The Kinetics and Dynamics of
*Schizosaccharomyces pombe* Riboflavin Kinase

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Abstract

Riboflavin kinase is an essential enzyme that catalyses the phosphorylation of riboflavin to form flavin mononucleotide. This reaction is first step in the synthesis pathway of flavin adenine dinucleotide, an important prosthetic group of metabolic enzymes. Although literature regarding the kinetic and thermodynamic parameters of the riboflavin kinase module of the prokaryotic bifunctional enzyme FAD synthethase is available, studies using a eukaryotic variant have not been previously reported. In this project three different techniques were employed to quantify the kinetics and thermodynamics of *Schizosaccharomyces pombe* riboflavin kinase: a spectrophotometric coupled-enzyme assay, NMR spectroscopy, and isothermal titration calorimetry. The $k_{cat}$ value was determined to be $0.014 \text{ s}^{-1}$, indicating a particularly slow rate of catalysis in comparison to other metabolic enzymes. Furthermore, the obtained $K_d^{ATP}$ value of $4.6 \mu\text{M}$ was well below expected intracellular ATP concentrations, typically found in the mM range. HQSC spectroscopy studies further revealed extensive conformational changes in the enzyme following the binding of ATP, which seem to corroborate a binding mechanism hypothesized in previous studies. In addition to kinetic studies, a method for expression and purification of highly pure, $^{15}$N-labeled riboflavin kinase, at amounts optimal for biophysical studies is also reported. The observations gathered throughout this work lay the ground work for future research into the dynamics of riboflavin kinase.
### List of abbreviations

<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>FMN</td>
<td>Flavin mononucleotide</td>
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<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
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<td>RFK</td>
<td>Riboflavin kinase</td>
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<tr>
<td>FMNAT</td>
<td>Flavin mononucleotide adenylyltransferase</td>
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<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<td>ADP</td>
<td>Adenosine diphosphate</td>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>ITC</td>
<td>Isothermal titration calorimetry</td>
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<tr>
<td>PEP</td>
<td>Phosphoenolpyruvate</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
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<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
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<tr>
<td>$^1$H</td>
<td>Hydrogen-1</td>
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<tr>
<td>$^{15}$N</td>
<td>Nitrogen-15</td>
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<tr>
<td>HSQC</td>
<td>Heteronuclear single quantum coherence</td>
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<tr>
<td><em>E. coli</em></td>
<td>Escherichia coli</td>
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<tr>
<td>LB</td>
<td>Lysogeny broth</td>
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<tr>
<td>OD</td>
<td>Optical density</td>
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<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
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<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>TEV</td>
<td>Tobacco etch virus</td>
</tr>
<tr>
<td>D$_2$O</td>
<td>Deuterium oxide</td>
</tr>
<tr>
<td>app</td>
<td>Apparent</td>
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<td>DP</td>
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1. Introduction

1.1 Enzyme kinetics

Enzymes are biological molecules that function as catalysts to reactions occurring in the cell. Reactions in which organic molecules (reactants) interact chemically to form one or more products typically require a certain amount of energy to begin, even if the reaction results in an overall decrease in the free energy of the system. The reason this starting energy, known as the activation energy, is required is to form an intermediate structure in which reactants are brought together in a configuration optimal for reaction to occur. The intermediate structure is chemically unstable (hence the energy requirement) and is referred to as the transition state of the reaction. The role of enzymes is to decrease the activation energy so that the reaction may occur at an adequate rate. Enzymes achieve this by creating an environment, the active site, where the transition state becomes energetically stable. The active site may for instance provide complementary charges to that of the transition state, or opportunities for hydrogen bonds between the transition state and the enzyme.

The process of enzymatic catalysis can be described by the following Michaelis-Menten reaction mechanism:

\[ E + S \overset{k_{on}}{\leftrightarrow} E \cdot S \overset{k_{cat}}{\leftrightarrow} E + P \]

