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Metal Binding Affinity of *Halobacterium salinarum* Cysteinyl-tRNA Synthetase

by

Lori Cobani

A thesis

submitted in partial fulfillment

of the requirements for the degree of

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Committee Approval

To the Graduate Faculty:

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Metal Binding Affinity of *Halobacterium salinarum* Cysteinyl t-RNA Synthetase

Thesis Abstract--Idaho State University (2018)

Cysteinyl t-RNA synthetase (CysRS), an enzyme that catalyzes the attachment of cysteine to its cognate tRNA, is an example of a protein with well characterized halophilic adaptations. Using the *Halobacterium salinarum* CysRS, we are investigating how the protein interacts with potassium, which seems to maintain its structure and stability. The effects of the metals on the protein were tested through equilibrium dialysis and atomic absorption spectroscopy (AAS). The goal was to determine the rate of diffusion over time and compare the results between the CysRS and *E.coli* CysRS, a negative control. It was observed that the halophilic protein binds to potassium faster than the nonhalophilic, *E. coli* protein. By characterizing this interaction, we estimated the binding constant of the protein, K_d, to the metal. The binding constant of the protein can contribute in designing techniques to be used in environmental remediation that could help us clean up metal contaminated sites.

Key Words: Halophile, Cysteinyl t-RNA synthetase, *Halobacterium salinarum*, Potassium.

Introduction

Archaea is one of the three domains of life, which includes inhabitants of some of the most extreme environments on the planet. Organisms that live in such conditions are called extremophiles and they thrive in physically or geochemically extreme conditions that would be detrimental to most life on Earth. One example is *Thermus thermophilus*, which grows optimally at 65 °C. At this temperature, many proteins from other organisms would denature, but the proteins found in *T. thermophiles* remain stable and functional. Because extremophiles require challenging and extreme growth conditions, like extreme cold, heat, salt, acid/base or pressure, they are also the least studied organisms. Extremophiles are divided into four branches: acidophiles, thermophiles, psychrophiles and halophiles. Many extremophiles grow under multiple conditions (cross over organisms), however, most of them have a primary adaptation that is within one of these branches [1].

Acidophiles are organisms with optimal growth below pH 5. Moderate acidophiles grow between pH 3-5, while extreme acidophiles grow optimally at pH 3 or below. For an organism to be considered an acid-tolerant species, it should grow above pH 5, but below 7. Acidophiles are often found in isolated environments, either naturally formed or people-made, which frequently form during mining operations. Acidophiles thrive in acidic solutions that contain concentrations of metals that are lethal to most life forms [2]. For example, *Picrophilus oshimae* is an extreme acidophiles found in hot springs with a pH of 2.2 [3]. While the adaptation of proteins to pH is still not fully understood, the activity of acidophilic proteins is attributed to the high number of acidic amino acids on the surface of these proteins [8].

Thermophiles are organisms that live between 45 °C to 122 °C, which is the upper temperature limit at which life is currently found. They grow optimally in hot springs,

hydrothermal vents and other geothermally heated areas [4]. These organisms face an incredible environmental challenge by adapting to the high amounts of thermal energy without their proteins losing their function. Therefore, their proteins have incorporated adaptations like an increased number of hydrophobic residues, a larger hydrophobic core, an increased number of disulfide bonds, and increased ionic interactions [1].

Psychrophiles are organisms capable of surviving at very low temperatures, which range from about -20 °C to 10 °C. They are found in cold places like the polar regions, glaciers or the deep sea. Most of these organisms are bacteria or archaea, although some eukaryotes such as snow algae, lichens and fungi are also classified as psychrophiles [5]. In order to survive, the proteins of psychrophiles increase kinetic energy, which increases the catalytic power of the enzyme to get reaction turnover [1]. Most psychrophilic proteins increase kinetic energy by increasing protein flexibility. They do this by increasing the amount of their hydrophobic surfaces, decreasing stabilizing interactions like salt bridges, disulfide bridges or hydrogen bonds, and having a greater overall negative charge [1].

Halophiles are the salt-loving organisms. While they require at least 0.2 M salt concentration for survival, their tolerance can go up to 5.1 M [6]. For comparison, the human body maintains a 0.05 M salt concentration. Halophiles are found in naturally and extremely salty environments like the Great Salt Lake and the Dead Sea. Under extremely ionic conditions caused by high concentration of ions like sodium, potassium and other metals, they have implemented various strategies to cope with the stress caused by this extreme environment [1]. They accumulate inorganic salts and small organic molecules like amino acids, sugars or polyols, in the cytoplasm until intracellular osmolarity equals the extracellular ion concentration, in order to avoid osmotic shock [1]. The proteins of halophiles rely on salt for proper folding in extreme

conditions. Some of their protein adaptations include an increased number of acidic residues on the protein's surface, a decreased amount of hydrophobic residues, and multiple peptide insertions in the protein that could increase enzyme flexibility [1].

The study of extremophiles has greatly expanded our knowledge of the diversity of life and our understanding on how microorganisms can adapt and thrive in hostile environments. These features make extremophiles and their proteins attractive sources for biotechnological research.

The organism of interest in this project is a well-studied archaeal extreme halophile called *Halobacterium salinarum* (*H.salinarum*). *H. salinarum* is a facultative anaerobe and requires 4.3 M NaCl, in addition to other salts, for optimal growth [7]. It is a rod-shaped and motile organism which possesses bacteriorhodopsin in the membrane that acts as a light driven proton pump. This complex gives the halophile a pink color and the energy to make carbohydrates. In order to thrive in extremely salty environments, it uses inorganic salts like K^+ or Na^+ to reduce osmotic stress. Unlike other microorganisms which pump salt out to counterbalance osmosis, halophiles like *H. salinarum* pump salt in to avoid osmotic shock. This gives them an internal salt concentration up to 3-4 M [8]. Failure to meet the salt requirement causes the cell to lyse [8]. By importing salt into its cytoplasm, its proteins have to be and are stable under high ionic conditions.

Proteins are polymers of amino acids. They perform different functions within an organism like catalyzing biochemical reactions, replicating DNA, transporting molecules and other vital metabolic processes. Non-extremophilic proteins are sensitive to the surroundings and are affected by temperature, pH and salinity. If placed in "harsh" conditions, they unfold and lose their enzymatic function. Halophilic proteins, however, are functional and structured under

extremely ionic conditions. This makes them interesting for potential biotechnological and industrial applications.

There are many areas where proteins from halophiles can contribute, as many industries produce highly saline waste. One example is the fabric dye industry which uses salt to set dyes and produces a toxic, salty waste product. The generated waste could be degraded with salt-stable enzymes to remove the toxicity. Another application could be the degradation of nonpolar organic solvents by enzymes. They form a system called reverse micelles which consists of three components: amphiphilic molecules, water and a nonpolar organic solvent [9]. In this system, the polar heads of the surfactant are directed towards the interior of the aqueous environment, whereas the aliphatic tails of the surfactant are directed towards the nonpolar organic solvent. The halophilic enzymes encapsulated in reverse micelles can be used in bioremediation applications [10].

Halophilic proteins might also improve water quality by binding to toxic metals like cadmium or lead and removing them from the water. Overall, halophilic proteins can be part of a new low cost and green biochemistry cleaning protocol. Halophilic enzymes also have potential applications for biocatalysis [11]. For example, halophilic hydrolases are used for commercial applications in textiles, baking, brewing and detergent industries. Produced by halophilic organisms, they are capable of working over a wide range of pH and are resistant to high temperatures and organic solvents [11].

Our protein of interest is the *H. salinarum* cysteinyl-t-RNA synthetase (Hs CysRS). The aminoacyl tRNA synthetases are divided into two classes, I and II. Class I aminoacyl-t-RNA synthetases contain a Rossman fold catalytic domain and are mostly monomeric, while class II have an anti-parallel beta-sheet fold flanked by alpha-helices [12]. The cysteinyl-tRNA

synthetases (CysRS) are class I synthetases. CysRS catalyzes the attachment of cysteine to its cognate tRNA, in a process called aminoacylation, in order to generate a substrate for the ribosome to make protein [8]. During aminoacylation, the activated amino acid is transferred to the 2'-OH group of a $tRNA^{Cys}$. This is a reaction that takes place in every cell, making the halophilic CysRS a highly conserved enzyme. Hs CysRS is stable between pH 5 to 9 and has an isoelectric point of 4.1 [8].

Based on the amino acid sequence of CysRS, an electrostatic potential surface model was developed for the Hs CysRS and *E.coli* CysRS, an example of a non-halophilic CysRS. The model shows that the surface of the halophilic CysRS is dominated by negatively charged amino acids which come from the many glutamic and aspartic acids in the protein.

