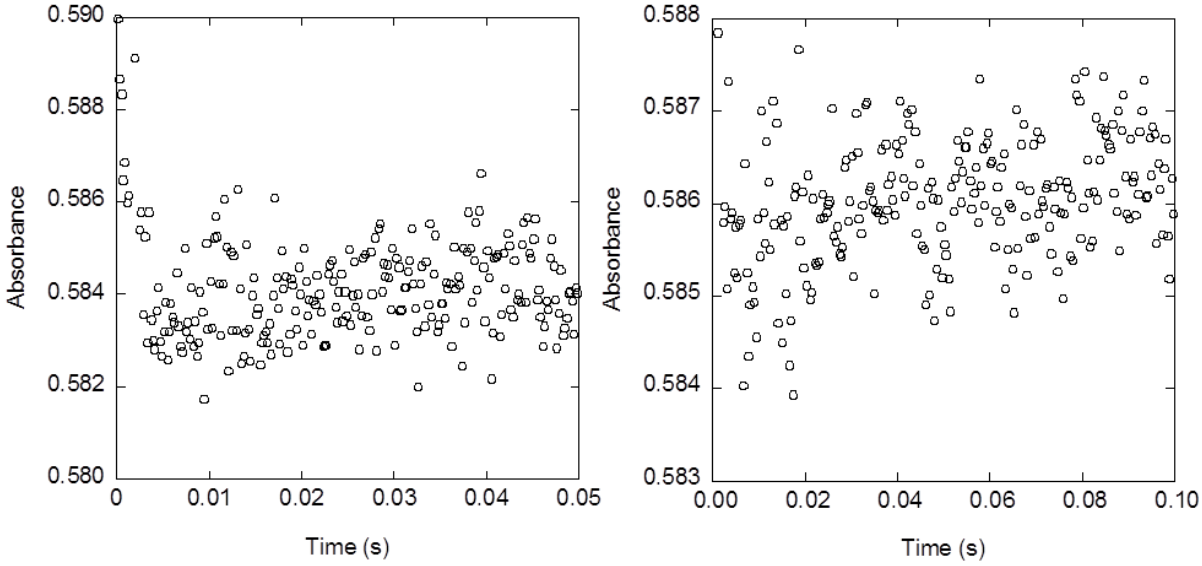


Figure A2. The stopped-flow absorbance spectra of a $k_{off\text{-}n}$ at 16.2^{0}C where the concentrations of hexokinase, TNP-ATP, and ATP were 4.0 μM, 3.8 μM, and 6 mM, respectively. The left diagram displays the time interval of 0.05 seconds and the right diagram displays the time interval of 0.1 seconds.