The reaction illustrates a process in which one substrate (S) is converted into one product (P) by an enzyme (E), but the general mechanism can also be applied to enzymes catalysing more complex reactions. The first step is naturally the binding of the substrate to the enzyme, resulting in the formation of the enzyme:substrate complex (E•S). The rate (in M⁻¹ s⁻¹) at which this process occurs is denominated \( k_{on} \). Because this is an equilibrium reaction, however, the reverse process where the complex dissociates into the free enzyme and unmodified substrate also occurs. This rate is designated \( k_{off} \) (not shown). It follows that the equilibrium constant in the direction of complex formation is given by the ratio \( k_{on}/k_{off} \). This value is known as the association constant, \( K_a \). More commonly, however, the inverse of the association constant, the dissociation constant \( K_d \), is used as a measure of affinity of an enzyme to its substrate(s). The reason is that the value of \( K_d \) is also the concentration (in M) at which the enzyme becomes half-saturated, that is, when half of the enzyme molecules in solution are in the ligand-bound state. The affinity of a particular enzyme may be different for each of its substrates. The free-energy change under standard conditions resulting from the binding of a ligand to an enzyme is given by \( \Delta G^o = -RT \ln K_d \). Once the enzyme:substrate complex is formed, catalysis takes place at a rate given by \( k_{cat} \), which may be thought of as a measure of the catalytic efficiency of the enzyme. Like the binding affinity, the rate of catalysis varies greatly between enzymes.

1.2 Protein dynamics

For over half a century after its postulation by Emil Fischer in 1894, the recognition and subsequent binding of specific substrates by enzymes had been thought to occur according to the lock-and-key model. According to this hypothesis, proteins adopt rigid conformations upon folding that remain unchanged throughout their life-spans. Thus, only molecules with a geometry that perfectly matches that of the enzyme’s binding site would be able to bind it and undergo catalysis. However, proteins have since been found to in fact possess a high degree of flexibility. Taking into account the intrinsic plasticity of enzymes, Daniel Koshland proposed in 1958 a new model to describe the process of ligand binding which became known as the induced-fit model. According to this theory, some degree of similarity is still present between ligand and the binding site, but the enzyme:substrate complex becomes fully cemented only
after the enzyme undergoes conformational changes to accommodate the substrate. In 1965 Jean-Pierre Changeux, building on the induced fit model, proposed yet another mechanism of substrate-binding. The model introduced by Changeux characterizes enzymes as existing in multiple conformations having similar but not equal free energies. In other words, every individual enzyme is constantly undergoing conformational changes, regardless of whether a ligand is present or not. When ligand is present, however, it binds to and stabilizes one particular conformation. This model is known as conformational selection and it has been corroborated by data gathered from experiments and computer simulations. It is important to note that the conformation stabilized by the ligand is not necessarily that with the lowest free-energy, but binding results in a change of free-energy to a level lower than that of any conformation existing in the absence of ligand.

Understanding and predicting the mechanisms behind the sampling of different conformational states by enzymes is one of the major challenges in biochemistry today.

1.3 Riboflavin kinase

Flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) are important biomolecules derived from riboflavin (vitamin B2) that function as prosthetic groups for a variety of proteins involved in cell metabolism. The synthesis of these molecules are closely related, as FMN itself is a precursor to FAD. In most eukaryotes, the complete conversion of riboflavin to FAD is performed in two steps by the enzymes riboflavin kinase (RFK) and FMN adenylyl transferase (FMNAT). The respective reactions are illustrated in figure 1.

![Figure 1. Synthesis pathway of flavin adenine dinucleotide. Riboflavin is first phosphorylated by the enzyme riboflavin kinase to form flavin mononucleotide. FMN and AMP are subsequently joined by FMN adenylyl transferase, forming FAD.](image)

In the present study we focus on *Schizosaccharomyces pombe* riboflavin kinase. This small protein located in the cytosol has a length of 163 amino acids and weight of 18939 kDa. As shown above, RFK catalyses the transfer of the gamma-phosphate from ATP to the terminal carbonyl-oxygen of riboflavin, resulting in the formation of FMN and ADP.

The three-dimensional structure of RFK has previously been determined by x-ray crystallography. There are two regions in the enzyme that are of particular interest, as they have been shown to undergo extensive conformational changes upon the binding of substrates. In this paper these regions shall be referred to as Flap I and Flap II, following the convention adopted in previous papers. The enzyme and its two major regions are shown in detail in figure 2. Further studies of the structure of prokaryotic RFK in both the presence and absence of substrates have revealed a mechanism where the binding of the two substrates is a highly cooperative process. Namely, binding of one substrate to its respective site induces conformational changes in the enzyme that affect the remaining site and facilitate the binding of the second substrate.
We initiated this project by studying the effects of expressing *Schizosaccharomyces pombe* $^{15}$N-labeled RFK under different conditions in transformed *E.coli* cells. After purification of the enzyme, we set out to investigate its kinetic and thermodynamic parameters using three alternative methods. In the first approach, a pyruvate kinase/lactate dehydrogenase coupled-enzyme assay was performed with the goal of determining the rate of catalysis, $k_{cat}$, of RFK.