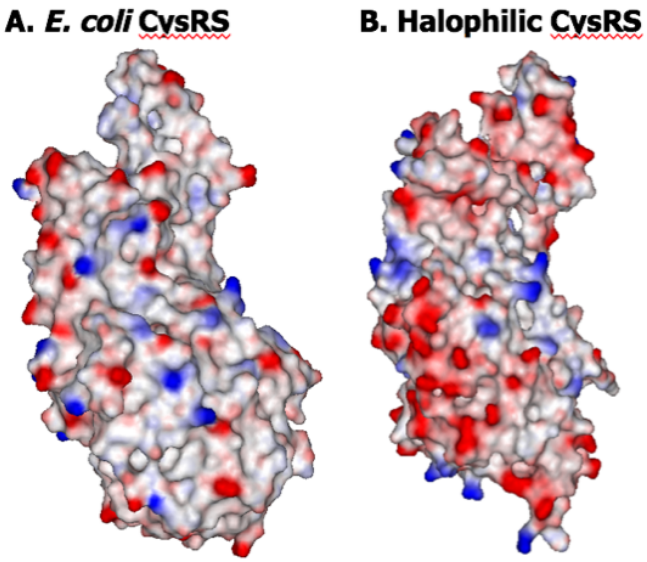

Figure 1. Coulombic potential surface models of two cysteinyl-tRNA synthetases (CysRS). (A) The crystal structure of E. coli CysRS (PDBid10UB) [13]. (B) A homology model of Hs CysRS. The amino acids in the proteins are shaded according their charge: red=negatively charged amino acids, blue= positively charged amino acids, white=uncharged areas. The Hs CysRS contains a greater number of negatively charged amino acids on its surface than *E. coli* CysRS [1].

It has been hypothesized that halophilic proteins bind group 1 metals using the carboxylic acid side chains of the aspartic and glutamic acids on the surface of Hs CysRS. These deprotonated carboxylic acids could chelate small cations like potassium and sodium, or potentially alkaline earth (group 2) and transition metals.

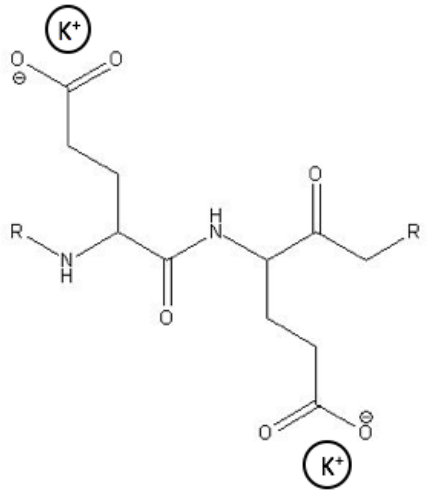

Figure 2. A short peptide of glutamic and aspartic acid showing its potential binding to potassium ions. The deprotonated carboxylic acids could chelate small metal ions.

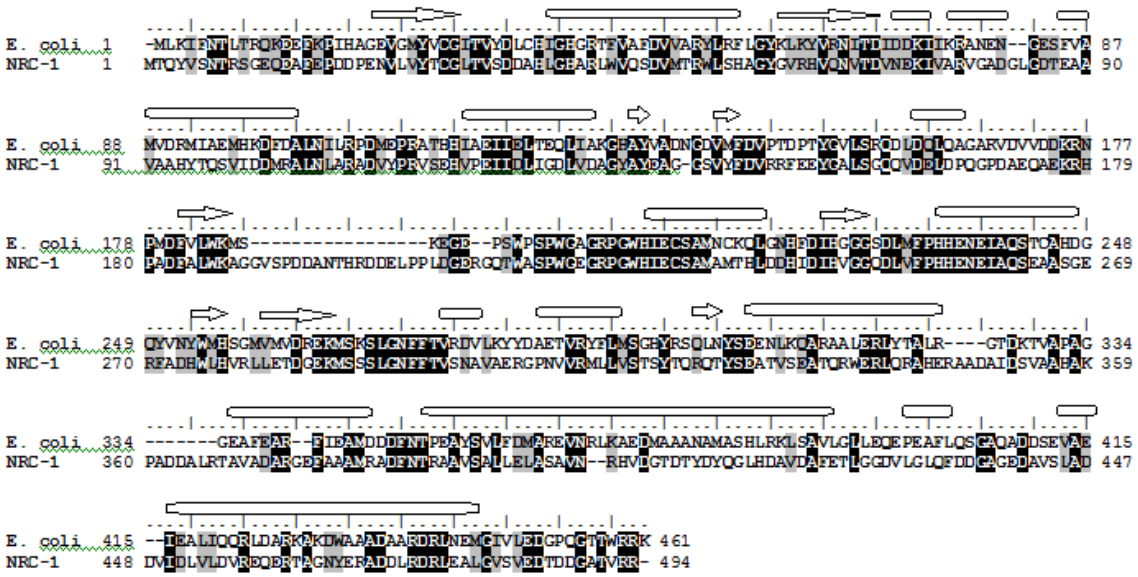

Figure 3. Amino acid sequence alignment for *E.coli* and Hs cysteinyl t-RNA synthetases [14]. The Hs CysRS is represented by NRC-1 in the figure.

Previous studies have been done on the structure and function of Hs CysRS [8]. According to Reed et al., group I salts induce structural changes in halophilic protein. Circular dichroism (CD) spectroscopy was used to detect the presence of secondary structure due to selective absorbance of one component of circularly polarized light by elements of the secondary structure, like α- helices, β-sheets and random coils. CD spectroscopy is a technique that measures the CD of molecules over a range of wavelengths. Far-UV circular dichroism and fluorescence spectroscopy were used to examine the effects of salt on the structure and stability of Hs CysRS and *E. coli* CysRS. A large change in the fluorescence emission spectra of CysRS in high salt compared to the non-halophilic *E.coli* CysRS suggests that the halophilic version is more structurally sensitive to its environment [8]. The structure of the protein was also affected by the type of the salt. Potassium ions, closely followed by sodium ions, were the strongest in inducing secondary structure in CysRS, however group I metals had the same effect, but slightly reduced. Limited group II metals were tried and had only a small effect on the structure of

CysRS. The specificity of the protein for the potassium and sodium ions suggests that strong protein-salt interactions are an evolutionary consequence from the prevalence of potassium in the cytoplasm of *Halobacterium salinarum* [8].

Our hypothesis is that the metal ions bind to the negatively charged amino acids and affect the structure and stability of this halophilic protein. Thus, environmental conditions can help the protein fold into its correct conformation. The focus of this work is to study the binding affinity of the halophilic CysRS with potassium, quantify it and develop a method that can be further used to test the binding affinity of other metals.

To measure metal binding, equilibrium dialysis was used. The goal of using equilibrium dialysis was to determine the rate of diffusion over time and compare and contrast the results between the Hs CysRS and *E.coli* CysRS, a negative control. This technique utilizes a size selective membrane made of cellulose and uses passive diffusion to allow specific binding between a receptor (CysRS) and ligand (K^+). The equilibrium dialysis apparatus used is made of a dialysis bag holder designed like an embroidery hoop system, with an inner ring and an outer ring that clamp together to hold the dialysis tubing open. Over time, the protein reaches equilibrium between the outside and inside of the membrane. Our experimental hypothesis is that when a halophilic protein is added inside a dialysis bag, the binding constant should be measurable and metal binding should be tight, while with a non-halophilic protein, little to no metal binding should be observed.

Potassium was chosen as a primary metal because of its high internal concentration inside the cell of *H. salinarum*. Atomic Absorption Spectroscopy (AAS) was used to determine the potassium concentration inside and outside the dialysis bag over time. AAS works by ionizing a metal in hot flame, which promotes its outer shell electrons to an excited state by absorbing a

specific quantity of energy. The high energy state is quickly depleted, emitting a photon, at a wavelength specific to the metal. A detector measures the absorbance of the light emitted at the specific wavelength. Standards with a known analyte concentration are used to establish a relationship between the analyte concentration and the measured absorbance using the principles of the Beer-Lambert law. The absorbance values were converted to parts per million (ppm) and used to calculate the concentration of potassium inside and outside the dialysis bag.

From these data, a binding constant (K_d) can be estimated. While metal ion binding has been observed for proteins, it has never been calculated for group 1 metals. The significance of measuring a K_d for our protein is to determine that halophilic proteins bind tightly and specifically to these ions, which is what evolution has directed them to do in order to adapt to their particular environment.

Materials and Methods

CysRS Production and Purification

The Hs CysRS protein clones used were propagated as such: 50 µL of Hh CysRS (NI) clone was streaked into LB-Amp plates and incubated for 18 hours at 37 °C.

Using the colonies from the plates, 3 mL LB-Ampicillin (LB-Amp) (100 µg/mL) was grown overnight at 37 °C. This growth was used to inoculate 500 mL of auto-induction media containing ampicillin [15]. This culture grew overnight and was harvested the next day.

The induced cells were chilled on ice for 15 minutes, then centrifuged at 8000 rpm for 20 minutes at 4 °C. The pellet was resuspended with resuspension buffer (100 mL of 50 mM Tris pH 7.5, 100 mM KCl, 0.5 mM β-mercaptoethanol (bME)) plus lysozyme and DNase I. After resuspension, the cells were lysed by 3 freeze-thaw cycles (liquid nitrogen then a warm water bath). To halt protease activity, a 1% PMSF (phenylmethylsulfonyl fluoride) solution was added after the freeze and thaw step.