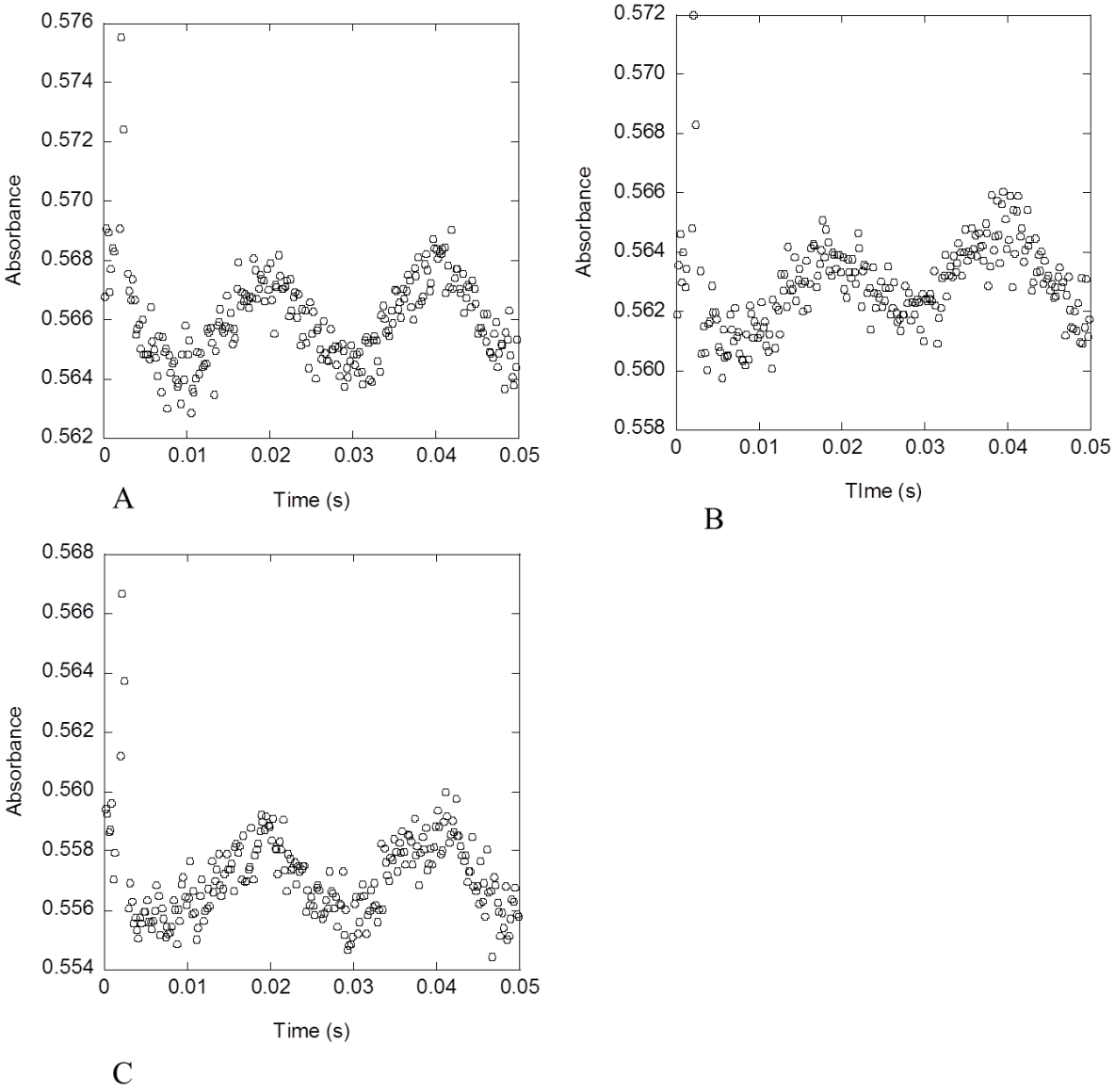


Figure A3. The stopped-flow absorbance spectra of three separate $k_{on\text{-}g}$ reactions with varying concentrations of hexokinase and TNP-ATP with 12.7 μM of glucose present at 16.6^{0}C over 0.05 seconds. Both hexokinase and TNP-ATP were either 1.0 μM (A), 0.5 μM (B), or 0.25 μM (C).

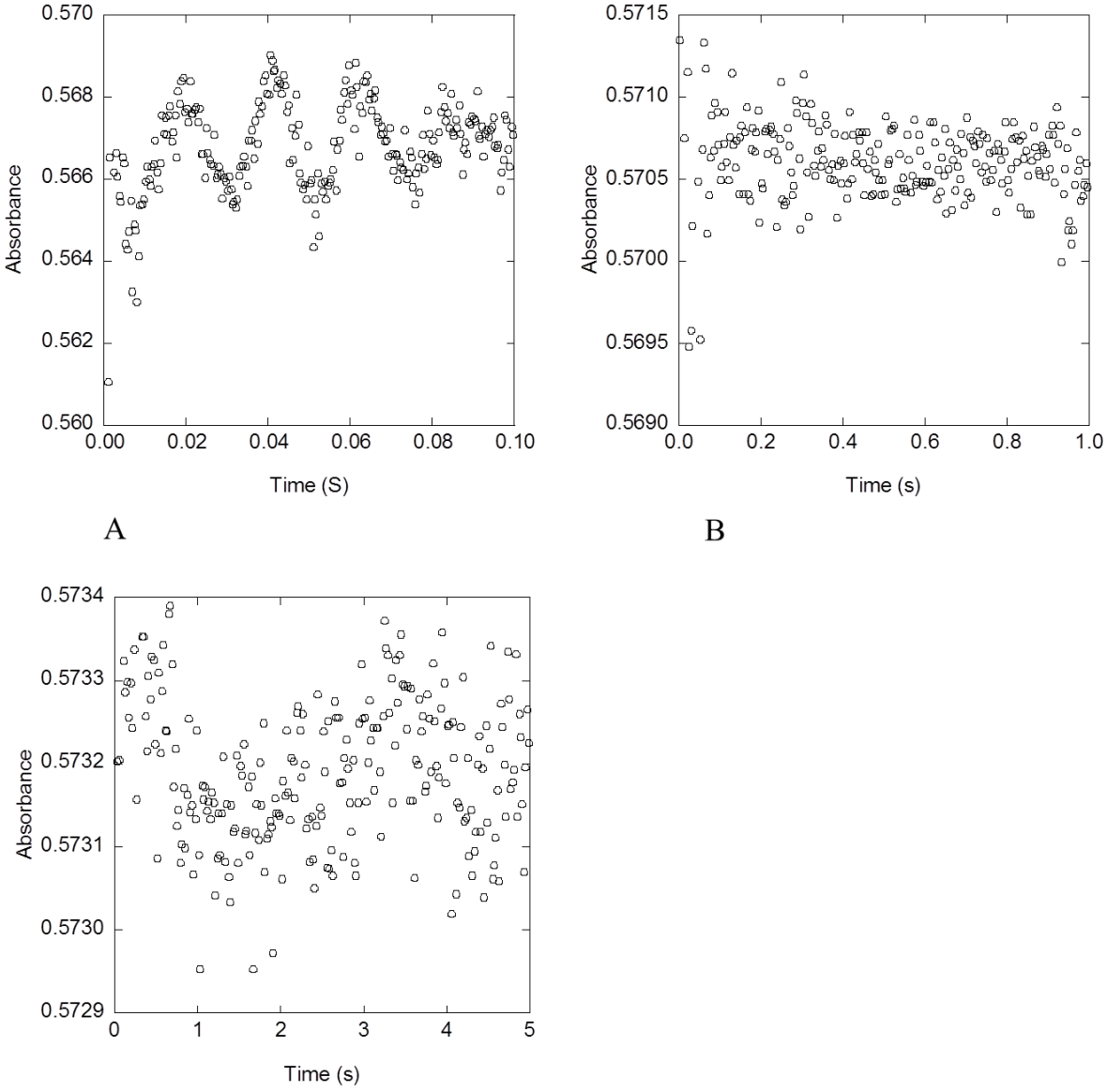


Figure A4. The stopped-flow absorbance spectra of the $k_{off\text{-}g}$ reaction with 10 μM of glucose present at 16.6°C over different time intervals where the concentrations of hexokinase, TNP-ATP, and ATP were 1.0 μM, 0.9 μM, and 100 μM, respectively. Time intervals observed were 0.1seconds (A), 1.0seconds (B), and 5.0seconds (C).