Following this experiment, a series of nuclear magnetic resonance (NMR) spectroscopy analysis were carried out in order to obtain a two-dimensional picture of the structure of the enzyme in both the absence and presence of substrates. The rate of catalysis was also determined from NMR measurements as a complement to the enzyme-coupled assay experiment. The small size of RFK make it an ideal protein for NMR studies. Finally, isothermal titration calorimetry (ITC) was employed to quantify the dissociation constant, $K_d$, as well as the enthalpy of binding, $\Delta H^o$ of RFK to ATP. One should note that the values obtained in these experiments apply solely under the conditions at which they were performed. Thus, the obtained rates and constants are classified as apparent, rather than definite ($k_{cat}^{app}$, $K_d^{app}$). Although similar kinetic measurements have been performed using prokaryotic RFK, kinetic studies using an eukaryotic variant of the enzyme have not been previously reported.

### 2. Aim

This project was performed in the larger context of understanding enzymatic catalysis from the motion of the enzyme itself. Thus, it aims to characterize the kinetics of *Schizosaccharomyces pombe* riboflavin kinase as to develop an adequate model for future studies in the field of protein dynamics.

### 3. Background

#### 3.1 Coupled-enzyme assays

Enzyme assays are a collection of methods used to quantify the activity of enzymes by monitoring the rate of formation of products. In spectrophotometric assays, monitoring is done by measuring the change in absorbance of the assay solution at a particular wavelength as the reaction progresses over time. The reaction of interest is not required to result in changes in
light absorbance itself. In such cases, a coupled-enzyme assay can be employed. In this type of assay, one or more reactions that lead to strong, easily detectable changes in absorbance are coupled to the reaction of interest by means of overlapping products and substrates. The progress of the reaction catalysed RFK cannot be monitored on its own by spectrophotometry. The absorbance spectra of riboflavin and FMN do not differ significantly given that the only structural distinction between these molecules is the presence of a phosphate group in the latter. Accordingly, an assay to measure the catalytic activity of RFK requires coupling with additional reactions. In this experiment, a coupled pyruvate kinase/lactate dehydrogenase assay was performed. Pyruvate kinase is the glycolytic enzyme that catalyses the transfer of a phosphate group from phosphoenolpyruvate (PEP) to ADP, forming pyruvate and ATP. Lactate dehydrogenase (LDH), in turn, catalyses the reduction of pyruvate, oxidizing NADH in the process. The products of this reaction are thus lactate and NAD\(^+\). NADH absorbs light strongly at 340 nm. Our reaction of interest results in the formation of ADP. Therefore, coupling this reaction with those described above will ultimately lead to the reduction of NADH to NAD\(^+\). By monitoring the change in absorption of NADH at 340 nm (\(\Delta A_{340}\)) over time (\(\Delta T\)), the rate of ADP formation, and therefore the rate of catalysis of RFK, can be quantified indirectly. First, the change in concentration (\(\Delta c\)) of NADH is determined by using the Beer-Lambert law equation:

\[
\Delta A = \varepsilon \times \Delta c \times l.
\]

Next, the change in concentration is divided by the change in time to obtain the rate of formation of ADP. Finally, the rate of formation of ADP is divided by the concentration of enzyme in solution to give the rate of catalysis of one individual enzyme molecule.

\[
\begin{align*}
\text{Riboflavin} + \text{ATP} &\xrightarrow{\text{Riboflavin kinase}} \text{FMN} + \text{ADP} \\
\text{Phosphoenolpyruvate} + \text{ADP} &\xrightarrow{\text{Pyruvate kinase}} \text{Pyruvate} + \text{ATP} \\
\text{Pyruvate} + \text{NADH} &\xrightarrow{\text{Lactate dehydrogenase}} \text{Lactate} + \text{NAD}^+ 
\end{align*}
\]

The method described above provides the rate of catalysis of the enzyme in the particular conditions of each solution. To determine the maximal rate of catalysis (\(V_{\text{max}}\)), several different measurements are carried out with increasing concentrations of one of the substrates (here either riboflavin or ATP) while maintaining the concentrations of all other molecules in solution constant. The rates obtained at each measurement are then plotted as a function of substrate concentration to generate a so-called Michaelis-Menten curve. At a certain substrate concentration, the enzyme will reach its maximum rate of catalysis upon which the addition of substrate no longer leads to an increase in rate of catalysis. The concentration at which half of this value is attained is known as the Michaelis constant, \(K_m\), and, analogous to \(K_d\), it is a measurement of the affinity of the enzyme for the particular substrate.