The cell extract was centrifuged at 10000 rpm for 30 minutes to harvest the protein pellet. It was resuspended in 50 mM HEPES, pH 7.5, 50 mM KCl, 1 mM bMe. Sodium deoxycholate was added to a concentration of 0.5%. After a gentle mix, the solution was centrifuged again for 30 minutes at 12000 rpm. The 80% protein pellet was resuspended in 8 M urea (in resuspension buffer) and allowed to incubate at 4 °C overnight. The solution was centrifuged and the supernatant was taken as the crude protein solution. This solution was dialyzed against HisLink buffer (50 mM HEPES, pH 7.5, 50 mM KCl, 1 mM bMe) overnight to remove the urea.

The protein was further purified using HisLink resin (Promega Inc.). 1.0 mL of resin slurry was mixed with 20 mL of resuspension buffer in a 15 mL plastic tube. The protein solution was added to the resin and put on a rocking platform for 1 hour. The supernatant was

transferred to another tube and the wash was repeated one more time. The protein was eluted from the resin using HisLink buffer plus 500 mM imidazole. Purity was confirmed by a 10% SDS-PAGE gel.

The protein concentration was determined using the Bradford Assay and bovine serum albumin as a standard. Before equilibrium dialysis, the protein solution was dialyzed against 1 L of 0.5 M EDTA solution to remove any metals that might be present, then dialyzed against water twice to remove the EDTA. The protein was used immediately to prevent degradation.

Equilibrium Dialysis

1 μM of protein was added to 10 mL of 18 MΩ water inside a 14 cm piece of a dialysis membrane with a molecular mass cut off of 12-14 kDa.

The dialysis bag holder in white, Figure 4C, was designed like an embroidery hoop system, with an inner ring that fits inside the tubing and an outer ring that slips on the outside tubing. The inner and outer rings that hold the dialysis bag were made using a 3D printer (J. Kuhlmeier). This design functions to hold the dialysis tubing open without removal from the system. Figure 4C shows the entire apparatus: the dialysis bag, the blue clip that seals the bag on the bottom, and the support bars that hold the bag steady for sampling. The aperture is wide enough for a standard yellow pipette tip (20-200 μL).

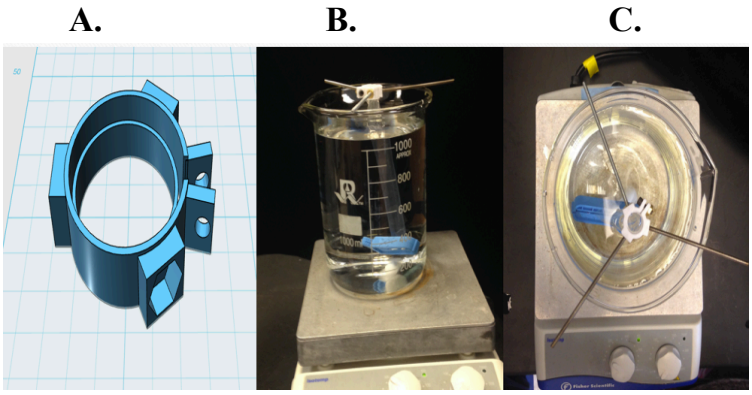

Figure 4. The equilibrium dialysis apparatus, designed by J. Kuhlmeier, showing the dialysis bag holder (A), the support bars and the beaker where KCl was added (B). (C) The view down the apparatus and dialysis bag.

The protein solution was placed inside the membrane bag and suspended in 18 MΩ water. 120 mL of 2.0 M KCl was added to the beaker containing the solution, but outside the dialysis bag. Three total concentrations of KCl were used: 0.27 M, 0.32 M, and 0.37 M. The concentrations of *E.coli* CysRS and Hs CysRS used in the experiment were 1 µM. The *E.coli* CysRS was used as a non-halophilic protein control. A blank, no protein experiment was also performed in which the dialysis bag was filled with 10 mL 18 MΩ water.

10 µL samples were taken from inside and outside the dialysis bag at different intervals for up to 4 hours, when the equilibrium was reached between the inside and outside of the dialysis bag. The sampling pattern is listed below:

0.5 min, 1 min, 2 min, 3 min, 4 min, 5 min, 7 min, 9 min, 11 min, 13 min, 15 min, 18 min, 21 min, 24 min, 27 min, 30 min, 35 min, 40 min, 45 min, 50 min, 55 min, 60 min, 90 min, 120 min, 150 min, 180 min, 210 min, 240 min.

After the samples were taken, they were transferred to 10 mL volumetric flasks, where they were mixed with 200 μL of concentrated HNO_3 and diluted with up to 10 mL with 18 MΩ water (total volume was 10 mL). The mixture was transferred to 15 mL plastic tubes.

Each run was repeated three times for the Hs CysRS, *E.coli* CysRS and the no-protein control.

Atomic Absorption Spectroscopy and Preparation of Standards

For the method of standard addition, six standards were made using the same analytical grade KCl that was used for the dialysis experiment. Standards' concentrations were 1.96 ppm, 3.91 ppm, 5.88 pm, 7.82 ppm, 10.0 ppm and 15.6 ppm. The standards were made in 250 mL volumetric flasks and then transferred to 5 different 50 mL plastic tubes for AAS run.

Varian Spectra AA 220 was used in analyzing the samples. Na/K hollow cathode lamp was used and the parameters for the instrument were set at 6 mA for the lamp current, 769.9 nm wavelength and 1.0 slit width. Eight replicates were taken for each sample. All data was processed and graphed in Microsoft Excel.

Standards	Average Absorbance
0 ppm	-0.0008
1.958 ppm	0.1201
3.910 ppm	0.2397
5.880 ppm	0.3756
7.820 ppm	0.5113
10.01 ppm	0.6585
15.64 ppm	1.0105

Table 1. The set of standards used to create the calibration curve.

A linear regression was run to convert the absorbance values of the unknown samples to concentration.

Linear regression			
slope	0.064776547	intercept	0
error	0.000375703	error	N/A
R^2	0.999798202	standard deviation of y	0.0080549
F-statistic	29726.63747	degrees of freedom	6
regression sum of squares	1.9287269	residual sum of squares	0.0003892934

Table 2. Linear regression of table 1 data, generated using the LINEST function in Microsoft Excel. The slope was used to convert the absorbance values obtained from the AAS to concentration in ppm.

All of the other sets were processed the same way, except the case where the salt concentration was 0.37 M. For this run, another set of standards with a higher concentration was prepared, in order to be consistent with the AAS limit of linearity.

Results

Halophilic proteins are known to be structured in high salt solutions. Therefore, they must have some affinity for the cations which are present in high concentrations in the environments where extremophiles grow. As previously determined in our lab, the Hs CysRS loses structure when placed in low salt solutions [8]. Our hypothesis is to determine if the Hs CysRS protein binds to salts tightly enough to measure a binding constant. If this is measureable, we will also determine approximately how many ions bind to the protein binding sites.

In order to answer this question, equilibrium dialysis was used. The dialysis bag contained 1 μM Hs CysRS which was placed in a beaker filled with 750 mL of 0.32 M KCl solution. 10 μL samples were taken from inside and outside the dialysis bag in intervals according to table 1. Using the procedure described in the Materials and Methods, the potassium concentration of the treated samples was measured using AAS. The absorbance values obtained from the instrument were converted to ppm using the method of standard additions.

Concentration in ppm was graphed versus time to observe the amount of potassium inside and outside the dialysis bag over a period of 4 hours. This was also done for a non-halophilic protein control, *E. coli* Cys RS, and a no-protein control, which was 18 MΩ water. These experiments were repeated twice more, with two different KCl concentrations: 0.27 M and 0.37 M. All results were graphed (figures 3-12).

To understand the behavior of potassium in the absence of protein in the dialysis bag, the no-protein control was graphed (figure 3 and 4). As you can see from the figures, this behavior is typical of equilibrium which was reached after 120 minutes.