### 3.2 NMR spectroscopy

Nuclear magnetic resonance spectroscopy is a widely employed technique used to map the electronic micro-environments of molecules from which the complete structure can subsequently be determined. When a molecule is subjected to a magnetic field, its nuclei possessing magnetic moment will tend to align their spin with the particular direction of the applied field, either in parallel or antiparallel fashion. If an electromagnetic pulse of the right frequency is applied to a molecule in such state, the active nuclei will absorb the energy (thus resonance) and as a result change their direction of spin. The energy necessary to achieve such change is known as the chemical shift. The chemical shift for a particular atom in a molecule
is highly dependent on the atoms immediately surrounding it. Thus, by applying pulses of different frequencies and recording those that induce chemical shifts, information about the micro-environments that form the molecule can be obtained, which can subsequently be combined to obtain a complete picture. Whether a particular atomic nucleus possesses a magnetic moment depends on its number of protons and neutrons. Some nuclei commonly used in NMR analysis are $^1$H, $^{13}$C, $^{31}$P, and $^{15}$N. The applications of NMR are vast, and this technique is often employed in studies of structural biology and biochemistry. The tertiary structure of proteins can be resolved by performing multidimensional NMR analysis, where the chemical shift of as many as three active nuclei are tracked simultaneously, allowing for bond lengths between atoms as well as their relative positions to be computed.

Enzyme dynamics can also be studied using NMR. As discussed above (see section 1.2), enzymes are flexible molecules with continually shifting structures. Following a similar principle to that used in the determination of the three-dimensional structure, subtle changes in the position of amino acids in an enzyme can be detected by recording changes in chemical shifts in nuclei present in each amino acid. A commonly used technique in such studies is the two-dimensional analysis using $^1$H and $^{15}$N nuclei known as heteronuclear single quantum coherence spectroscopy (HSQC). The shifts experienced by these atoms, corresponding to individual amino acids, are measured and plotted in a two-dimensional plane. Multiple analysis are carried out under different conditions allowing for potential structural responses in the enzyme to be detected. In this way, protein dynamics may not only be observed qualitatively, but also quantified using computational methods. An important application of this technique is in the study of drug-binding to proteins in pharmaceutical research.

### 3.3 Isothermal titration calorimetry

Isothermal titration calorimetry is a technique used to quantify the thermodynamics of ligand-binding of enzymes. This method originates from the fact that the binding of a substrate to an enzyme is a thermodynamic process that results either in overall releasing (exothermic) or intaking (endothermic) of energy. A typical ITC system is comprised of two cells: a reference cell containing pure water, and a sample cell, where the binding reaction of interest takes place. Before any measurement is carried out, the two cells are heated to the same temperature. Next, the protein is injected to the sample cell followed by the addition of small aliquots of the ligand. The energy released or absorbed in the binding reaction process causes the solution in the sample cell to increase/decrease. The rise/decline in temperature is significantly enough for a sensor in the system to detect a thermal imbalance between the two cells. The system responds by increasing the temperature of the reference cell as to match that of the sample cell, or vice-versa. The heat necessary to bring the cells to thermal equilibrium after the addition of each aliquot is recorded. When the binding sites of all enzyme molecules in the solution have been occupied, the solution becomes saturated with ligand molecules and the temperature of the sample cell ceases to shift following the addition of further aliquots, indicating the end of the binding reaction. The heat released/absorbed throughout the reaction is plotted as a function of the molar enzyme:substrate ratio in solution. From the resulting isotherm, the $K_d$ of the enzyme for the substrate can be derived by determining the concentration at which the solution is half-saturated. The molar enthalpy of binding may also be obtained by computing the amount of heat released/absorbed per mole of enzyme in solution.

### 4. Methods

#### 4.1 Transformation of E.coli and overexpression of RFK

100μL of *E.coli* BL21 pLysS (DE3) recombinant cells were transformed by heat shock with 1μL of Pet-His-RFK1-163 plasmid. The transformed cells were plated on a kanamycin- and
chloramphenicol-enriched CB plate and allowed to grow overnight. Four cultures were harvested from the plate and each was suspended in 10 mL LB-medium with 50 μg/mL kanamycin and 34 μg/ml chloramphenicol. The cultures were then incubated overnight at 37°C. Following incubation in LB medium, the cells were pelleted by centrifugation and resuspended in a 2-litre solution of 15N-enriched M9 medium containing 50μg/mL kanamycin and 34μg/mL chloramphenicol. The culture was incubated at 37°C until it reached an OD₆₀₀ = 0.6. The culture was then divided into two flasks of equal volume (1 litre) and 1 mM IPTG was added to each. The cultures were then incubated overnight, one at 16 °C and one at 37 °C. Following incubation, the cells were harvested by centrifugation at 6000 rpm for 20 minutes and resuspended in 50 mM Tris buffer pH 6.0.

4.2 RFK purification

His trap chromatography
Each culture (one grown at 16°C and one grown at 37°C) was sonicated 3x1 minute. The cultures were then centrifuged for 40 minutes at 18000 rpm. Column chromatography was then performed using a His trap™ FF column (Buffer A: 50 mM Tris pH 6.0. Buffer B (gradient elution): 50 mM Tris, 0.5 M imidazole, pH 6.0). Portions that displayed high absorption at 280 nm were sampled for SDS-PAGE analysis. Upon verification of the presence of RFK by SDS-PAGE, the relevant samples were pooled together.

Polyhistidine-tag digestion
The removal of the polyhistidine-tag from RFK was performed by adding TEV protease to the RFK solution for a final w/w ratio of 1:100 (TEV:RFK). The solution was then dialysed against 50 mM Tris, 150 mM NaCl, 1 mM β-mercaptoethanol, pH 7.0 for 22h at room temperature. Following dialysis, the TEV and the polyhistidine-tag were removed from the solution by performing a new Histrap column chromatography (Buffer A: 50 mM Tris pH 6.0. Buffer B (gradient elution): 50 mM Tris, 0.5 M imidazole, pH 6.0).

Gel filtration (size-exclusion chromatography)
RFK was concentrated to a volume of approximately 1.5 mL using Amicon™ Ultra-15 centrifugal filters. Gel filtration was then done using a GE Superose 12 10/300 GL column (Buffer: 30 mM MOPS, 50 mM NaCl, pH 7.0). Following gel filtration, the protein was concentrated and flash-frozen with liquid N₂. The purified protein was then stored at -18 °C for future use.

4.3 Enzyme-coupled Assay
Spectroscopic measurements were made with solutions of increasing riboflavin concentration in buffer (100 mM Tris, 80 mM KCl, 2 mM MgCl₂, 200 μM ATP, 100 μM, 400 μM PEP, pH 7.5). To each solution, 10 units of Sigma-Aldrich pyruvate kinase/lactic dehydrogenase solution and 3 μM riboflavin kinase were added. Absorption at 340 nm was measured immediately following the addition of riboflavin kinase to the solution.

4.4 NMR Spectroscopy
For the ATP titration experiment NMR spectroscopy measurements were made using a Bruker’s 850 MHz Avance III HD equipped with a 5 mM HCN cryoprobe. For the reaction assay a 600 MHz Avance III HD equipped with a BBO broadband cryoprobe was used.
Samples were prepared in 30 mM MOPS, 50 mM NaCl, pH 7.0 buffer. Assay samples contained 1 mM ATP, 150 μM RF, 0.2 mg/mL bovine serum albumin, and 1 μM RFK. The volume was adjusted to 500 μL with 7% of the final volume consisting of D₂O in every sample. ATP titration samples contained the same concentrations as in the assay samples but with varying concentrations of ATP, 50, 100, and 500 μM.

4.5 Isothermal titration calorimetry
The ITC experiment was carried out using a GE Microcal™ Auto-ITC200 system. The reaction cell containing 12 μM RFK (Buffer: 30 mM MOPS, 50 mM NaCl, pH 7.0) was titrated with 30 x 2 μL aliquots of 1.1 mM ATP.