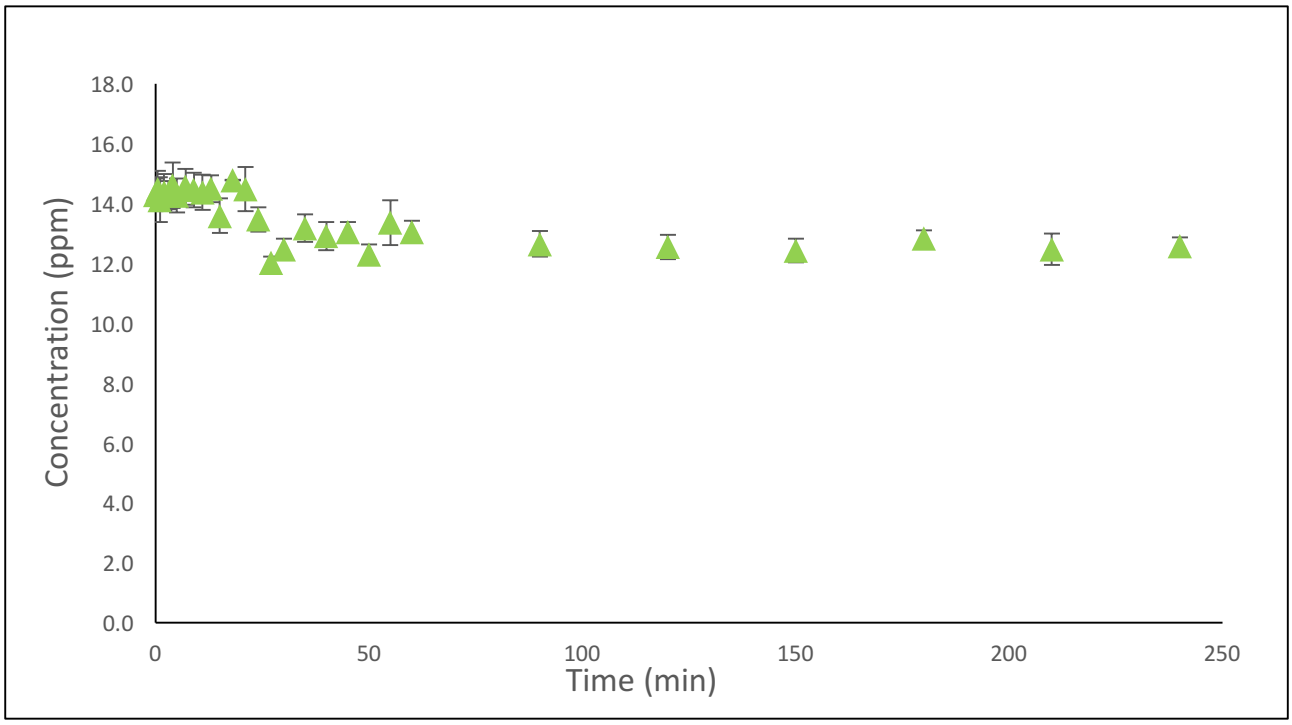

Figure 5. The concentration, in ppm, of the potassium ions outside the dialysis bag for the no-protein control. The concentration of potassium decreases with time, because some ions enter the dialysis bag and stay there.

As can be seen in figure 5, there are some fluctuations of the potassium concentration outside the dialysis bag. There are two reasons to explain this phenomenon. First, there is the big difference between the volume of the solution in the beaker, which was 750 mL and the volume of the dialysis bag, which was only 10 mL. The 1:75 ratio between the volume in the dialysis bag and the volume of the solution in the beaker does not allow for a significant decrease of the potassium concentration outside the dialysis bag.

The other reason is the sampling method. Only one sample was taken at a time and the samples were taken assuming the solutions were homogeneous all the time, which, in reality, might not be true.

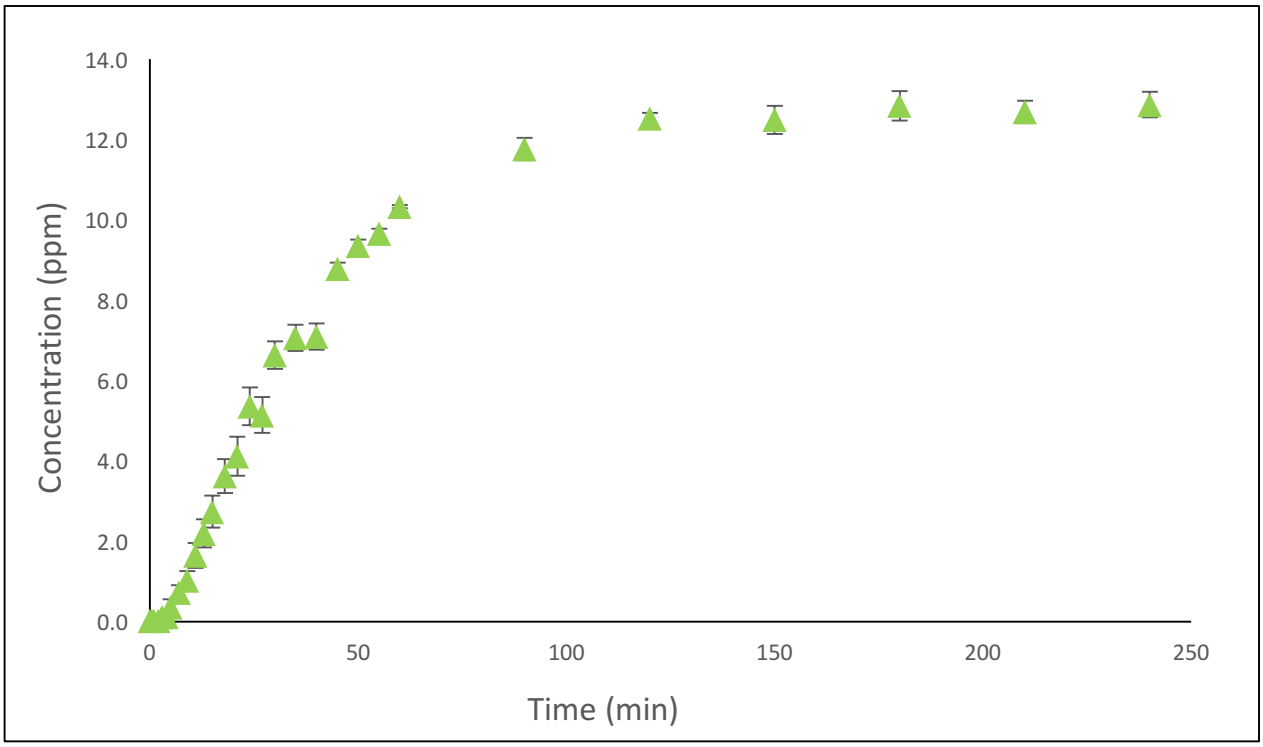

Figure 6. The concentration of the potassium ions inside the dialysis bag for the no-protein control. The amount of potassium increases with time until it reaches equilibrium after about 2 hours.

According to figure 6, the concentration of the cation increases rapidly inside the dialysis bag. Initially, the concentration was 0 ppm inside and during the first hour, there is a fast influx of potassium inside the dialysis bag. It slows down until there is an equal concentration inside and outside the bag, at around 12.5 ppm.

The same equilibrium trend is observed for the other control, the non-halophilic protein, *E. coli* CysRS. While the *E. coli* CysRS run looks essentially the same as the no protein control, there is a small decrease of approximately 1 ppm potassium outside the bag. On the other side, there is a logarithmic increase inside the bag, until it reaches equilibrium at around 12 ppm, as seen in figure 7.

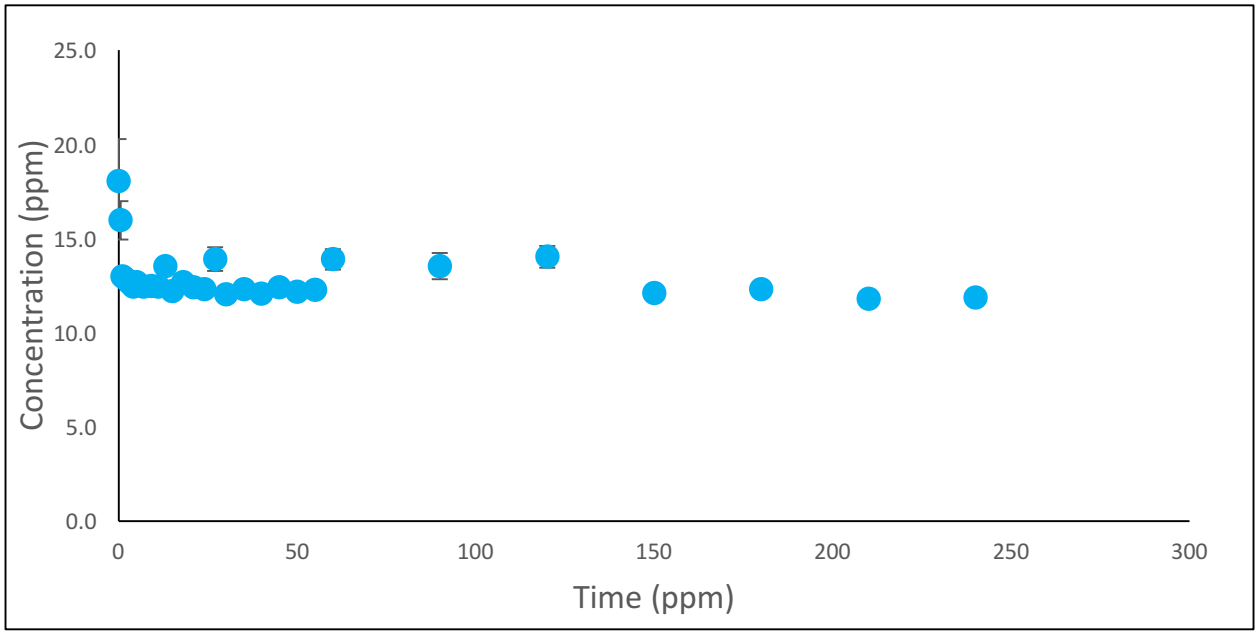

Figure 7. The concentration of the potassium ions outside the dialysis bag for the *E.coli* CysRS, non-halophilic protein control. Despite the fluctuations at the beginning of the run, the concentration of potassium slightly decreases and reaches equilibrium after 120 minutes.

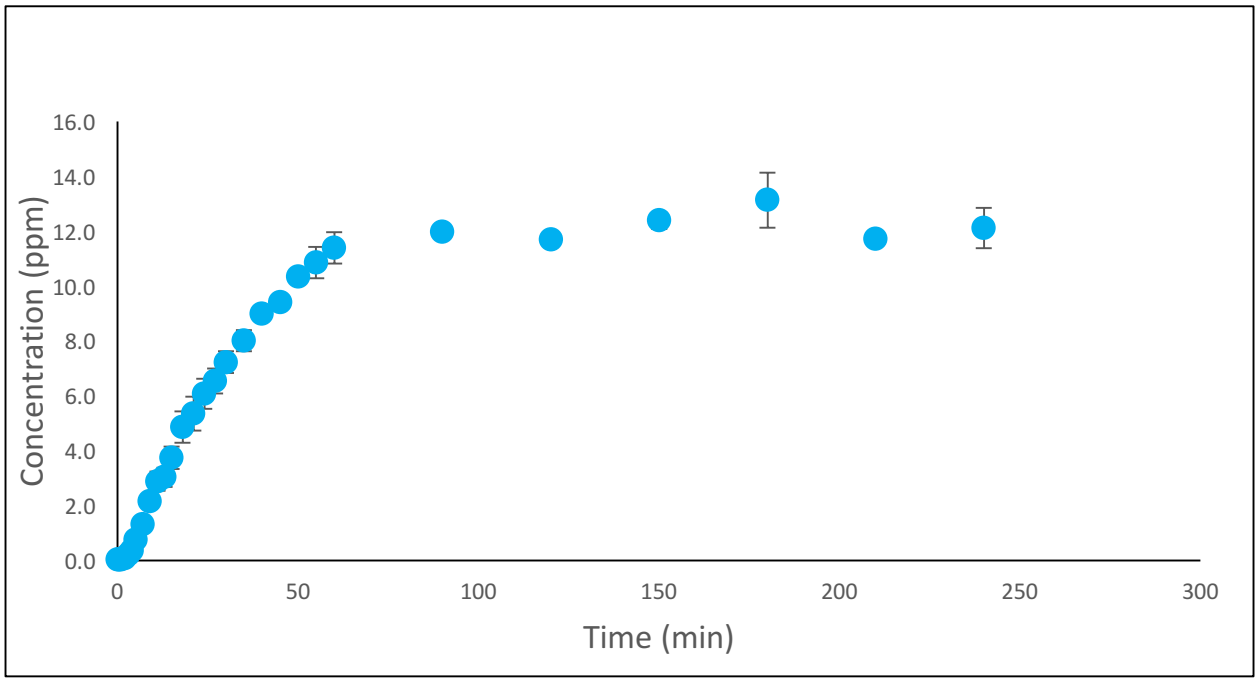

Figure 8. The concentration of the potassium ions inside the dialysis bag for the *E.coli* CysRS control. The potassium ions enter the dialysis bag, which increases the concentration until the equilibrium is reached.

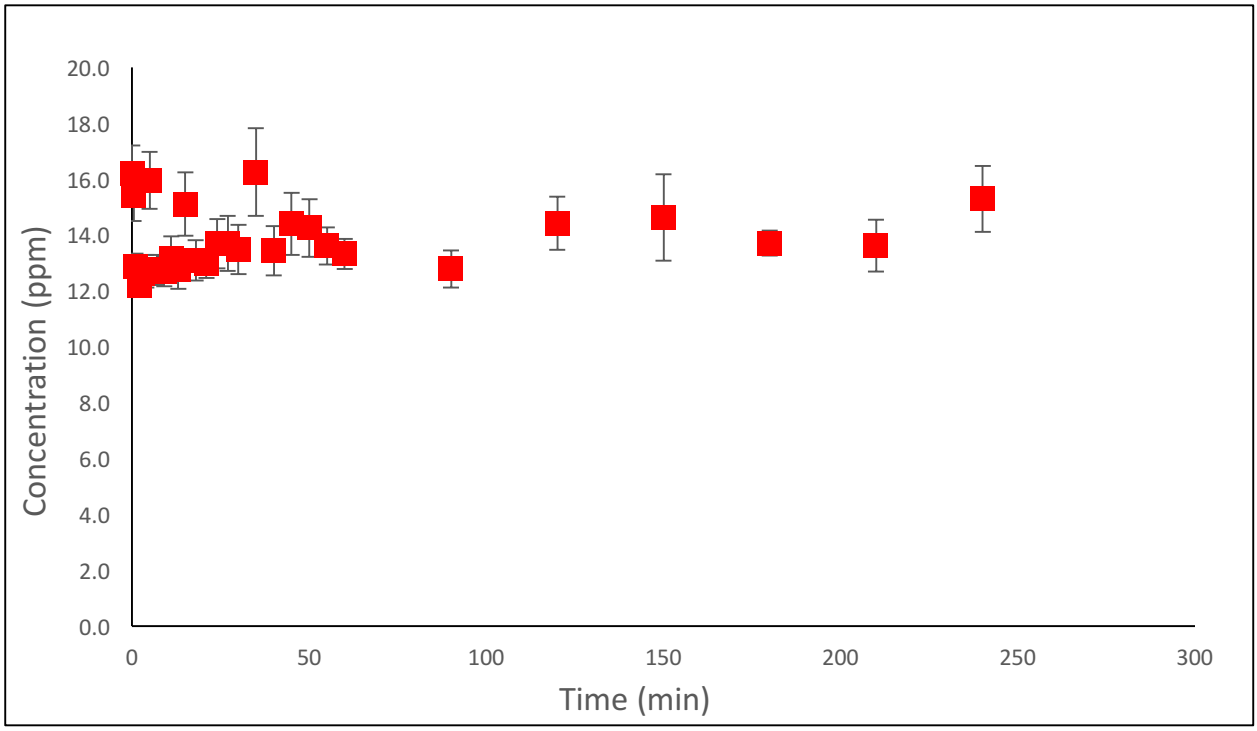

Figure 9. The concentration of the potassium ions outside the dialysis bag when the Hs CysRS protein is added in the dialysis bag. There are larger fluctuations in the concentration of potassium, unlike the other two controls.

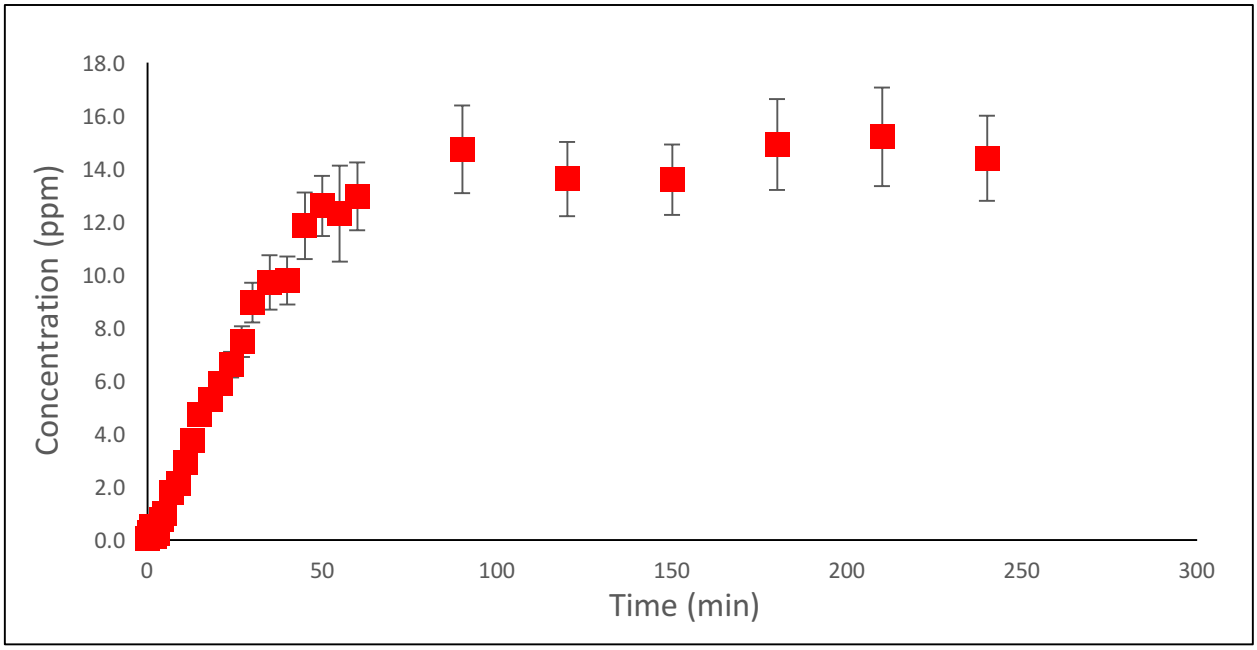

Figure 10. The concentration of the potassium ions inside the dialysis bag for the Hs CysRS protein. The cations increase their concentration with time inside the dialysis bag, until the equilibrium is reached.

All the results presented in figures 6, 8 and 10 are summarized in figure 11, in order to observe the difference of the K^+ in all three runs.