5. Results
5.1 RFK purification
5.1.1 Effect of temperature in the expression of RFK
An initial objective of this project was to optimize the expression and purification process of RFK, building on previously reported methods. It has been reported that proteins expressed under low temperature conditions may have increased solubility and lower tendency to form inclusion bodies. Aggregated proteins are not easily retrievable by purification. Thus, a low ratio of soluble proteins to inclusion bodies in solution leads to lower yields of purified protein. To verify the effect of temperature in the expression of Schizosaccharomyces pombe RFK, transformed E.coli cells were induced to express the enzyme at different temperatures, 16°C and 37°C. Following expression, each culture was subjected to centrifugation. During centrifugation, inclusion bodies present in solution are collected at the bottom of the vial together with other large cellular components. Thus, by the end of the process, the supernatant solution consists mostly of free proteins. By performing an SDS-analysis of the supernatant solutions of both cultures, it is possible to verify the amount of free RFK in each, and thus assess whether either of the expressing conditions had any significant effect in solubility. Figure 3 shows SDS-gel analysis performed at different points during the expression and purification process. In figure 3A the presence of RFK in culture solutions following overnight incubation in M9 media is indicated by the large, sharp bands under both 16°C and 37°C. Both bands are of similar size and intensity, indicating similar amounts of RFK. The gel in figure 3B shows the composition of the supernatants as well as pellets in both solutions after centrifugation. The band corresponding to RFK was more intense in the supernatant of the 37 relative to the 16°C solution. Accordingly, the pellet composition of this solution shows a fainter, nearly imperceptible RFK band. The RFK band visible in the pellet of the 16°C solution, however weak, indicates the presence of a larger amount of inclusion bodies. Together, these observations suggest that higher solubility of RFK is achieved when the enzyme is expressed at 37, relative to 16°C.

5.1.2 Cleavage of the poly-histidine tag by TEV protease
The plasmid with which the E.coli cells were transformed in this experiment contained a slightly modified gene of RFK. This modification results in the expressed protein having a so-called polyhistidine-tag, a short sequence of histidine amino-acids, attached to one of its terminals. The addition of polyhistidine-tags to proteins is a common practice used to facilitate the purification process. The expressed RFK also contains the required sequence for cleavage by protein TEV protease at a strategic location that allows for the removal of the polyhistidine-tag. A further goal of this experiment was to assess the efficiency of the removal of the polyhistidine-tag of RFK by TEV protease under room-temperature conditions. Figure 3C displays SDS-gel analysis carried out at different incubation times of RFK with TEV protease.
After five hours of incubation, most, but not all, RFK in solution is already in a tag-free state. This is evidenced by the weight-shift observed in relation to the protein in solution preceding the addition of TEV protease. After 22 hours of incubation, the presence of tagged enzyme in solution is barely detectable visually. Thus, we conclude that an incubation time of at least 22 hours at room-temperature is optimal for nearly complete cleavage of the polyhistidine-tag by TEV protease.

Figure 3. SDS-gel analysis at different stages of RFK purification. (A) Composition of culture solutions before and after the addition of IPTG and overnight incubation. (B) Composition of supernatants and pellets of the solutions following overnight incubation and expression. A sharper band in the supernatant of the 37°C solution suggests a higher RFK content. (C) RFK at different stages of poly-histidine tag cleavage process. Cleavage is evidenced by a shift in the weight of the enzyme. (D) RFK obtained at the end of the purification process. Absence of additional bands indicate a high level of purity.

5.2 Coupled-enzyme assay
The fluorescence of seven solutions with varying concentrations of riboflavin, ranging from 25 μM to 175 μM, was analysed in order to obtain the rate of catalysis of RFK as described under section 2. Linear fits of the change in absorbance in the solutions are shown in figure 4. At
concentrations below 75 μM, the change in absorbance was too subtle for any meaningful computation to be performed. It is evident from figure 3 that at a riboflavin concentration of 75 μM the catalysed reaction occurs at a faster rate than at higher concentrations. We thus assume that maximal rate of catalysis is reached under such conditions. Thus, from the change in absolute absorbance observed at this concentration, a $k_{cat}^{app}$ of 0.0095 s$^{-1}$ is obtained. Because measurements at lower concentrations were not feasible, however, it wasn’t possible to derive the $K_m$ value of the enzyme using this technique. Furthermore, given the considerably low rate of catalysis of RFK evidenced by the data, as well as stability issues with the spectrophotometer system, the coupled-enzyme assay method was deemed impractical for our current purposes. Measurements with varying concentrations of the second substrate, ATP, were therefore not performed.

![Graph showing absorbance change for different riboflavin concentrations](image)

**Figure 4. Relative change in absorbance for solutions of different riboflavin concentration.** Linear fits were performed on the data and plotted over reaction time. The change in absorbance observed in the 75 μM riboflavin solution occurs at a clearly faster rate. From the absolute change in absorbance (not shown), a $k_{cat}$ of 0.0095 s$^{-1}$ was obtained.