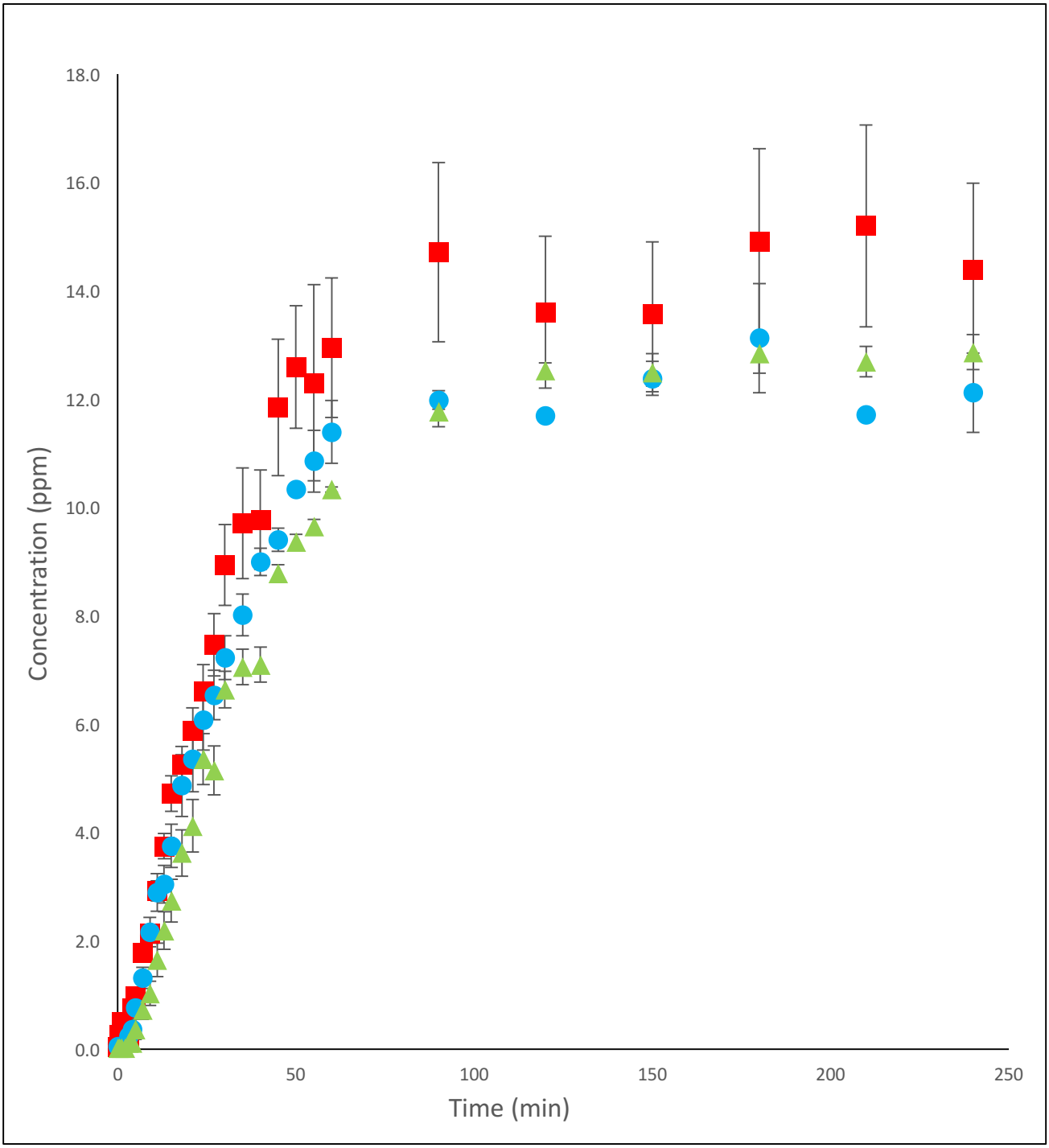

Figure 11. The concentration of potassium inside the dialysis bag for: Hs CysRS, *E.coli* CysRS and no-protein control, when the initial salt concentration in the beaker was 0.32 M.

From figure 11, it can be seen that the potassium stays inside the Hs CysRS dialysis bag longer, which we attribute to the halophilic protein binding to the potassium ions tighter. Because Hs CysRS has a steeper slope, the potassium binding must be faster. The difference in ppm between the plateaus (data after 120 minutes of sampling) shows the presence of binding sites.

The *E. coli* CysRS shows no significant difference in comparison with the no-protein control. This means that the non-halophilic protein does not bind to the potassium ions specifically and stably, unlike Hs CysRS.

These experiments were repeated two more times using two different KCl concentrations, 0.37 M and 0.27 M, in order to get more data points for the calculation of the binding constant.

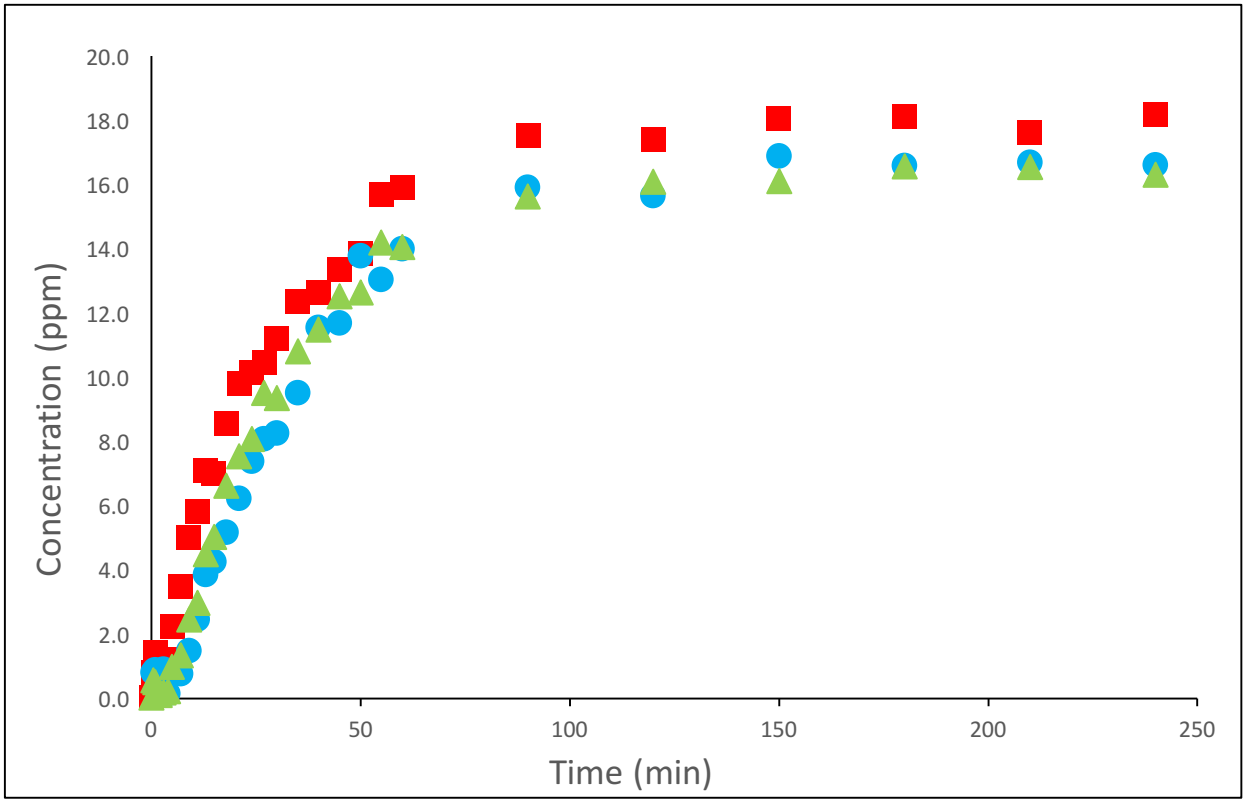

Figure 12. The concentration of potassium inside the dialysis bag for: HsCysRS, *E.coli* CysRS and no-protein control, when the initial salt concentration in the beaker was 0.37 M.

The same trend is observed even when more salt was added in the beaker and the KCl concentration was brought up to 0.37 M. Like the 0.32 M experiment, the potassium concentration was higher when Hs CysRS was added in the bag. There is no significance difference between *E.coli* CysRS and the no-protein control. Their values cross with each, indicating there is no specific binding of the potassium ions to the non-halophilic protein.

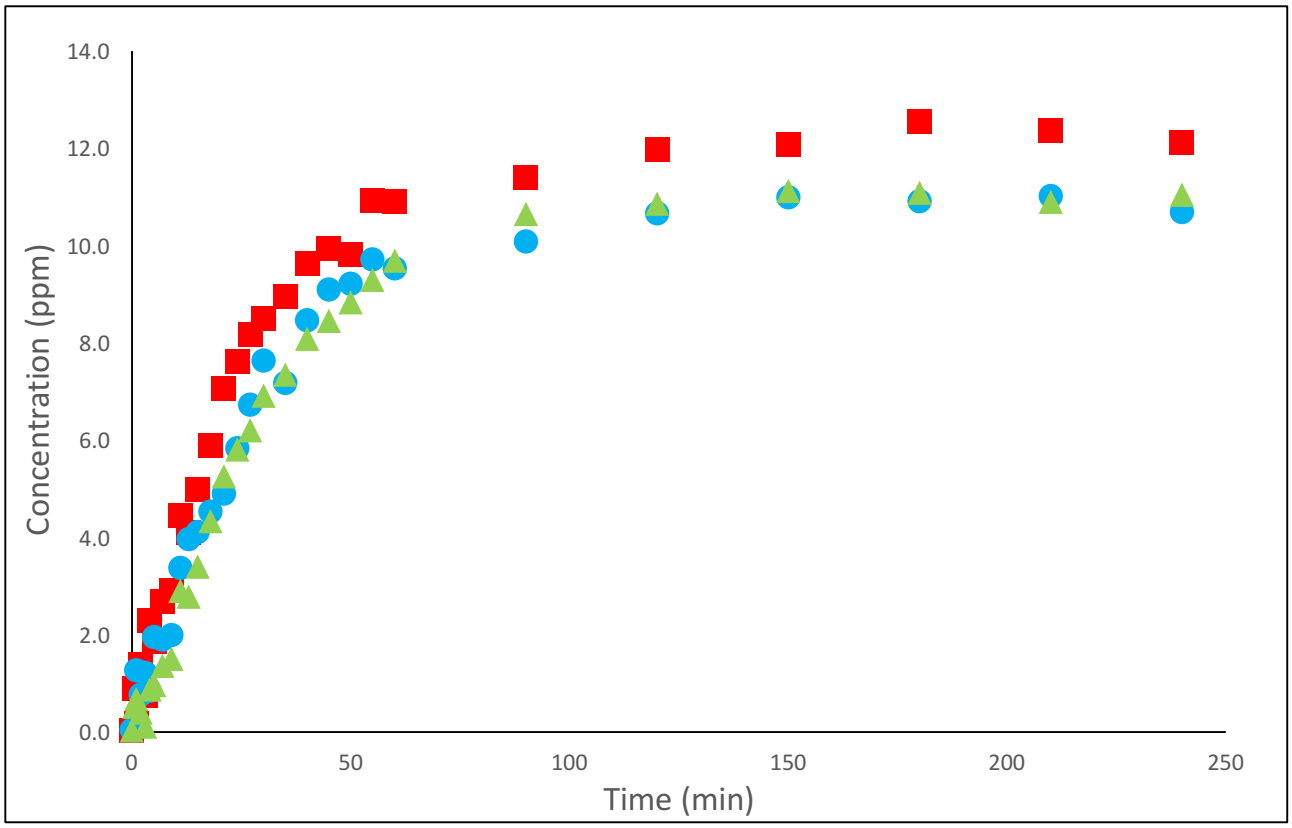

Figure 13. The concentration of potassium inside the dialysis bag for: HsCysRS, *E.coli* CysRS and no-protein control, when the initial salt concentration in the beaker was 0.27 M.

In the case when less salt was added in the beaker, the same trend was seen: more potassium inside the dialysis bag when Hs CysRS is present. No difference was observed between the two controls.

The concentration of potassium versus time was graphed for Hs CysRS, *E.coli* CysRS and the no-protein control, inside and outside the dialysis bag, for three KCl concentrations: 0.27 M, 0.32 M, and 0.37 M. The results are shown in figure 14.

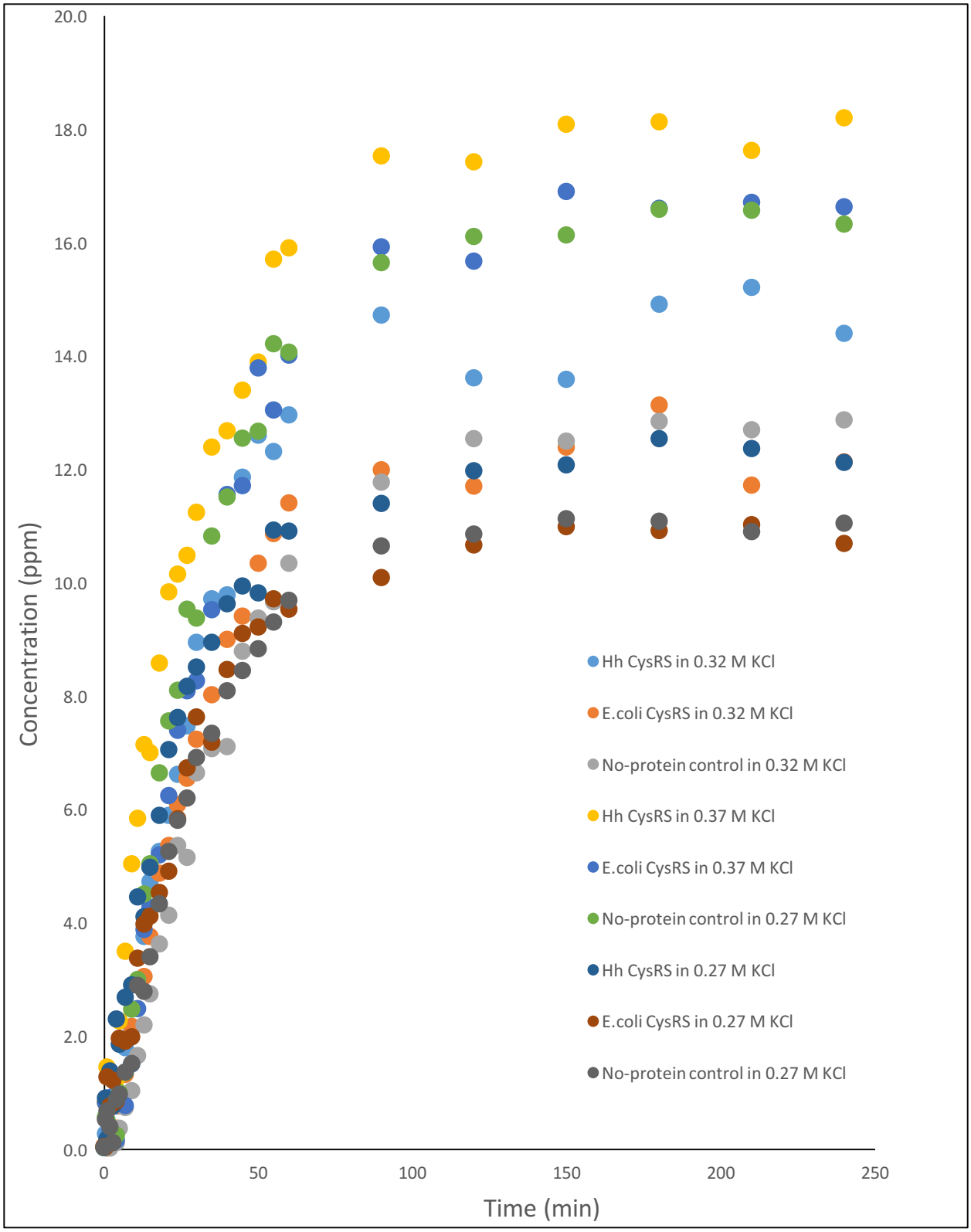

Figure 14. The concentration of potassium inside the dialysis bag for: Hs CysRS, *E.coli* CysRS and no-protein control, for all three salt concentrations: 0.27 M, 0.32 M, 0.37 M.

Calculation of the Binding Constant

A lot of biological functions involve the interactions of small molecules that serve as metabolites, regulators, and signals with the specific surfaces of the macromolecules that carry out cellular processes [16]. Therefore, an understanding of the mechanism of such an interaction is necessary at the molecular level. The binding constant, K_d, represents the affinity between a ligand and receptor. For our purposes, the potassium ions represent the ligand and the Hs CysRS represents the receptor. Using these assumptions, we will calculate how tight the potassium ions bind to the Hs CysRS.

In this case, we are assuming that all binding sites of the protein are equivalent and independent. This means that all sites on the macromolecule will have the same affinity for the ligand as any other and the binding is noncooperative, meaning that the affinity of any site is independent of whether or not other sites are occupied.

In a protein-ligand complex, the equilibrium dissociation constant can be written as:

$$Kd = \frac{[PA]}{[P][A]}$$

where [P] is the protein concentration, [A] is the ligand concentration and [PA] is the protein – ligand concentration. $\bar{\upsilon}$ is the fraction of protein molecules bound by the ligand. When there is only one binding site per molecule, $\bar{\upsilon}$ can be expressed as:

$$\bar{\upsilon} = \frac{[K^+ \; in \; tubing]}{[K^+ \; in \; tubing + K^+ \; in \; beaker]}$$

$$\bar{\upsilon} = \frac{[PA]}{[P]+[PA]} = \frac{Kd[P][A]}{[P]+Kd[P][A]} = \frac{Kd[A]}{1+Kd[A]}$$

When there is more than one binding site, as in the case of Hs CysRS, the equilibrium constant of all the reaction complexes equals the product of the equilibrium constants of every single ligand-protein interaction, so K_d can be expressed as: $K_n = K_1K_2K_3...K_n$, where n is the

number of the binding sites of the molecule. In this case, the average fraction of bound ligands per protein, $\bar{\upsilon}$, equals:

$$\bar{\upsilon} = \frac{\sum_{i=1}^{n} nKi[P][A]n}{\sum_{i=0}^{n} Ki[P][A]n}$$

This is known as the Adair equation [16] and is used to calculate the binding affinity. Theoretically, it can be used to describe any number of binding sites and situations. It can be simplified assuming all binding sites are equivalent and independent of each other.

$$\bar{\upsilon} = \frac{nKd[A](1 + Kd[A])^{n-1}}{(1 + Kd[A])n} = \frac{nKd[A]}{1 + Kd[A]}$$

$\bar{\upsilon}$ versus [A] will be a logarithmic function. A double reciprocal plot is obtained after inverting the function $\bar{\upsilon}$ versus [A].

$$\frac{1}{\bar{\upsilon}} = \frac{1}{n} + \frac{1}{nk[A]}$$

The slope of a graph $1/\bar{\upsilon}$ versus 1/[A] is $1/nK_d$ and 1/n is its y-intercept. After finding the y-intercept from the graph, its reciprocal equals n. The value of n, together with the slope, can be used to solve for K_d.