### 5.3 NMR spectroscopy

#### 5.3.1 Determination of RFK rate of catalysis

A $^{31}$P NMR assay containing RFK and its substrates was implemented with the goal of determining the rate of catalysis of the enzyme as a complement to the coupled-enzyme assay.
experiment reported above. In the spectra in figure 5A the emergence and subsequent evolution of a peak representing the product FMN demonstrates the progress of catalysis over time.

![Image of NMR spectra](image)

**Figure 5. Progress of the reaction catalysed by RFK revealed by NMR spectroscopy.** (A) Overlay of chromatograms taken from the same assay solution at different time periods of the reaction, from the initial (green) to the final (blue) stage. The intensity of the peaks representing the three phosphate groups of ATP are seen to decrease as the peak representing FMN emerges and increases in intensity. (B) Change in concentration of ATP in solution over time. The exponential decrease indicates the consumption of the molecule. (C) Change in concentration of FMN in solution over time. (D) Change in concentration of ADP in solution over time. As with FMN, the exponential increase mirrors the decrease observed for ATP. (E) Observed rate of catalysis of RFK in three assays of equal composition. The rate was obtained by dividing the rate of consumption of ATP by the concentration of enzyme molecules in solution.

The remaining peaks in the spectrum arise from the phosphates groups in ATP. These intensity of these peaks is seen to decrease accordingly, as the molecule is consumed over time. Integrating the area of a peak gives the concentration of that particular species in solution. The reaction was allowed to occur until completion whereupon the concentration of products and
substrates over time was plotted as a function of reaction time. Figure 5B shows the exponential decrease in ATP concentration in solution. Conversely, the exponential increase in FMN and ADP concentrations is seen in figures 5C and 5D, respectively. The data readily provides a method for quantifying the rate of catalysis of RFK. The rate of consumption (decline in concentration) of ATP was determined and the value divided by the total concentration of enzyme in solution, giving a mean rate of catalysis of 0.014 s⁻¹ (Figure 5E).

5.3.2 Conformational changes in RFK
In addition to the determination of the rate of catalysis, an HSQC experiment was performed with the goal of obtaining a two-dimensional view of the structure of RFK both in a substrate-free state (apoenzyme) and in an ATP-bound state. Figure 6 shows the two-dimensional spectrum of the apoenzyme (A) and a superimposition of the spectra of apoenzyme and ATP-bound enzyme at a saturating concentration of ATP (B). Such a high concentration of substrate ensures that all enzyme molecules in solution are in the bound-state. As discussed in section 2, conformational changes are expected to occur in the enzyme upon the binding of a substrate. The superimposed spectra displayed in figure 6B reveals the extent of such changes in RFK. Chemical shifts are observed for several, in fact most, amino acids forming the enzyme. This wide-spread effect suggests a binding mechanism of ATP that results in a generalized conformational change affecting the entire molecule rather than a localized effect restricted to the binding site.

It is possible to quantify the binding affinity of enzyme to its substrates by HSQC spectroscopy. This is achieved by performing measurements in assay solutions of increasing concentrations of substrate. Ideally, such procedure will lead to gradual changes in chemical shifts in the affected amino acids until saturation point is reached whereupon shifts cease to occur. Such experiment was performed using RFK in an attempt to obtain the dissociation constant of the enzyme for ATP. However, the resulting changes in chemical shifts were sudden rather than continuous. This effect can be seen in figure 5C where shifts occurring in the solution of 50 μM ATP are as pronounced as those in the 500 μM solution. This observation indicates that saturation of the RFK:ATP complex in solution is achieved at lower concentrations than 50 μM, suggesting tight binding and thus high affinity of the enzyme for ATP. In such cases the binding affinity of the enzyme becomes impossible to quantify using this method. Therefore, to achieve this goal, isothermal titration calorimetry was employed instead.
Figure 6. HSQC spectra of RFK in apo and ligand-bound form. (A) Apo RFK. (B) Overlay of apo and RFK:ATP complex at saturating concentration of ATP. Differences in chemical shifts are observed in several amino-acids throughout the entire molecule indicating generalized conformation changes. (C) Overlay of apo RFK and RFK:ATP at different concentrations of ligand. The changes in chemical shifts are immediate rather than gradual, making it impossible to determine the dissociation constant, $K_d$. (D) Idealized depiction of transitions in chemical shifts that would allow for the determination of $K_d$. 
5.4 Isothermal titration calorimetry