The concentration of potassium inside the dialysis bag for the no-protein control from 120 minutes to 240 minutes, was subtracted from same time points for the Hs CysRS. This timeline was chosen because the equilibrium was established by this point and the values are close to each other, meaning the change between the outside and inside environments was insignificant. The results are presented in table 2.

	0.32 M		0.37 M		0.27 M	
	No- protein control	Hs CysRS	No- protein control	Hs CysRS	No- protein control	Hs CysRS
120 min	16.1	17.4	12.5	13.6	10.9	12.0
150 min	16.1	18.1	12.5	13.6	11.1	12.1
180 min	16.6	18.1	12.8	14.9	11.1	12.5
210 min	16.6	17.6	12.7	15.2	10.9	12.4
240 min	16.3	18.2	12.9	14.4	11.0	12.1
Average	16.3	17.9	12.7	14.3	11.0	12.2

Table 3. Average potassium concentration in ppm for all three K^+ concentrations after the equilibrium was reached. This is the data before subtraction of the no-protein control from Hs CysRS.

	$\bar{\upsilon}$ average fraction of bound ligands per protein	[A] free ligand concentration
0.27 M	1212 ppm	10440 ppm
0.32 M	1650 ppm	12512 ppm
0.37 M	1550 ppm	14597 ppm

Table 4. The difference of the potassium concentration between Hs CysRS and the no-protein control adjusted for the dilution factor of 1000.

The values from table 4 were converted from ppm to μM in order to be consistent with the unit for the amount of protein, which was 1 μM. The results are summarized in Table 5.

	$\bar{\upsilon}$ (μM)	[A] (μM)	1/[A] (1/μM)	1/$\bar{\upsilon}$ (1/μM)
0.27 M	30997	267000	$3.75*10^{-6}$	$3.23*10^{-5}$
0.32 M	42199	320000	$3.13*10^{-6}$	$2.37*10^{-5}$
0.37 M	39642	373332	$2.68*10^{-6}$	$2.52*10^{-5}$

Table 5. Average fraction of bound ligands per protein ($\bar{\upsilon}$) and free ligand concentration [A] for all three different KCl concentrations.

From table 5, the double reciprocal plot of $\frac{1}{\bar{\upsilon}} = \frac{1}{n} + \frac{1}{nKd[A]}$ was generated to calculate n and K_d (figure 15).

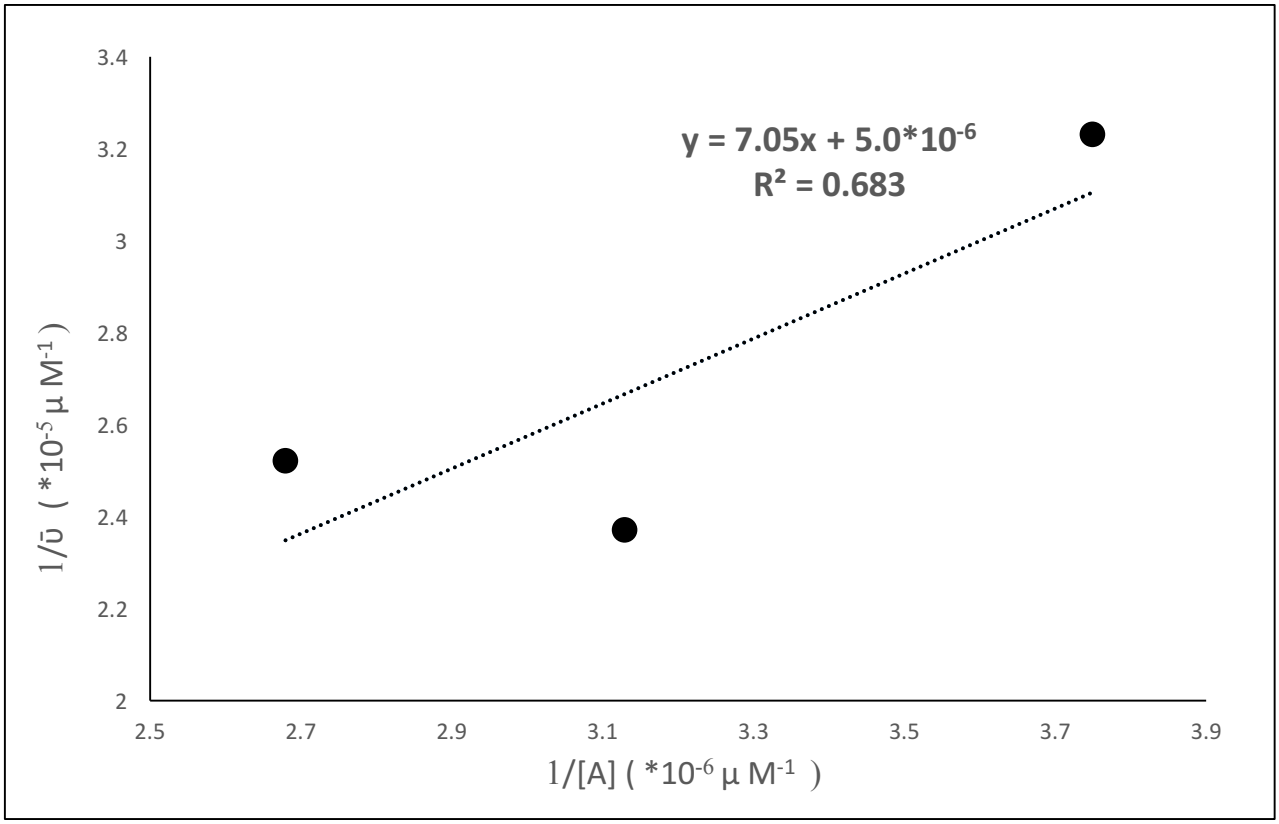

Figure 15. A graph of $\frac{1}{\bar{\upsilon}} = \frac{1}{n} + \frac{1}{nKd[A]}$. Graphing 1/ῡ versus 1/[A] gives a value for the slope and y-intercept, which are further used to calculate, first the number of binding sites n, and then the binding constant K_d.

The equation obtained from this graph was used for the calculation of the binding constant, K_d and the number of binding sites, n.

$$\frac{1}{\bar{\upsilon}} = \frac{1}{nKd[A]} + \frac{1}{n}$$

$$y = 7.05x + 5.0*10^{-6}$$

Since the y-intercept is $5.0*10^{-6}$, the reciprocal gives a value of n = 200,000. The slope of the graph was $1/nK_d$, so the value of K_d is $7.10*10^{-7}$ μM^{-1}.

Discussion and Conclusion

A difference was observed in the metal concentration between the outside KCl solution and the protein solution inside the dialysis bag. According to our data, the concentration of the potassium ions is greater inside the bag. The metal appears to be tightly binding to the protein, since Hs CysRS has a steeper slope as shown in figures 11, 12, 13 and 14. The plateau after the first hour of sampling shows the presence of multiple and specific binding sites. It also represents the difference in potassium concentration between the halophilic protein and the two controls. The *E.coli* CysRS and no protein controls show no significant difference in binding to the potassium ions. The same trend is repeated even when the concentration of KCl is changed to 0.27 M and 0.37 M.

From the results, n=200,000, meaning that there are 200,000 binding sites on the molecule of Hs CysRS. This number seems too large, since the total number of the negatively charged amino acids in the protein is only 93. There are 55 Asp and 38 Glu in Hs CysRS and for comparison, *E.coli* CysRS has 38 Asp and 34 Glu. One possible reason for the impossibly large number of possible binding sites is that we used only three points for the graph in figure 12. Future work on this project will fill in with at least 2 more points. Another reason might be that not all of the cations inside the bag bind to the protein. There could be cations binding to the dialysis bag itself, although this is unlikely, as this should also happen with the *E. coli* CysRS and the no protein control. The value of the binding constant, K_d, which is $7.10*10^{-7}$ μM means that the binding of the potassium ions and the protein is strong. The accuracy of these values can be improved by repeating the experiment with at least 2 more salt concentrations, below 0.27 M, in order to get more data points for the graph shown in figure 15. The current R^2 value of 0.683 indicates that there is some error in the trendline.

In conclusion, potassium was observed to have a strong effect on the *Halobacterium salinarum* ssp. NRC-1 cysteinyl-tRNA synthetase, by helping the protein fold properly and remain stable in extreme environments. Our results support these data by showing fast, tight and specific binding of potassium which is stable over time. Our future work will be to repeat these data, and test the behavior of Hs CysRS at different protein concentrations, as well as to try other group I metals. We hope this work will give a better understanding on how metals interact with proteins, particularly halophilic proteins, and influence their stability.

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