The ITC data displayed in figure 6 reveals the thermodynamic features of the binding process of ATP to RFK. The heat released by the reaction is indicated by the peaks with negative values of differential power (DP) between the two cells in figure 6A. A gradual decrease is observed in these peaks until complete disappearance at around 1600 seconds, indicating ligand saturation in the solution. Peaks with positive value are also observed throughout the entire experiment. The origin of such peaks could possibly be traced to the heat of dilution of enzyme and ATP molecules in solution, which arise from the breaking and forming of bonds between solvent and solute. The heat of binding of the reaction is thus obtained by subtracting the heat of dilution from the overall heat of the process. The corrected values plotted against the RKF:ATP molar ratio in solution are shown in figure 6B. The data indicates a $K_d$ value of 4.6 μM and an $\Delta H$ of -2.9 kcal/mol.

6. Discussion

We began this project with the aim of characterizing the kinetics and dynamics of Schizosaccharomyces pombe riboflavin kinase as to gain insight into the relationship between protein motion and catalysis, and to pave the way for further studies using the enzyme. The expression and purification procedure presented provides a method for obtaining highly pure RFK at sufficiently high amounts as to enable biophysical studies. The principal objective of this experiment, however, was to verify whether the solubility of the enzyme would increase to any significant degree at lower temperature conditions of expression, as it has been previously reported. Curiously, our data revealed the very opposite. As it can be seen from figure 3B the expression of soluble RFK (that is, present in the supernatant) was stronger at the higher temperature of 37 °C. The lower temperature conditions reported in previous studies were in the 20-30 °C range. The temperature of 16 °C used in this study may have affected protein synthesis to the point of negating the benefits of increased solubility. Experimenting with the lower temperature limit is therefore crucial for the optimization of expression in future studies.

The investigation on the catalytic rate of RFK proved to be more demanding than previously expected. The remarkably low rate of catalysis of the enzyme as evidenced by the initial coupled-enzyme assay experiment required a more sensitive approach. A definitive $k_{cat}$ of
0.01412 s⁻¹ was only achieved by NMR spectroscopy measurements. This value is nonetheless in reasonable accordance with that of 0.0095 s⁻¹ obtained via the former method. Previous studies have reported catalytic rates ranging from 0.042 to 7.33 s⁻¹⁹,1⁰. Such measurements, however, were done on the RFK module of the prokaryotic enzyme FAD synthethase, a bifunctional enzyme which catalyses the full conversion of riboflavin to FAD+. The deviation from the reported values might thus illustrate differences in catalytic efficiency of prokaryotic and eukaryotic RFK.

The NMR experiments also revealed an extensive structural effect in RFK following the binding of ATP (figure 6). Referring to figure 2A, the observed conformational changes might be interpreted in the context of the three-dimensional structure of the enzyme. As previously described, the binding site of ATP is located in the region denominated Flap I. In the absence of this ligand, the loop and helix forming this region have previously been found to be in an ‘open’ conformation. That is, collapsed outwards, away from the core of the enzyme⁶. Flap II, the binding site of the second substrate, riboflavin, is arranged in a similar manner in the absence of this ligand. The widespread conformational changes observed (figure 6B) indicate that the binding of ATP may trigger the simultaneous inwards shift of both regions, Flap I and II. Thus, the enzyme is only brought to a catalytic conformation upon the binding of this ligand. These observations seem to corroborate a binding mechanism previously hypothesized based on solved crystal structures of RFK⁶,⁷.

Within the context of the previous NMR spectroscopy experiments, the dissociation constant of RFK for ATP obtained by ITC measurements turned out to be surprisingly high. Given the earlier data (figure 5C), a $K_d$ value in the 0.1 – 1 μM range seemed likely. The obtained value of 4.6 μM is nonetheless plausible and it is in accordance with the previously reported data⁹,2³.

7. Conclusions
In this project we have successfully accomplished the main objective of characterizing the fundamental kinetic parameters of Schizosaccharomyces pombe riboflavin kinase. Additionally, we have described and addressed important questions regarding the process of expression and purification of the enzyme. Three independent techniques were employed in this study, which in combination were used to generate an ample view of RFK. Initial investigations in the binding dynamics of the enzyme were also achieved, which seem to be in good accordance with the available literature. The work presented here may therefore be used as a framework for future studies in protein dynamics using eukaryotic riboflavin kinase.

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9. References


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